



SEDE AMMINISTRATIVA: UNIVERSITÀ DEGLI STUDI DI PADOVA  
DIPARTIMENTO DI AGRONOMIA ANIMALI ALIMENTI RISORSE  
NATURALI E AMBIENTE

CORSO DI DOTTORATO IN SCIENZE ANIMALI E AGROALIMENTARI  
INDIRIZZO: PRODUZIONI AGROALIMENTARI  
CICLO XXX

**GENETIC AND NONGENETIC VARIATION OF MILK PROCESSING  
CHARACTERISTICS IN IRISH AND ITALIAN DAIRY CATTLE**

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## *Abstract*

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Milk processing characteristics describe the aptitude of milk to be transformed into different dairy products. Milk intended for cheese production should coagulate early in order to generate a strong curd; cheesemaking is favored by low milk pH, small casein micelle, and high content of minerals such as Ca, P, and Mg. Small casein micelle, high milk pH, and low inorganic  $\text{Ca}^{2+}$  favor the stability of milk protein to heat treatments, which is a fundamental characteristic for the production of dried milk. The quantification of such milk quality traits is important at the dairy industry level in order to define more precisely the best use of milk prior to processing. The overall objectives of the present thesis were: i) to develop mid-infrared spectroscopy (MIRS) prediction models for a fast and on-line measurement of milk processing characteristics; ii) to study the effect of stage of lactation, parity, breed, heterosis and recombination loss on a large number of predicted processing phenotypes; and iii) to estimate (co)variance components of milk technological aspects in order to potentially breed for improved milk processability.

The effectiveness of MIRS as a fast and cost-effective technique to predict rennet coagulation time, curd-firming time, curd firmness at 30 and 60 min after rennet addition (milk coagulation properties), heat coagulation time, casein micelle size, pH and minerals (Ca, K, Mg, Na, and P) was evaluated. The proportion of variance explained by the prediction models in external validation ranged from 13% (casein micelle size) to about 70% (minerals, with the exception of Na, and pH).

Factors associated with the phenotypic variation of MIRS-predicted milk coagulation properties, heat coagulation time, casein micelle size, and pH, and minerals was evaluated on large spectral datasets. Milk coagulation properties were more favorable (i.e., short rennet coagulation time and strong curd firmness) for

cheese manufacturing in early lactation, concurrent with the lowest values of both pH and casein micelle size, and the greatest milk mineral content; milk was more stable to heat in mid-lactation. Milk yielded by primiparae was more adapted for both cheese and milk powder production. Jersey cows, on average, yielded milk more suitable for cheese rather than milk powder production. Simmental cows produced milk with the greatest content of Ca and Na, and milk of Holstein-Friesian had the lowest P content.

Genetic parameters for milk processing characteristics were studied. Heritability estimates ranged from 0.16 (heat coagulation time) to 0.54 (Ca). Within-trait genetic correlations were weaker than 0.40 only at the peripheries of the lactation. On average, more than 80% of the additive genetic variation in each trait was associated with the height of the lactation profile for all traits. Milk processing traits were generally antagonistically correlated with milk production suggesting that emphasis should be placed to milk processability to halt any deterioration.

## *Riassunto*

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Le qualità tecnologiche del latte descrivono l'attitudine di questa materia prima ad essere trasformata in prodotti lattiero-caseari. Il latte destinato alla produzione di formaggio dovrebbe coagulare in tempi relativamente brevi e generare un coagulo consistente; in quest'ambito la coagulazione del latte è favorita da valori bassi di pH ed alta acidità titolabile, micelle caseiniche piccole e alto contenuto di minerali come Ca, P e Mg. Micelle caseiniche piccole, valori di pH alti e un basso contenuto di  $\text{Ca}^{2+}$  favoriscono la stabilità del latte a trattamenti termici, una caratteristica fondamentale per la produzione di latte in polvere. La determinazione delle caratteristiche tecnologiche del latte è un aspetto rilevante nell'industria lattiero-casearia al fine di definire in maniera più efficiente la destinazione e l'utilizzo dello stesso durante la trasformazione. Gli obiettivi generali della presente tesi sono: i) sviluppare modelli di predizione utilizzando la spettroscopia nel medio-infrarosso (MIRS) per la determinazione routinaria di caratteri tecnologici del latte; ii) studiare l'effetto di stadio di lattazione, ordine di parto, razza, eterosi e ricombinazione su un elevato numero di fenotipi predetti; iii) stimare le componenti di (co)varianza degli aspetti tecnologici del latte al fine di verificare la possibilità di migliorarli geneticamente.

È stata valutata l'efficacia del MIRS quale tecnica rapida e relativamente economica per predire il tempo di coagulazione, il tempo di rassodamento e la consistenza del coagulo a 30 e 60 minuti dall'aggiunta del caglio (proprietà coagulative del latte), la stabilità al calore, la dimensione delle micelle caseiniche, il pH e i minerali (Ca, K, Mg, Na e P). La proporzione di varianza spiegata dai modelli di predizione in validazione esterna varia dal 13% per la dimensione delle micelle caseiniche a circa il 70% per i minerali (con l'eccezione di Na e pH).

I fattori associati alla variazione fenotipica delle predizioni MIRS delle proprietà coagulative del latte, della stabilità al calore, della dimensione delle micelle caseiniche, del pH e del contenuto di minerali sono stati valutati utilizzando dataset di spettri di popolazione. Le proprietà coagulative del latte sono più favorevoli (breve tempo di coagulazione e elevata consistenza del coagulo) per la produzione di formaggio all'inizio della lattazione, contestualmente a valori bassi di pH e della dimensione delle micelle caseiniche, e ad alte concentrazioni di minerali; la stabilità risulta più elevata a metà lattazione. Il latte prodotto da bovine primipare è maggiormente adatto alla produzione sia di formaggio sia di latte in polvere. Soggetti di razza Pezzata Rossa producono un latte con i più alti contenuti di Ca e Na, mentre il latte di Frisona ha i valori più bassi di P.

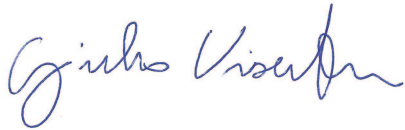
Infine, sono stati determinati i parametri genetici per i caratteri tecnologici menzionati in precedenza. Le stime di ereditabilità variano da 0.16 (stabilità al calore) a 0.54 (Ca). Le correlazioni genetiche entro carattere sono inferiori a 0.40 solamente ad inizio e fine lattazione. In media, più dell'80% della varianza genetico-additiva risulta associata con l'altezza del profilo di lattazione di tutti i caratteri studiati. In generale, i caratteri tecnologici del latte sono correlati antagonisticamente con la produzione di latte. Alla luce dei risultati si ritiene opportuna una concreta applicazione di tali caratteri negli attuali programmi di miglioramento genetico.



## *Declaration*

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I declare that the present thesis has not been previously submitted as an exercise for a degree at University of Padova, or any other University, and I further declare that work embodied is my own.



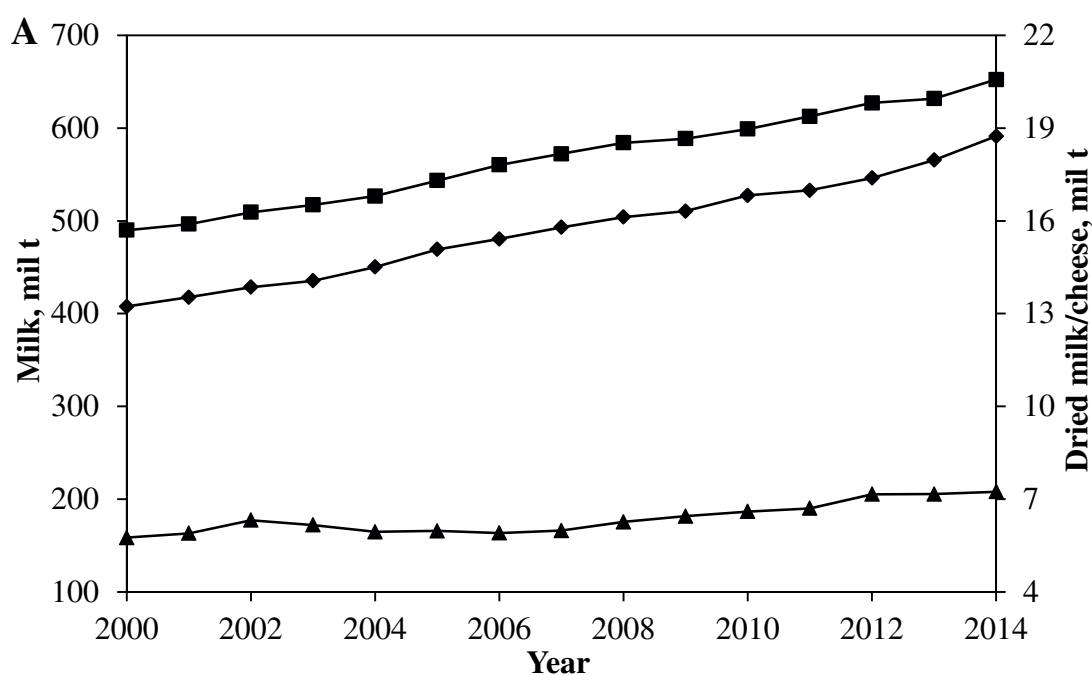
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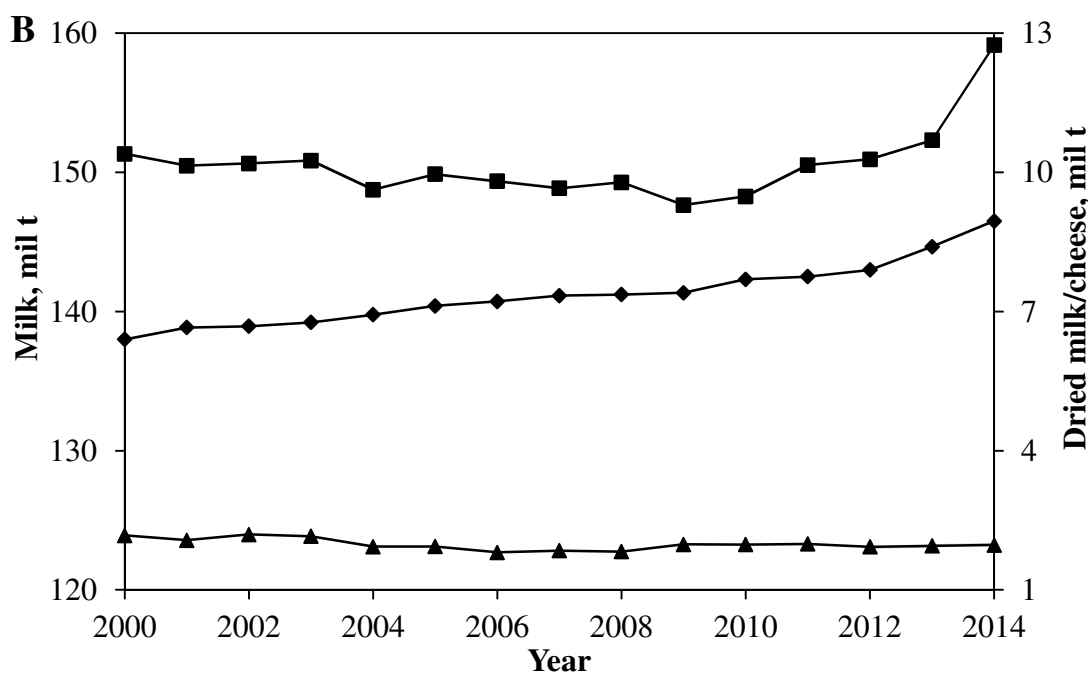


### MILK PROCESSING AND BEST USE OF MILK

#### *Milk and dairy products market*

Global bovine milk production increased constantly between 2000 and 2014, moving from 490 mil t in 2000 to 652 mil t in 2014 (Figure 1; FAO, 2017). Within the same period, such a positive trend was not evident in the European Union, where milk production remained constant at about 150 mil t because of the milk quota regime (EEC Regulation 856/1984) which was abolished in March 2015 (Figure 1). On average, global milk and cheese production increased since 2000 at an annual growth rate of 2.02% and 2.45%, respectively. Cheese production in the European Union increased from 6 mil t in 2000 to 9 mil t in 2014, representing half of the global cheese production; in 2013 and 2014 the annual growth rate of cheese production, within the same geographical context, was around 6% (Figure 1).





**Figure 1.** Production of bovine milk (—■—), cheese (—◆—), and dried milk (—▲—) globally (A) and in the European Union (B; FAO, 2017).

The annual growth rate of milk powder was 1.59% and -0.83% in the world and European Union, respectively, between 2000 and 2014.

However, the production of milk powder did not constantly grow over time (Figure 1). Regarding other important dairy products such as condensed milk and butter, between the years 2000 and 2014 the global annual growth rate was 2.26% and 0.74%, respectively (FAO, 2017). However, for these two last products, the annual growth trend was not constant over the analyzed period, similar to the trend of dried milk production. The evolution of the market price for milk and dairy products is more affected by the geographical location considered. In the European Union, the market price of whole milk has increased by 31.7% between July 2016 and July 2017. The market price of cheese has a positive trend as well, although it is influenced by the type of cheese considered. For example, in September 2017, the price of Parmigiano Reggiano cheese in Italy, increased by 15% compared to September 2016,

whereas the market price of Edamer cheese, in Germany increased by 27.7% in the same period. Also, the market price of dried milk and butter increased by 32.2% and 60.3%, respectively, from September 2016 to September 2017 (CLAL, 2017). Almost all the milk in the national and international market needs to be processed prior to human consumption. Therefore, characteristics describing milk processability – that is, the ease and the capacity to transform milk into different dairy products – are extremely important for processors.

In the present thesis, two main categories of traits defining milk processability were studied: i) traits important for cheesemaking, and ii) traits important for milk powder production.

### ***Traits important for cheesemaking***

These traits describe the capacity of milk to react to rennet addition and to generate the curd. Ideally, milk for cheesemaking should coagulate within a short interval of time, should have a fast firming rate, and should produce a firm curd. If these three criteria are fulfilled, then the amount of cheese produced per unit of milk (generally called cheese yield, expressed as kg of cheese/100 kg of milk processed), and consequently the industry profit, is maximized (Aleandri et al., 1989; Formaggioni et al., 2008; Pretto et al., 2013). Traits important for cheesemaking are generally referred to as milk coagulation properties. Another important feature for the cheesemaking is milk acidity, expressed as titratable acidity or pH. High milk pH is not a desired feature for the cheesemaking (Ikonen et al., 2004; Pretto et al., 2013). Finally, casein micelle size (**CMS**), which is the diameter of the casein micelle, should be small in order to favor rennet activity (Glantz et al., 2010). Besides the casein content and the fat to casein ratio (Formaggioni et al., 2005), the milk mineral

content has an important role during cheesemaking (Lucey and Fox, 1993; Malacarne et al., 2014).

### ***Traits important for the production of milk powder***

These traits describe the ability of milk to withstand high temperature treatments without visible coagulation or gelation (Singh, 2004). The most common way to measure milk heat stability is the determination of heat coagulation time (HCT), which measures the time needed to identify visible floccules of protein aggregate in milk when subjected to high temperature treatments. Small CMS is a desired characteristic to process milk into dried milk (Chen et al., 2014), as well as high pH (Singh, 2004). The mineral profile of milk plays an important role for the determination of heat stability; for example, a reduction in Ca ion activity promotes milk heat stability (Fox and Brodkorb, 2008).

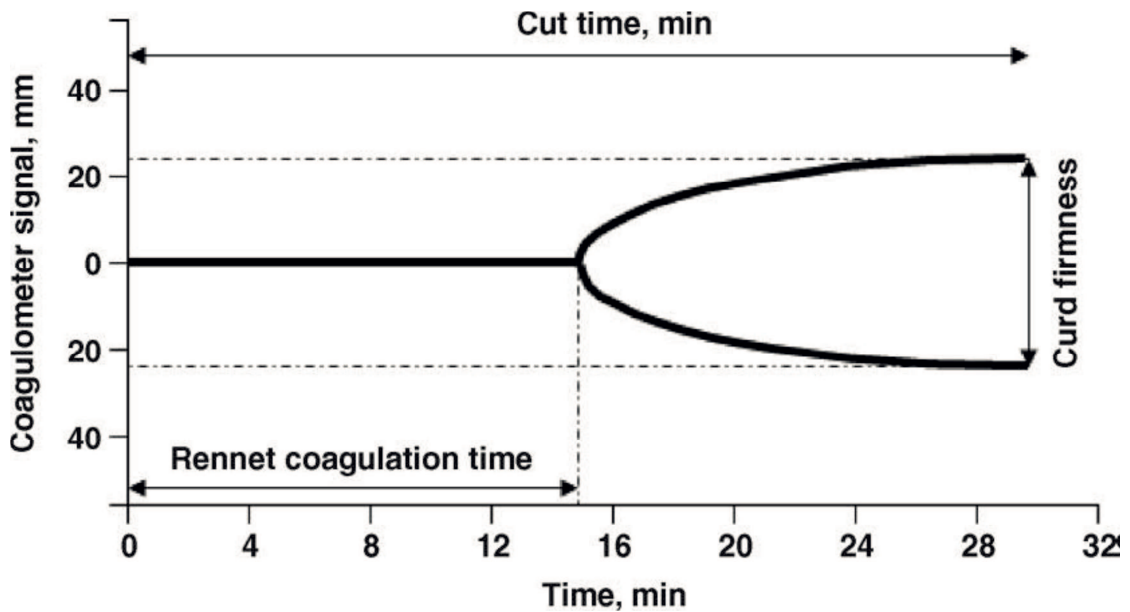
The concept of best use of milk is therefore strictly related to the capacity of transforming milk to different dairy products within the dairy plant (Chen et al., 2017). Given a series of milk processing features measured on a milk sample, which dairy product can be produced more efficiently? Being able to answer to this question can be an important decision-support tool for the dairy processor to identify the best dairy product which can be obtained more efficiently prior to manufacturing.

## **ASSESSMENT OF MILK PROCESSING TRAITS**

### ***Milk coagulation properties***

Commonly used methods to measure milk coagulation properties include mechanical instruments such as the Formagraph (Foss Electric A/S, Hillerød, Denmark; McMahon and Brown, 1982). In mechanical instruments, a given amount

of rennet solution is added to the milk, which is then brought into contact with an oscillating pendulum. When the viscosity of milk starts to increase because of the coagulation, a force is back transmitted to the pendulum. Therefore, the output of a mechanical analysis is a graph of curd firmness against time, as depicted in Figure 2.



**Figure 2.** Typical output of the Formagraph (from De Marchi et al., 2009).

Milk coagulation properties can be identified from the visual analysis of Figure 2. Rennet coagulation time (**RCT**) is the time (min) from the beginning of the test (which corresponds to the moment of the rennet addition) to the start of milk coagulation; the rate of curd firming, or curd-firming time (**k<sub>20</sub>**), is the time (min) from the beginning of the coagulation to the achievement of a 20-mm spread in the graph; curd firmness is represented as the spread (mm) in the graph at a given time from the beginning of the test, which is generally measured at 30 (**a<sub>30</sub>**) or 60 min (**a<sub>60</sub>**). Other methods to measure milk coagulation are the computerized renneting meter (Polo Trade, Monselice, Italy; Dal Zotto et al., 2008; Cassandro et al., 2008) or the Optigraph (Ysebaert SA, Frépillon, France; Cipolat-Gotet et al., 2012).

### ***Casein micelle size***

The hydrodynamic diameter of casein micelle is normally determined using photon correlation spectroscopy (Devold et al., 2000; Glantz et al., 2010) with a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). Casein micelle can be measured in both raw or skimmed milk, following milk dilution in deionized water, using noninvasive backscatter optics. The mean particle size of the volume distribution is normally the parameter considered to define the CMS.

### ***Milk acidity***

The acidity of milk can be assessed using a pH-meter (pH) or using the method by Anonymous (1963). This last method measures the titratable acidity of milk expressed in Soxhlet-Henkel degrees. High milk pH means low titratable acidity given the strong and negative correlation between these two features (Cassandro et al., 2008).

### ***Milk minerals***

The quantification of minerals in milk is carried out using inductively coupled plasma optical emission spectrometry. This method can detect elements present at ppm level (mg/kg); sample preparation may include a pre-digestion in acid in order to increase the element detection and quantification. However, this process significantly increases the time of samples analysis (Soyeurt et al., 2009; Toffanin et al., 2015).

### ***Heat stability***

The assessment of milk heat stability is normally carried out using a hot oil bath (Davies and White, 1966a). Milk samples are sealed in a glass tube which is then



immersed into hot oil heated at a given temperature (normally 120-140°C) in an oscillating rack; this method permits the measurement of HCT. There are also less commonly used methods to measure milk heat stability, such as the ethanol test or the protein sedimentation test (Davies and White, 1966b; Singh, 2004).

All the reference methods to measure milk processing characteristics have two common features. Firstly, the reference methods are time-demanding, allowing the measurement of milk processability on only few samples in a relative long-time interval (with the only exception of the measurement of milk pH); secondly, they are expensive (Table 1). These aspects have negative implications as processors are lacking an on-line method to constantly monitor milk technological characteristics, and therefore they may not be aware of the best use of milk in the production plant. Moreover, from a genetics point of view, setting up a breeding program to genetically improve milk processing features is not feasible given the high phenotyping costs associated with the measurement of these characteristics on a large-scale level. A possible way to overcome these issues is the development of mid-infrared spectroscopy (MIRS) prediction models.

**Table 1.** Summary of the methods to measure milk processing characteristics, number of analyzable samples, time and cost for each sample.

Trait	Method	Samples	Time (min)	Cost (Euro)
Milk coagulation properties	Formagraph	10	30 to 60	53
Heat stability	Hot oil bath	7	30	50
Casein micelle size	NanoSizer	1	7	35
pH	pH-meter	1	1	16
Minerals	ICP-OES	15	60	42

### ***Mid-infrared spectroscopy***

Mid-infrared spectroscopy is based on the capacity of all molecules to reflect, transmit, and absorb a proportion of the incidence energy (De Marchi et al., 2014). Therefore, in the specific case of milk, when the sample is hit by the incident radiation in the mid-infrared region ( $5,000$  to  $900\text{ cm}^{-1}$ ), the result is a plot called a spectrum which quantifies the amount of the energy transmitted at each specific wavenumber through the relation  $T = \frac{P}{P_0}$ , where  $T$  is the transmittance,  $P$  is the intensity of the incident radiation, and  $P_0$  is the intensity of the energy not absorbed by the molecules in the milk sample. Transmittance and absorbance are related through the formula  $A = \log_{10} \frac{1}{T}$ , where  $A$  is the absorbance (the amount of energy retained by the sample) and  $T$  is the transmittance. The Lambert-Beer law relates the absorbance to the concentration of a molecule as  $A = \epsilon bc$ , where  $\epsilon$  is a constant which is wavelength-dependent,  $b$  is the length of the path of the incident energy in the sample, and  $c$  is the concentration of the analyte in the sample. Mid-infrared spectroscopy is routinely used in milk quality labs to quantify the content of protein, casein, lactose, and fat (De Marchi et al., 2014). However, several studies have demonstrated the effectiveness of this technique in predicting other milk quality characteristics, including milk coagulation properties and minerals (De Marchi et al., 2014), but also animal features such as feed efficiency (McParland and Berry, 2016) and health and fertility of dairy cows (Bastin et al., 2016). Because of the strong collinearity between adjacent spectra wavelengths, ordinary least squares regression cannot be used to predict a particular trait using mid-infrared spectra because of the presence of singularities in the incidence matrix. The statistical technique normally used to develop the prediction models is called partial least squares regression. The aim of partial least squares regression is to maximize the covariance between the dependent variable and a new

set of orthogonal predictors called latent factors or principal components. The main difference with principal component calculated by principal component regression is that in partial least squares the latent factors are calculated by projecting both the response(s) and predictors to a new space, and not only the predictors.

## **SOURCES OF VARIATIONS OF MILK PROCESSING FEATURES**

The identification of the sources of variation of milk processing characteristics is important as they can support processors to manage more efficiently their product portfolio so that milk can be partitioned to different production plants based on its processability. Moreover, the quantification of exploitable genetic variation is the necessary process after which a breeding decision can be potentially proposed. Sources of variation and genetic parameters of milk processing characteristics have been studied mainly for milk coagulation properties and pH, and to a lesser extent for milk minerals content. Because of the amount of resources required to quantify milk processability on a large scale, all the studies in the literature are characterized by limited size of samples and animals involved.

### ***Non-genetic variation in milk processability***

Within and across lactation variability has been mainly studied for milk coagulation properties and pH (Ikonen et al., 2004; Vallas et al., 2010; Poulsen et al., 2013; Penasa et al., 2014). These studies concluded that milk coagulation properties tended to worsen when approaching the peak of milk production, and to improve only at the end of the lactation. Moreover, milk produced by pluriparous cows is less suitable for cheese production (i.e., it exhibits worse milk coagulation properties and higher pH) than milk from primiparous animals. The profile of milk minerals such as

Ca and P over the lactation has an opposite trend to that of milk yield (Carroll et al., 2006; Van Hulzen et al., 2009), whereby Ca and P content deteriorates with cow parity number (Kume et al., 1998).

### ***Genetic variation in milk processability***

Differences due to cow breeds are known to exist. High yielding dairy breeds, such as Holstein and Brown Swiss, are generally characterized by poorer milk coagulation properties (De Marchi et al., 2007; Poulsen et al., 2013), and lower milk minerals content (Carroll et al., 2006). Additive genetic variation has been estimated for milk coagulation properties and pH (Tyrisevä et al., 2004; Cassandro et al., 2008; Vallas et al., 2010; Poulsen et al., 2015) as well as milk minerals (Van Hulzen et al., 2009) using repeatability animal models. Such type of models assumes that the within-trait genetic correlation in each day in milk (DIM) is unity. However, for traits measured multiple times across a trajectory, it may be beneficial to use random regression animal models, as more precise breeding strategies can be implemented to genetically improve milk processability.

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## *Aim of the thesis*

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The general aim of the thesis was to develop prediction models using mid-infrared spectroscopy for milk processing features, and to quantify the sources of phenotypic and genetic variation in such traits.

The specific aims were:

1. to evaluate the accuracy of mid-infrared spectroscopy in predicting milk coagulation properties, CMS, HCT, and pH using a dataset of milk samples from multiple cow breeds fed predominantly a grazed grass diet (Ireland);
2. to evaluate the accuracy of mid-infrared spectroscopy in predicting major mineral contents using partial least squares regression coupled with uninformative variable elimination (Italy);
3. to identify the factors associated with milk coagulation properties, CMS, HCT, and pH, predicted by mid-infrared spectroscopy, in a large database of seasonal calving grass-based dairy cows (Ireland);
4. to identify factors associated with milk minerals predicted by mid-infrared spectroscopy in a large database of dairy and dual-purposes cattle breeds (Italy);
5. to quantify the genetic variation in milk coagulation properties, HCT, CMS, and pH, and their correlations with milk-related performance traits, using random regression models in grazing dairy cows (Ireland);
6. to quantify the genetic variation in milk minerals, and their correlations with milk-related performance traits, using random regression models in Italian Holstein-Friesian dairy cows (Italy).



**Prediction of bovine milk technological traits from mid-infrared spectroscopy  
analysis in dairy cows**

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## ABSTRACT

Rapid, cost-effective monitoring of milk technological traits is a significant challenge for dairy industries specialized in cheese manufacturing. The objective of the present study was to investigate the ability of mid-infrared spectroscopy to predict rennet coagulation time, curd firming time, curd firmness at 30 and 60 min after rennet addition, heat coagulation time, casein micelle size, and pH in cow milk samples, and to quantify associations between these milk technological traits and conventional milk quality traits. Samples ( $n = 713$ ) were collected weekly from 605 cows from multiple herds; the samples represented multiple breeds, stages of lactation, parities, and milking times. Reference analyses were undertaken in accordance with standardized methods, while mid-infrared spectra in the range of  $900\text{-}5,000\text{ cm}^{-1}$  were also available for all samples. Prediction models were developed using partial least square regression, and prediction accuracy was based on both cross and external validation. The proportion of variance explained by the prediction models in external validation was greatest for pH (71%), followed by rennet coagulation time (55%), and milk heat coagulation time (46%). Curd firmness 60 min from rennet addition and casein micelle size prediction models, however, were poor, explaining only 25% and 13%, respectively, of the total variance in each trait within external validation. On average, all prediction models tended to be unbiased ( $P > 0.05$ ). The linear regression coefficient of the reference value on the predicted value varied from 0.17 (casein micelle size regression model) to 0.83 (pH regression model) but were all different ( $P < 0.05$ ) from one. The ratio performance deviation of 1.07 (casein micelle size prediction model) to 1.79 (pH prediction model) for all prediction models in the external validation was less than two, suggesting that none of the prediction models could be used for analytical purposes. With the exception of casein

micelle size and curd firmness at 60 min after rennet addition, the developed prediction models may however be useful as a screening method, since the concordance correlation coefficient ranged from 0.63 (heat coagulation time prediction model) to 0.84 (pH prediction model) in the external validation.

**Keywords:** milk coagulation properties, milk heat stability, casein micelle size, milk acidity, grass

## INTRODUCTION

World dairy food production from cow milk has increased consistently by 2% annually (FAOSTAT, 2014). Rapid and low-cost tools to quantify milk quality processability can provide the dairy industry with a more effective monitoring strategy, to aid in segregating milk prior to manufacturing. Rennet coagulation time (**RCT**), curd firming time (**k<sub>20</sub>**) and curd firmness after 30 or 60 min from rennet addition (**a<sub>30</sub>** and **a<sub>60</sub>**, respectively) reflect milk coagulation properties, which are milk characteristics that describe the reactivity of milk after rennet addition. Shorter coagulation time and greater curd firming capacity are correlated with greater cheese yield and improved sensory properties (Aleandri et al., 1989; Martin et al., 1997; Pretto et al., 2013) as well as quality (Ng-Kwai-Hang et al., 1989; Malacarne et al., 2014). Other milk technological traits of interest for cheese production include casein micelle size (**CMS**), expressed as the diameter of the colloidal particles (Fox and Brodtkorb, 2008), heat coagulation time (**HCT**), defined as the time required to induce milk coagulation at a given temperature (Davies and White, 1966), and both pH and titratable acidity.

Mid-infrared spectroscopy (**MIRS**) is a rapid, low cost technique that measures the interaction between physical matter and electromagnetic radiation in the region between 900 and 5,000  $\text{cm}^{-1}$ . Mid-infrared spectroscopy is currently used to routinely quantify milk composition such as protein, fat, casein, and lactose in individual cow milk aliquots sampled at herd milk testing. Recent research has documented the ability of MIRS to predict novel milk quality traits (Soyeurt et al., 2011; De Marchi et al. 2014) and animal characteristics (McParland et al., 2011; De Marchi et al., 2014). The ability to predict milk technological traits with MIRS could be advantageous, since all individual cow milk samples are subjected to MIRS analysis and thus the marginal cost in predicting these new traits is negligible. Few studies, however, have reported the ability of MIRS to predict milk coagulation properties in dairy cows and these studies have been largely confined to Italian Holstein Friesian (Dal Zotto et al., 2008; De Marchi et al., 2013) and Brown Swiss (De Marchi et al., 2009) cows. To our knowledge, no study has attempted to predict either CMS or HCT from milk MIRS; only De Marchi et al. (2009) investigated the potential of MIRS to predict milk pH.

The objectives of the present study therefore were to (i) evaluate the accuracy of MIRS in predicting milk coagulation properties, CMS, HCT and pH using a dataset of milk samples from multiple cow breeds fed predominantly a grazed grass diet, and (ii) quantify the associations between these milk technological traits and traditional milk quality traits.



## MATERIALS AND METHODS

### *Data*

A total of 713 individual milk samples from 605 cows were collected weekly between August 2013 and August 2014. Samples originated from 7 different Irish research herds, and consisted of Holstein-Friesian, Jersey, Norwegian Red cows as well as their respective crosses. Milk samples were from different stages of lactation, different parities, and both morning and evening milking. Cows were on different experimental treatments based on a grazed-grass basal diet but were also, at times, supplemented with concentrate and grass silage. Milk aliquots (50 mL) were stored at 4 °C after sampling. Within 48 h of collection, samples were analyzed in the laboratory of Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork, Ireland) for milk chemical composition (protein, fat, casein, urea, total solids), determined using a MilkoScan FT6000 (Foss Electronic A/S, Hillerød, Denmark). The resulting spectrum, containing 1,060 transmittance data in the mid-infrared region between 900 and 5,000  $\text{cm}^{-1}$ , was stored. All samples were then preserved with Broad Spectrum Microtab II, containing 8 mg of Broponol and 0.3 mg of Natamycin (D&F Control System Inc., Norwood, MA, USA), and kept at refrigerator temperature for further analysis. For logistical reasons, not all technological milk quality parameters were quantified on every sample.

### *Gold standard analysis for milk coagulation properties*

Milk coagulation properties were quantified using a Formagraph (Foss Electronic A/S, Hillerød, Denmark) on the preserved individual samples within 5 d of collection. The Formagraph is a mechanical instrument, where an aliquot of milk, following inclusion of raw rennet diluted in distilled water, is brought into contact

with an oscillating loop pendulum. Initially, no force is transmitted from the uncoagulated milk to the pendulum. When coagulation begins, the viscosity of the milk increases, resulting in a force applied to the pendulum. The output of the Formagraph is a graph of a value of curd firmness against time. A 10 mL aliquot of milk was warmed to 35 °C. Subsequently, 1 mL of raw rennet (Chy-Max Plus, 190 International Milk Clotting Units/mL, Christian Hansen A/S, Denmark) was diluted in 20 mL of distilled water (1:20 vol/vol). The level of coagulant added to milk samples was adjusted based on protein concentration of the milk sample (O'Callaghan et al., 1999; 2001) and testing began immediately after rennet addition.

The measured traits were (i) RCT, defined as the number of minutes taken from rennet addition to the beginning of the coagulation, (ii)  $k_{20}$ , which is the time from the gel development to a width of 20 mm in the graph, and (iii)  $a_{30}$ , measured at the width of the graph after 30 minutes from enzyme addition. To facilitate the determination of RCT for non-coagulating milk samples (i.e., samples whose rennet coagulation time was longer than 30 min; Ikonen et al., 2004), the duration of the analysis was extended from 30 to 60 min, as suggested by De Marchi et al. (2013). Thus, curd firmness after 60 min from rennet addition ( $a_{60}$ , mm) was determined as the width of the graph at the end of the analysis for all the samples.

### ***Gold standard analysis for casein micelle size, heat stability, and pH***

The hydrodynamic diameter of the casein micelles was determined using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 25 °C using the Non-Invasive Backscatter optics at 173°. Samples were analysed within 24 h of collection and diluted with MilliQ® water prior to measurements (approximately 1 in 30), which is sufficient for dynamic light

scattering measurements (attenuator of 4 to 5). The cumulative method was used to determine the mean particle size (diameter) that corresponded to the mean of the volume distribution of the main peak. The viscosity of the solvent was assumed to be the same as water, given the low concentration of protein.

Heat coagulation time was tested using the method outlined by Davies and White (1966), within 48 h of samples collection and all the analyses were undertaken by the same operator. Briefly, an aliquot of 3.4 g of each milk sample was transferred into an individual glass tube suitable for the Elbanton BV (Kerkdriel, The Netherlands) hot oil bath. The oil temperature was set at 140 °C with an oscillator speed of 8 RMP. The heat coagulation time was recorded as the time when each sample started to flocculate based on visual assessment. The test time was set to 30 minutes (Davies and White, 1966); therefore samples that did not coagulate within this time period range (n = 15) did not provide any information about their heat susceptibility. Their inclusion in the calibration dataset was not feasible, because regression models of reference values on predicted values cannot be performed on observations with missing true values of HCT. Therefore, these samples were classified as heat non-coagulating and discarded from further statistical analysis. Repeatability information of the present methods are reported by Davies and White (1966).

Milk pH was assessed, within 24 h of samples collection, with a SevenCompact<sup>TM</sup> pH-meter S220 (Mettler Toledo AG, Switzerland).

### ***Development of the MIRS prediction models***

Development of the prediction models was undertaken using SAS software (ver. 9.3, SAS Institute Inc., Cary, NC, USA). Spectral data, expressed in transmittance, were transformed to absorbance by taking the  $\log_{10}$  of the reciprocal of

the transmittance. The prediction models were developed using only the spectra that did not include high noise level regions (Hewavitharana and Brakel, 1997) which are part of the spectra related to water absorption (Heuer et al., 2001). As a consequence, spectra regions between 1,580 and 1,710  $\text{cm}^{-1}$  and between 2,990 and 3,690  $\text{cm}^{-1}$  were discarded before the chemometric analysis. Principal component analysis (PROC PRIN COMP; SAS Institute Inc., Cary, NC, USA) was performed on the raw spectra, providing principal components which were used to detect similarities and differences among individual spectra, and to identify spectral outliers. The first 2 principal components explained 66.38% and 18.78% of the total spectral variation, respectively. Both individual sample Mahalanobis distances and a visual inspection of the plot of the first principal component against the second principal component did not indicate outliers and therefore no samples were discarded.

The distribution of each trait, as well as the identification of outlier reference values, was determined using PROC UNIVARIATE (SAS Institute Inc., Cary, NC, USA). If the distribution of a trait was non-normal, based on visual inspection and the Shapiro-Wilk test statistic, the reference values were transformed using a natural logarithm transformation. As a consequence, HCT and  $k_{20}$  were log-transformed. Outliers were defined as reference values  $> 3$  standard deviations from the mean. Based on this definition, 1 observation for RCT, 2 observations for log-transformed HCT, 6 observations for log-transformed  $k_{20}$  and pH, and 34 observations for CMS were identified as outliers and subsequently discarded. The relative high number of outliers values for CMS existed mainly for two reasons: i) error in laboratory processing ( $n = 17$ ), and ii) CMS values deviating more than three standard deviations from the mean ( $n = 17$ ). Further examination of these data points did not indicate any

trend in date of measurement and characteristics (e.g., parity, breed, stage of lactation) of the cows that contributed the samples.

Partial least square regression (PROC PLS; SAS Institute Inc., Cary, NC, USA) was undertaken to generate the prediction models. Before performing the analysis, the initial dataset was divided randomly in two different subsets for each trait separately, representing a model calibration dataset (80% of the entire dataset) and a validation dataset (the remaining 20% of the entire dataset). The calibration dataset was used to generate the prediction models. The validation dataset was considered as an independent dataset, since the samples were not used to calibrate the prediction models. Developed prediction models were applied to the validation dataset to quantify their predictive ability. The mean, standard deviation and range of the milk technological traits in the validation and calibration dataset were similar. Mathematical pre-treatments (Savitzky-Golay 1<sup>st</sup> and 2<sup>nd</sup> derivative) were applied to the raw spectra but no improvement on the prediction models accuracy was detected; therefore the untreated spectra were used to generate the models.

The optimal number of partial least square factors was defined as the minimum number of factors to achieve the lowest root mean predicted residual sum of squares. The goodness-of-fit statistics considered in the present study were the coefficient of determination in the cross-validation (i.e., one-at-a-time cross-validation) and external validation ( $R^2_C$  and  $R^2_V$ , respectively) datasets and the standard error of prediction in the cross-validation and external validation ( $SEP_C$  and  $SEP_V$ , respectively) datasets. To evaluate the practical utility of the prediction models, the ratio performance deviation (**RPD**) and concordance correlation coefficient (**CCC**) were calculated. The RPD was calculated as the ratio of the

standard deviation of the trait and the standard error of prediction from the prediction model (Williams, 2007). The CCC was calculated as (Lin, 1989):

$$CCC = \frac{2 * COV(\hat{Y}; Y)}{\sigma_{\hat{Y}}^2 + \sigma_Y^2 + (\mu_Y - \mu_{\hat{Y}})^2}$$

Where  $COV(\hat{Y}; Y)$  is the covariance between the reference ( $Y$ ) and predicted values ( $\hat{Y}$ ),  $\sigma_Y^2$  is the variance of the reference values,  $\sigma_{\hat{Y}}^2$  is the variance of the predicted values, and  $\mu_Y$  and  $\mu_{\hat{Y}}$  represent the mean of the reference and predicted values, respectively. Bias for each prediction model was calculated as the average of the difference between the reference value and the respective predicted value for each sample; a t-test was used to determine if this was significantly different from zero. Reference values for each prediction model were also linearly regressed on the predicted values to obtain the linear regression coefficient; a t-test was used to determine if the linear regression coefficient was different from one.

Pearson correlations were calculated between both reference and predicted values for the technological traits and other measures of milk quality traits (i.e. protein, casein, fat, total solids, and urea concentration).

## RESULTS

### *Summary statistics of reference traits*

The DIM when milk samples were taken varied from 5 to 375; 19.27% of milk samples ( $n = 138$ ) were from the first 60 DIM while 50% (i.e.,  $n = 356$ ) were from between 61 and 200 DIM. Milk samples were available from first to eleventh parity cows. Morning milking samples represented almost 70% of the total samples collected ( $n = 497$ ). A total of 146 samples were from two herds with autumn calving

cows, whose diet was partially grass-silage based; the remainder of data originated from six herds with spring-calving cows that were fed predominantly grazed grass.

The most represented breed was Holstein-Friesian (n = 443), followed by Jersey (n = 109) and Norwegian Red (n = 17); 144 samples were from crossbreed cows. Non-coagulating milk samples represented 15.71% of the total dataset (n = 88), of which the majority (n = 62) were from Holstein-Friesian cows.

Descriptive statistics of the milk technological traits are in Table 1. The coefficient of variation of all traits, with the exception of pH, varied from 18.13 (CMS) to 70.76% (HCT); the coefficient of variation for pH was only 1.18%. The Shapiro-Wilk value for both  $k_{20}$  and HCT suggested that neither trait was normally distributed but, following transformation with the natural logarithm, the Shapiro-Wilk value increased from 0.87 to 0.97 for  $k_{20}$ , and from 0.87 to 0.98 for HCT. This transformation reduced the coefficient of variation from 64.26 to 37.50% for  $k_{20}$  and transformed  $k_{20}$ , respectively, and from 70.76 to 35.59% for HCT and transformed HCT, respectively.

**Table 1.** Number of samples (n), mean, standard deviation (SD), range, coefficient of variation (CV), and Shapiro-Wilk value (W) for normality distribution test of reference values for rennet coagulation time (RCT), curd-firming time ( $k_{20}$ ), curd firmness ( $a_{30}$ ,  $a_{60}$ ), heat coagulation time (HCT), casein micelle size (CMS), and pH.

Trait	N	Mean	SD	Range	CV	W
RCT, min	560	20.70	8.93	47.25	43.15	0.98
$k_{20}$ , min	523	6.12	3.93	18.50	64.26	0.87
$k_{20}$ , $\log_e$ min	523	1.63	0.61	2.76	37.50	0.97
$a_{30}$ , mm	467	32.17	15.61	72.88	48.54	0.98
$a_{60}$ , mm	559	31.54	12.23	70.68	38.79	0.95
HCT, min	492	8.75	6.19	27.02	70.76	0.87
HCT, $\log_e$ min	492	1.94	0.69	3.16	35.59	0.98
CMS, nm	654	175.36	31.79	231.08	18.13	0.98
pH	702	6.68	0.11	0.66	1.63	0.99

### ***Prediction model accuracy***

The goodness-of-fit statistics for MIRS prediction models are summarized in Table 2. The number of factors included in the prediction models varied from 14 (HCT and CMS) to 17 (pH), with the exception of the  $a_{60}$  prediction model where 7 factors were used. The most accurate prediction model was for pH ( $R^2_C$  and  $SEP_C$  of 0.73 and 0.06, respectively), followed by RCT ( $R^2_C$  and  $SEP_C$  of 0.61 and 5.64 min, respectively). The accuracy of predicting  $a_{60}$  and CMS was poor, with a respective  $R^2_C$  of 0.26 and 0.23, and  $SEP_C$  of 10.33 mm and 28.11 nm. The RPD values in external validation ranged from 1.07 (CMS) to 1.79 (pH), while the CCC in the external validation dataset was between 0.28 (CMS) and 0.84 (pH). The mean bias of prediction (i.e., the average of the difference between the gold standard and predicted



values for each individual sample) was not different ( $P > 0.05$ ) from zero in external validation. The slope of the linear regression of the reference on the predicted values of each technological trait in the external validation varied from 0.17 (CMS) to 0.83 (pH); all were different from unity ( $P < 0.05$ ).

Model predictive ability was also evaluated on back transformed values for both HCT and CMS. The model fit statistics on the back transformed predicted HCT and CMS were less accurate than the fit statistics using natural logarithm transformed HCT and CMS. For example, the coefficient of determination in external validation was 0.29 for HCT and 0.43 for  $k_{20}$ , whereas the  $SEP_v$  was 3.01 min for  $k_{20}$  and 5.64 for HCT. The slope of the linear regression coefficient of the original (i.e., untransformed) reference values on the back-transformed predicted values for both models was different from unity ( $P < 0.05$ ), while the bias was different from zero only for HCT (-1.31 min). Neither prediction model was useful for analytic purposes (RPD of 1.17 and 1.32 for HCT and  $k_{20}$ , respectively) and the models were characterized as “moderate predicting ability” (CCC of 0.49 and 0.59 for HCT and  $k_{20}$ , respectively).

**Table 2.** Fitting statistics<sup>1</sup> of prediction models in cross and external validation for rennet coagulation time (RCT), curd firming time ( $k_{20}$ ), curd firmness ( $a_{30}$ ,  $a_{60}$ ), heat coagulation time (HCT), casein micelle size (CMS), and pH. Prediction models and results for curd firming time and heat coagulation time are the natural logarithm values.

Trait	Cross Validation						External Validation						
	n	#L	SEP <sub>C</sub>	R <sup>2</sup> <sub>C</sub>	RPD	CCC	N	Bias	Slope (SE)	SEP <sub>V</sub>	R <sup>2</sup> <sub>V</sub>	RPD	CCC
RCT, min	450	16	5.64	0.61	1.59	0.76	110	-0.10	0.55 (0.05)	5.85	0.55	1.49	0.71
$k_{20}$ , log <sub>e</sub> min	414	15	0.39	0.59	1.56	0.74	109	-0.08	0.52 (0.05)	0.42	0.51	1.43	0.67
$a_{30}$ , mm	378	15	11.32	0.50	1.41	0.66	89	-0.85	0.53 (0.06)	10.38	0.46	1.35	0.66
$a_{60}$ , mm	458	7	10.33	0.26	1.16	0.41	101	1.23	0.23 (0.04)	11.49	0.25	1.15	0.37
HCT, log <sub>e</sub> min	389	14	0.46	0.55	1.48	0.71	103	0.04	0.47 (0.05)	0.51	0.46	1.36	0.63
CMS, nm	535	14	28.11	0.23	1.14	0.37	119	0.75	0.17 (0.04)	28.75	0.13	1.07	0.28
pH	553	17	0.06	0.73	1.92	0.84	149	0.00	0.83 (0.04)	0.06	0.71	1.79	0.84

<sup>1</sup> n = number of samples; #L = number of model factors; SEP<sub>C</sub> = standard error of prediction in cross validation; R<sup>2</sup><sub>C</sub> = coefficient of determination in cross validation; RPD = ratio performance deviation; CCC = concordance correlation coefficient; Bias = average difference between the reference value and the respective predicted value; Slope = linear regression coefficient of reference values on predicted reference values; SEP<sub>V</sub> = standard error in external validation; R<sup>2</sup><sub>V</sub> = coefficient of determination in external validation.

### ***Correlations with milk composition traits***

Pearson correlations between technological traits and milk composition traits are in Table 3. Correlations between the reference technological traits and the milk chemical composition traits were similar to the correlations between the predicted traits and the milk chemical composition traits. In general, the greater the accuracy of the MIRS prediction model, the more comparable the correlations with the milk composition traits for either the reference or predicted technological traits. Rennet coagulation time had a strong positive correlation with  $k_{20}$  (correlations of 0.77 and 0.86 for the reference and MIRS-predicted values, respectively) and was strongly negatively correlated (-0.73 to -0.82 for the reference and MIRS-predicted values, respectively) with  $a_{30}$ . Rennet coagulation time was associated with milk composition, particularly with protein and casein concentrations. Correlations between RCT and protein were -0.46 (reference RCT and protein %) and -0.50 (MIRS-predicted RCT and protein %), while correlations between RCT and casein were -0.44 (reference RCT and casein %) and -0.49 (MIRS-predicted RCT and casein %). Milk coagulation properties were weakly correlated with the other milk technological traits, with the exception of RCT and pH (correlations between 0.63 and 0.74 for either the reference or MIRS-predicted values). Casein micelle size was not associated with milk composition; correlations ranged from -0.01 to 0.08, and were not different from zero ( $P > 0.05$ ). Weak correlation existed between reference CMS and reference RCT (0.23). Predicted CMS was weakly correlated with predicted  $a_{60}$  (0.31), predicted pH (-0.07), and milk protein (0.20) and casein (0.15) levels. Heat coagulation time had the strongest correlations with milk urea (0.48) and protein (0.22) concentration. Heat coagulation time was also weakly associated with milk coagulation properties, with correlations ranging from -0.22 ( $k_{20}$ ) to 0.34 ( $a_{30}$ ).

**Table 3.** Pearson correlations<sup>1</sup> between reference (below diagonal) and MIRS-predicted (above diagonal) rennet coagulation time (RCT), curd-firming time ( $k_{20}$ ), curd firmness ( $a_{30}$ ,  $a_{60}$ ), heat coagulation time (HCT), casein micelle size (CMS), pH, and MIRS-predicted milk protein (PRT), fat, total solids (TS), urea, and casein (CN) concentration. Correlations within both curd-firming time and HCT are based on back-transformed values.

	RCT	$k_{20}$	$a_{30}$	$a_{60}$	HCT	CMS	pH	PRT	Fat	TS	Urea	CN
RCT	-	0.86	-0.82	-0.40	-0.32	0.09	0.74	-0.50	-0.46	-0.42	-0.49	-0.49
$k_{20}$	0.77	-	-0.90	-0.55	-0.38	-0.05	0.67	-0.64	-0.47	-0.46	-0.51	-0.65
$a_{30}$	-0.73	-0.79	-	0.62	0.46	0.09	-0.60	0.62	0.38	0.37	0.50	0.62
$a_{60}$	-0.29	-0.38	0.50	-	0.65	0.31	-0.19	0.60	0.01	0.04	0.55	0.58
HCT	-0.21	-0.22	0.34	0.28	-	0.02	-0.12	0.29	0.00	-0.02	0.55	0.23
CMS	0.23	0.21	-0.07	-0.01	-0.11	-	-0.07	0.20	0.02	0.04	-0.02	0.15
pH	0.63	0.51	-0.39	-0.09	-0.09	0.04	-	-0.41	-0.49	-0.47	-0.33	-0.42
PRT	-0.46	-0.54	0.52	0.36	0.22	0.08	-0.34	-	0.42	0.54	0.42	0.90
Fat	-0.36	-0.35	0.27	0.01	0.01	0.01	-0.43	0.42	-	0.93	0.12	0.44
TS	-0.33	-0.35	0.28	0.05	-0.01	0.02	-0.40	0.54	0.93	-	0.08	0.60
Urea	-0.38	-0.37	0.38	0.27	0.48	-0.01	-0.31	0.41	0.11	0.07	-	0.40
CN	-0.44	-0.56	0.53	0.37	0.18	0.05	-0.36	0.90	0.44	0.60	0.39	-

<sup>1</sup> Correlations <|0.11| were not different from zero ( $P > 0.05$ ).

## DISCUSSION

Fundamental to the successful development of an accurate prediction model, is the incorporation of extensive variability in the calibration dataset, representative of the population in which the model will be used (De Marchi et al., 2014). In the present study this was achieved. The comparison of the results of the present study with previous international studies is, nonetheless, difficult due to differences in production system as well as potential differences in methodologies of analysis (i.e., different gold standard methods). Previously documented coefficients of variation for milk coagulation properties in international populations of between 37 (RCT) to 45% ( $a_{30}$ ) (Ikonen et al., 2004; Cassandro et al. 2008) were lesser than observed in the multi-breed population of cows used in the present study. Moreover, compared to the present study, less variation in milk coagulation properties was detected by O'Brien et al. (1999) over a 12-month study period of Irish herd bulk milk samples. Few studies (Auld et al., 2004; De Marchi et al., 2013) have considered  $a_{60}$  in their analysis since normally during cheese manufacture, curd is cut after 30 minutes from rennet addition (Cassandro et al., 2008). Auld et al. (2004), studying coagulation ability of New Zealand Holstein and Jersey cows, and De Marchi et al. (2013), studying coagulation ability of Italian Holstein-Friesian cows, reported a coefficient of variation for  $a_{60}$  of 10 and 62%, respectively. The variability in casein micelle size and pH documented by Chen et al. (2014) using milk samples from 550 lactating Holstein cows in the UK, was low, yet comparable to that observed in the present study. On average, HCT values of the present study was consistent with Holt et al. (1978) based on bulk milk samples over a period of 15 months in south-west Scotland; moreover, as expected results from our study had greater variability for this trait. Irish Holstein-Friesian was the breed with the greatest prevalence of non-

coagulating milk samples (13.99 %). Such proportion was determined also by Ikonen et al. (2004) on Finnish Ayrshire and by Poulsen et al. (2013) on Swedish Red, although these last authors defined “non-coagulating” milk samples that did not coagulate within 60 minutes. High prevalence of non-coagulating milk samples is a serious concern and results in a negative impact on dairy industry profitability.

### ***Prediction model practical utility***

For a prediction model to be considered useful for analytic purposes, an RPD value of greater than two is desired (Williams, 2007). No prediction model in the present study had an RPD in external validation that surpassed this threshold. Nonetheless, the CCC of the prediction equations in external validation demonstrated that some models could at least be used for screening purposes. Concordance correlation coefficient of between 0.21 and 0.40 indicates fair predictive ability, between 0.41 to 0.60 indicates moderate predictive ability, between 0.61 and 0.80 indicates substantial predictive ability, and between 0.81 and 1.00 indicates accurate predictive ability (Lin, 1989; De Marchi et al., 2014). Of the traits investigated in the present study, the pH prediction model (CCC = 0.84) had “accurate predictive ability”, while the prediction models for RCT, transformed  $k_{20}$ ,  $a_{30}$ , and transformed HCT were characterized by “substantial predictive ability” (CCC of 0.71, 0.67, 0.66, and 0.63 for RCT,  $k_{20}$ ,  $a_{30}$ , and HCT, respectively). Poor predictive ability existed for CMS and  $a_{60}$ . No prediction model in the present study, on average, significantly over-estimated or under-estimated the reference values for any of the traits investigated. Although not necessarily important for breeding purposes, significant bias in estimation could impact milk price, if a milk pricing system was based on milk technological traits. To our knowledge, this is the first study that attempted to quantify

this bias in coagulation as well as acidity traits. The lesser than unity linear regression coefficient of the reference value on the predicted value suggested a re-scaling in variance between the reference and predicted values, and therefore the extent of the difference between reference samples is not being fully reflected in differences in their predicted value. This could have implications for breeding programs, for example, where the true variance in milk coagulation properties may actually be underestimated using predicted rather than reference values; thus the breeding goal trait should be the gold standard trait with its respective variance and the MIR-predictor trait included in the selection criterion. Nonetheless, if the number of records in the calibration dataset used to develop the prediction models, as well as the variability in the reference values, could be increased, then possibly the prediction accuracy of the models may also improve.

### ***Prediction of rennet coagulation traits***

Although previous studies have already highlighted the effectiveness of MIRS in predicting milk coagulation properties (Dal Zotto et al., 2008; De Marchi et al. 2009), only De Marchi et al. (2013) included non-coagulating milk samples in the calibration dataset and used the same instrument (i.e., Formagraph) as used in the present study. The identification of non-coagulating samples could be an extremely useful tool, since extended RCT has repercussions on the profitability and efficiency of the dairy industry. Indeed, milk characterized by poor reactivity to rennet addition increases the time of the entire cheese-making process. For instance, the implementation of MIRS prediction models at the dairy processor level could be advantageous to segregate milk for use in either cheese production (good reactivity to

rennet addition, i.e. short RCT) or fluid consumption (poor reactivity to rennet addition, i.e. long RCT).

To our knowledge, no study has investigated the ability of MIRS to predict milk coagulation properties in a grazing production system, as exist in Ireland, New Zealand, Southern Australia, and elsewhere. Nevertheless, comparison with other studies is difficult do to different reference procedures adopted. In fact, even if the reference instrument for measuring milk coagulation properties was the Formagraph, as exists both in the present study and that of De Marchi et al. (2013), the methodology used in the present study considered an adjustment of the level of added rennet based on the protein concentration of each individual milk sample. Therefore, the amount of coagulant was not the same for samples with different protein levels. In contrast, De Marchi et al. (2013) kept the quantity of rennet at a constant level (200  $\mu\text{L}/10\text{ mL}$  of milk sample) for determining milk coagulation properties. Moreover, previous research undertaken in Italian herds of Holstein and Brown Swiss cows (Dal Zotto et al., 2008; De Marchi et al., 2009) used the Computerized Renneting Meter (Polo Trade, Monselice, Italy) as the reference analysis instrument. As described by Pretto et al. (2011), differences in gold standard methodologies have to be considered when comparing the results for milk coagulation properties, since different instruments and methodologies can yield different milk coagulation properties values on the same samples.

Overall, prediction models in the present study were more accurate than reported by Dal Zotto et al. (2008) and De Marchi et al. (2009), but less accurate than reported by De Marchi et al. (2013). In particular, Dal Zotto et al. (2008), who attempted to evaluate the effectiveness of milk MIRS to predict milk coagulation properties using 83 samples collected from Italian Holstein Friesian cows, obtained



$R^2_C$  ranging between 0.29 and 0.31 for RCT and  $a_{30}$ , respectively. De Marchi et al. (2009), using 1,200 Brown Swiss milk samples from 37 Italian herds, obtained  $R^2_C$  of 0.62 and 0.37 for RCT and  $a_{30}$ , respectively. Finally, based on milk samples from 335 Italian Holstein Friesian cows, De Marchi et al. (2013) developed robust MIRS prediction models for RCT ( $R^2_C = 0.76$ ),  $k_{20}$  ( $R^2_C = 0.72$ ) and  $a_{30}$  ( $R^2_C = 0.70$ ). These prediction models are currently used in several milk laboratories in Italy to routinely provide milk coagulation phenotypes for genetic evaluations (Tiezzi et al., 2013; Penasa et al., 2014).

The prediction of some innovative milk traits is quite difficult, especially when these traits are not strictly related to the mid-infrared energy absorption of specific chemical functional bounds (i.e., N-H bounds in protein or C-H bounds in lipids). This is the case of milk coagulation traits, where several milk constituents affect the MIRS predictions. As reported by De Marchi et al. (2009), loadings variation across spectra demonstrated that several parts of the spectra contributed to the MIRS prediction models. Indeed, specific peaks across spectra, related to mid-infrared energy absorption of protein as well as lipid chemical bonds were observed both in the present study and in De Marchi et al. (2009). The difficulty of MIRS in predicting the technological traits considered in the present study is also confirmed by the optimum number of models factors included in the partial least squares prediction model, which were greater in the prediction models for milk coagulation properties compared to other milk quality traits (i.e., casein fractions, fatty acids composition) (De Marchi et al., 2014).

### ***Prediction of HCT, CMS, and pH***

The present study is the first, to our knowledge, to quantify the effectiveness of MIRS to predict both HCT and CMS. The observed predictive ability of HCT in the present study is very promising, despite the subjective nature of the HCT reference measurement. Heat coagulation time is of great importance for the dairy industry since all milk intended for human consumption is subjected to a heat treatment, and milk with high heat susceptibility (i.e., low HCT) is not suitable for milk processability, especially for the production of milk powder (i.e. mechanical obstruction of machinery for milk powder). Anyway, MIRS was demonstrated to be effective in predicting  $\beta$ -lactoglobulin genotypes (Rutten et al., 2011), and  $\beta$ -lactoglobulin A was demonstrated to be more susceptible to heat treatments (Hill et al., 1997). The predictive ability of CMS was poor and unsatisfactory; only 23 and 13% of the total variance in CMS was explained by the prediction model in cross and external validation, respectively. Although the repeatability of dynamic light scattering measurements with the reference instrument used in the present study was high (error  $\pm 2\%$ , Malvern, 2014), the poor predictive ability of CMS prediction could be due to a limitation of the MIRS instrumentation in detecting small particles dimensions. These results need further investigation in an independent population.

Milk acidity is usually measured as pH and titratable acidity (expressed as Soxhlet-Henkel degrees) in dairy factory and it affects the aggregation rate of paracasein micelles, the reactivity of rennet, and the rate of syneresis (Toffanin et al., 2015). With the exclusion of hyper acidity milk types, great milk acidity milk is considered more favorable for cheese making; indeed the relationship between milk acidity and coagulation traits have been stressed in several studies (Cassandro et al., 2008; Pretto et al., 2013). Even if the measurement of pH during the cheese-making

process is quite easy, the possibility to predict milk pH on individual cow milk samples using MIRS, combined with the existence of potential genetic variation in this characteristic (Ikonen et al., 2004), could facilitate genetic improvement at the population level. Results from our study demonstrated that MIRS could be used to predict milk pH and the accuracy of prediction in the present study was greater than the only other study that evaluated the potential of milk MIRS to predict milk pH (De Marchi et al., 2009) ( $R^2_c$  of 0.59 vs. 0.73).

### ***Correlations between the technological traits and milk composition***

The similarity in correlations with milk composition traits irrespective of whether the reference values or predicted values of the technological traits was considered is somewhat expected, given the relatively good MIRS prediction accuracy of these traits. Milk technological traits were correlated with several traditional milk traits, particularly protein, casein, and urea concentration. Previous research (Ikonen et al., 2004; Cassandro et al., 2008) evaluating both phenotypic and genetic correlations between coagulation traits and milk chemical composition reported weak correlations between RCT with both protein and casein levels. Strong correlations between milk coagulation traits and protein and casein contents were, nonetheless, expected in the present study, considering the rennet correction on protein concentration was used for the determination of milk coagulation traits. Therefore, comparison with previous literature is not completely correct because of differences in the gold standard methods used; this was supported by O'Callaghan et al. (1999) who demonstrated that different concentrations of coagulant yielded different RCT values.

The present study demonstrated that, of all milk constituents, the nitrogen fraction (i.e., protein and casein concentrations) had the strongest correlation with

RCT. Such a conclusion was expected since casein is the only milk constituent susceptible to rennet addition. Therefore, curd formation happens because of the aggregation of paracasein micelles, which subsequently, depending on their rate of aggregation, can incorporate other milk constituents, such as milk fat.

Lower milk pH values were associated with more favorable milk coagulation properties which is consistent with several previous studies (Ikonen et al., 2004; Cassandro et al., 2008) that considered different measurements of acidity (e.g. pH, titratable acidity). The lack of a correlation between CMS and protein is in direct contrast with both Chen et al. (2014), who analyzed 550 individual milk samples for Holstein cows in UK. These authors, however, had different laboratory methodologies both for the determination of protein concentration as well as for the determination of CSM.

The positive correlation between RCT and  $k_{20}$ , and the negative correlation between RCT and  $a_{30}$  were expected because if coagulation begins within a short time period, the paracasein micelles have more time available to aggregate and to create a stronger curd, when measurement of curd firmness is detected at 30 minutes from rennet addition. In the same way, milk samples with short RCT achieved 20 mm of curd firmness ( $k_{20}$ ) earlier relative to those that were characterized by poor reactivity to rennet addition (i.e. long RCT). The positive correlation between CMS and RCT is because major mineral constituents (i.e. calcium and phosphorus concentration) influence both CMS and RCT (Chen et al. 2014; Gustavsson et al., 2014).

## **CONCLUSIONS**

Results from the present study demonstrated that MIRS, combined with PLS regression on untreated spectra, could be a useful screening tool to acquire new and

innovative milk technological phenotypes rapidly and at a low cost. Such advancements could be important for the dairy industry to better monitor milk quality prior to processing, and to discriminate more accurately milk more adapted for cheese manufacturing whether for direct human consumption, or stocking (milk powder production). MIRS-predicted technological quality traits could be an important source of routine information on a very large population of animals from which to generate estimated breeding values for milk technological traits for exploitation in breeding programs. More effort, however, should be made to expand the numerosity in the calibration dataset to increase the robustness of the prediction models.

### **ACKNOWLEDGEMENTS**

Funding from EU project LowInputBreed and Breed Quality, and laboratory technical supports by Bernard Corrigan, Jim Flynn, and Paula O'Connor are gratefully acknowledged.

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**Predictive ability of mid-infrared spectroscopy for major mineral composition  
and coagulation traits of bovine milk by using the uninformative variable  
selection algorithm**

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## ABSTRACT

Milk minerals and coagulation properties are important for both consumers and processors, and they might aid in increasing milk added value. However, large scale monitoring of these traits is hampered by expensive and time-consuming reference analyses. The objective of the present study was to develop prediction models for major mineral contents (Ca, K, Mg, Na, and P) and milk coagulation properties (MCP: rennet coagulation time, curd-firming time, and curd firmness) using mid-infrared spectroscopy. Individual milk samples (n = 923) of Holstein-Friesian, Brown Swiss, Alpine Grey, and Simmental cows were collected from single-breed herds between January and December 2014. Reference analysis for the determination of both mineral contents and MCP was undertaken with standardized methods. For each milk sample, the mid-infrared spectrum in the range from 900 to 5,000  $\text{cm}^{-1}$  was stored. Prediction models were calibrated using partial least squares regression coupled with a wavenumber selection technique called uninformative variable elimination, in order to improve models accuracy, and validated both internally and externally. The average reduction of wavenumbers used in partial least squares regression was 80%, which was accompanied by an average increment of 20% of the explained variance in external validation. The proportion of explained variance in external validation was about 70% for P, K, Ca, and Mg, and it was lower (40%) for Na. Milk coagulation properties prediction models explained between 54% (rennet coagulation time) and 56% (curd-firming time) of the total variance in external validation. The ratio of standard deviation of each trait to the respective root mean square error of prediction, which is an indicator of the predictive ability of an equation, suggested that the developed models might be effective for screening and collection of milk minerals and coagulation properties at the population level.

Although prediction equations were not accurate enough to be proposed for analytic purposes, mid-infrared spectroscopy predictions could be evaluated as phenotypic information to genetically improve milk minerals and MCP on a large scale.

**Keywords:** mid-infrared spectroscopy, dairy cattle, milk mineral, milk coagulation property

## INTRODUCTION

Milk quality is crucial to maximize milk added value and it contributes to increase the profitability of the entire dairy chain. Traditional quality traits have been mainly referred to milk chemical composition, particularly protein and fat quantity and concentration, as confirmed by selection indices of several cattle breeds worldwide (Miglior et al., 2005). However, the concept of milk quality is often interpreted differently by processors and consumers. For example, under a processor point of view, adequate milk quality is translated into adequate processing ability, whereas the consumer's perception of milk quality deals more with health aspects.

Minerals represent a relatively small part of cow milk, close to 10 g/L, and they are divided into two categories, based on their concentration (Cashman, 2006): macro minerals (normally expressed in mg/kg) and trace elements (normally expressed in µg/kg). Macro minerals include calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), and phosphorus (P), which are important for the homeostasis of both infant and adult people. Indeed, Ca and Mg are involved in bones and teeth health, and in muscular and cardiac contractility (Cashman, 2006; Haug et al., 2007). A deficiency of these minerals is associated with osteoporosis and muscular disorders, and Ca deficiency in the diet might be partially responsible of a

greater incidence of hypertension, colon cancer and obesity (Huth et al., 2006). Potassium is known as the most important intracellular cation, playing a fundamental role in the maintenance of homeostasis (Young et al., 1995; He and MacGregor, 2008). However, K is found also as extracellular element and it participates to the transmission of nervous impulses, muscle contraction, and regulation of blood pressure (He and MacGregor, 2008). In particular, an increase of dietary K, coupled with a reduction of dietary Na, limits the hypertension risk (Whelton and He, 2014). Recently, Uribarri and Calvo (2014) claimed that, although considered an essential nutrient, high dietary P intake is a risk factor for bone and cardiovascular diseases. Besides health aspects, Ca and P are essential components of casein micelles and thus they are directly involved in milk coagulation process. Malacarne et al. (2014) reported that high content of inorganic P affects positively casein micelle reactivity to rennet.

Milk coagulation properties (**MCP**), namely rennet coagulation time (**RCT**, min), curd-firming time (**k<sub>20</sub>**, min), and curd firmness (**a<sub>30</sub>**, mm), are currently used to measure milk quality during the cheese-making process, and they are measured by several laboratories for breeding purposes and milk quality payment systems (Tiezzi et al., 2013; Penasa et al., 2015). A number of studies demonstrated that milk characterized by short RCT and firm curd results in greater cheese yield and thus it increases the efficiency of the entire cheese-making process (Comin et al., 2005; Malacarne et al., 2006; Pretto et al., 2013).

Reference methods normally used to measure milk mineral composition and coagulation traits are expensive and time-consuming. Mid-infrared spectroscopy (**MIRS**) is a rapid, non-destructive, and cost-effective laboratory technique that allows the (a posteriori) prediction of innovative phenotypes from milk samples (De

Marchi et al., 2014; McParland and Berry, 2016), but the prediction of both MCP (De Marchi et al., 2013; Visentin et al., 2015) and mineral contents (Soyeurt et al., 2009; Toffanin et al., 2015) are still a big challenge. To improve the accuracy of MIRS prediction models two paths should be considered: i) to increase the accuracy of reference methods, and ii) to use different statistical approaches coupled with multivariate analyses, including partial least squares (**PLS**) regression. Recently, Gottardo et al. (2015) demonstrated that uninformative variable elimination (**UVE**) can increase the prediction accuracy of MIRS models, by reducing the number of uninformative spectral regions. This process is extremely advantageous when models have to be applied subsequently to large spectral dataset for the prediction of novel phenotypes, since a lower number of spectral wavelengths used for PLS regression reduces the computational time. Therefore, the aim of the present study was to develop MIRS prediction models for major mineral contents and MCP using PLS coupled with UVE for the application of these prediction models on spectral data.

## **MATERIALS AND METHODS**

### ***Data***

***Samples Collection.*** From January to December 2014, 923 individual cow milk samples were collected in 60 single-breed herds. This dataset, subsequently used to develop MIRS prediction models, comprised the 4 major cattle breeds reared in the Alpine area of Bolzano province (Italy), where all animals were sampled. Cow breeds considered in the present study were Holstein-Friesian (**HF**, n = 237), Brown Swiss (**BS**, n = 223), Alpine Grey (**AG**, n = 223), and Simmental (**SI**, n = 240). The sampling protocol aimed at covering as much biological variability as possible, for both MCP and major mineral composition. For each cow, two 50 mL aliquots were

collected, immediately added with preservative (Bronysolv; ANA.LI.TIK Austria, Vienna, Austria) and kept at refrigerating temperature.

***Milk Chemical Composition and Spectra Determination.*** Both aliquots were transferred to the laboratory of the South Tirol Dairy Association (Bolzano, Italy) and one aliquot was processed the same day of sampling according to the International Committee for Animal Recording (ICAR, 2014) recommendations. For each milk sample, both traditional milk quality traits (pH and contents of protein, casein, fat, lactose, and urea) and MIRS spectra were determined using a MilkoScan FT+ (Foss Electric A/S, Hillerød, Denmark). Each individual spectral information, containing 1,060 infrared transmittance data in the region between 900 and 5,000  $\text{cm}^{-1}$ , was stored. Somatic cell count was measured using Fossomatic (Foss Electric A/S, Hillerød, Denmark) and then converted to SCS through the formula  $\text{SCS} = 3 + \log_2(\text{SCC}/100,000)$ . The other 50 mL aliquot was transferred (within 24 h from collection) at refrigerating temperature to the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padova (Legnaro, Italy). This aliquot was subsequently split in two sub-aliquots: one was stored at  $-20^\circ\text{C}$  and the other one was delivered the same day to the laboratory of the Breeders Association of Veneto Region (ARAV, Padova, Italy).

***Reference Analysis of Major Mineral Contents.*** Milk content of Ca, K, Mg, Na, and P was determined on 251 milk samples in the laboratory of DAFNAE (Legnaro, Italy) and each of the 4 cattle breeds (HF, BS, AG, and SI) was represented in equal proportion. In the present study it was chosen to perform a mineralization of milk samples prior to their quantification, as recommended by Soyeurt et al. (2009). A nitroperchloric mineralization by MILESTONE START D microwave (1,200 watt, Milestone Srl Sorisole, Bergamo, Italy) was used. The microwave contained a SK-10



rotor at high pressure (64 bar) and control systems with temperature probe and software. Milk samples stored at -20°C were thawed in water at 35°C and homogenized before sampling. A total of 2.5 g of sample was introduced in Teflon vessels at high pressure, and 2 mL of H<sub>2</sub>O<sub>2</sub> 30% and 7 mL of HNO<sub>3</sub> 67% were added. The vessel was hermetically sealed and placed in the microwave. The process of mineralization involved three steps: i) the sample was heated to 200°C in 15 min; ii) the sample was kept at 200°C for 18 min; and iii) the sample was cooled down to 35°C. The mineralized sample was added with demineralized water to reach a final volume of 25 mL.

Content of Ca, K, Mg, Na, and P was quantified using Inductively Coupled Plasma Optical Emission Spectrometry (**ICP-OES**), Ciros Vision EOP (SPECTRO Analytical Instruments GmbH, Kleve, Germany). This method was employed to determine Ca at wavelength 315.887 nm, K at wavelength 766.491 nm, Mg at wavelength 279.079 nm, Na at wavelength 589.592 nm, and P at wavelength 178.287 nm. Instrument operating parameters were optimized for acid solution and calibration standards were matched with nitric acid 5% “suprapure” grade. The elements to be determined were added from single element solutions (Inorganic Ventures, Christiansburg, VA, USA). The concentration range of the calibration solutions was between 0 and 100 mg/kg for all elements. The accuracy and precision of this method were investigated by analyzing the certified reference material BCR<sup>®</sup> - 063R “Skim milk powder” [(Institute for Reference Materials and Measurements (IRMM), Geel, Belgium)]. These standards of “Skim milk powder” were mineralized according to the protocol previously described. The measured and the certified values were in excellent agreement for all the elements ( $R^2$  of calibration curves for all minerals > 0.99).

**Reference Analysis of Milk Coagulation Properties.** Reference analysis was assessed through the Formagraph (Foss Electric A/S, Hillerød, Denmark), using the method proposed by McMahon and Brown (1982), in the laboratory of ARAV (Padova, Italy) within 1 d from samples arrival. A 10 mL aliquot of each preserved milk sample was heated to 35°C and then added with 200 µL of a rennet solution (Hansen Standard 160; Pacovis Amrein AG, Bern, Switzerland) diluted to 1.6% (w/v) in distilled water. At the start of the analysis, an oscillating loop pendulum is brought into contact with the milk; when the coagulation begins, the viscosity of milk increases and so a force is back-transmitted to the loop. The output of the Formagraph is therefore a graph of curd firmness against time. Measured traits were: i) RCT, the time required, from the beginning of the test, to induce milk coagulation; ii)  $k_{20}$ , the time from RCT to the achievement of a 20 mm width in the graph; and iii)  $a_{30}$ , the width of the graph at the end of the test, which lasted 30 min. Milk samples that did not coagulate within the testing time (about 12% of total samples) were classified as non-coagulating and labeled as missing values.

### **Chemometric Analysis**

**Data Editing.** The normality of each trait and the identification of any possible reference outliers were ascertained both through visual inspection of the histogram/density plot and by carrying out the Shapiro-Wilk normality test in R software (R Development Core Team, 2015). Traits that are not normally distributed are characterized by low and statistically significant ( $P < 0.05$ ) Shapiro-Wilk value, and they require normalization. In the present study, both milk minerals and MCP were normally distributed, and thus no data transformation was needed. Reference outliers were defined as samples whose values deviated more than 3 SD from the

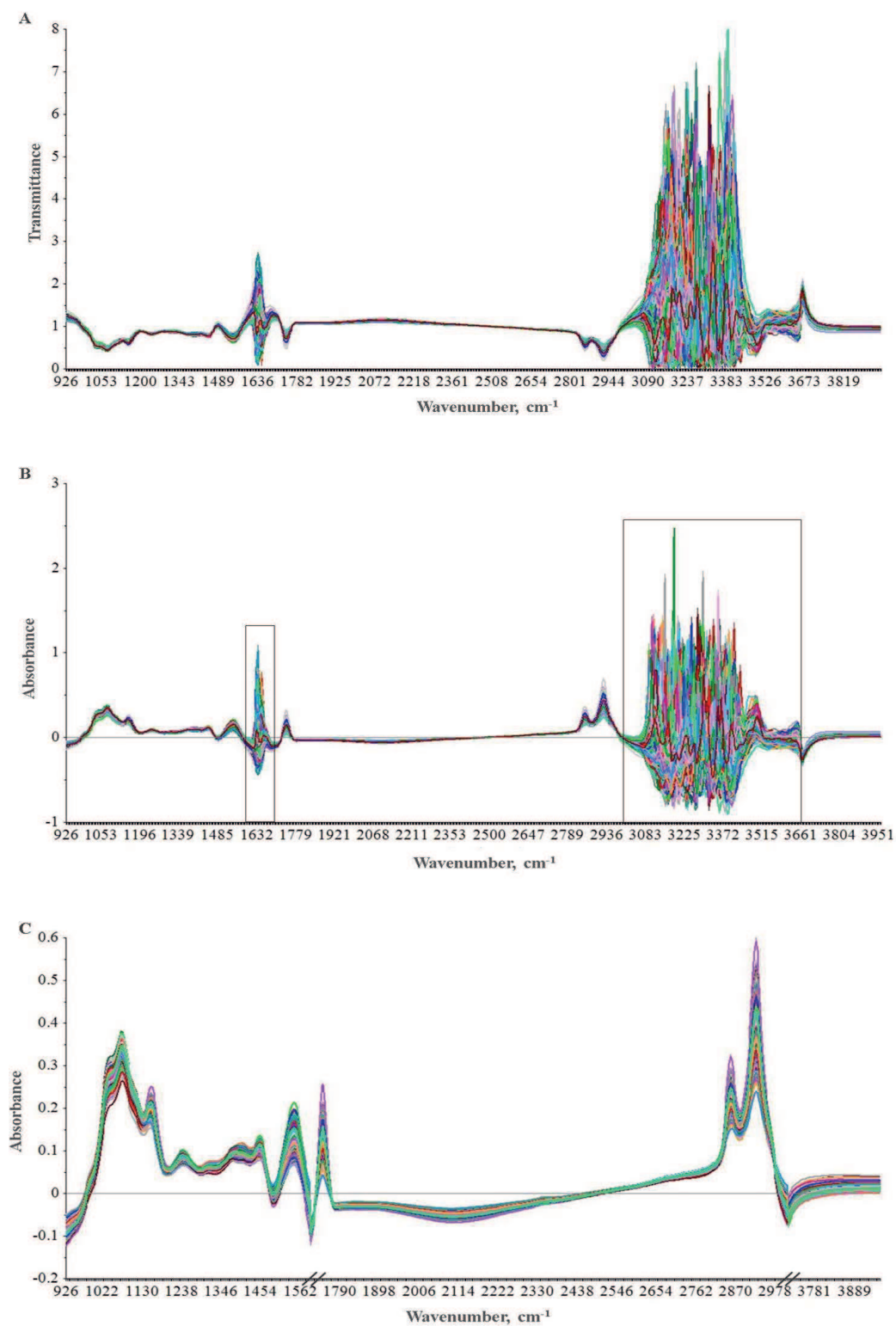
mean of each trait (McDermott et al., 2016a). Based on this definition, none observations for  $a_{30}$  and K, 1 observation for RCT,  $k_{20}$ , and Mg, 2 observations for Na and P, and 3 observations for Ca were discarded prior to the development of prediction models.

Spectral data expressed in transmittance were transformed to absorbance by taking the  $\log_{10}$  of the reciprocal of the transmittance (McDermott et al., 2016b; Figure 1). Two high noise level spectral regions related to water absorption (1,600 to 1,700  $\text{cm}^{-1}$ , and 3,040 to 3,660  $\text{cm}^{-1}$ ) were discarded prior to multivariate analysis (Hewavitharana and van Brakel, 1997; Figure 1). Principal component analysis was carried on raw spectral data to identify similarities and differences between spectra, in order to detect any possible spectral outlier. Principal component analysis is a method of data compression which produces a new matrix of uncorrelated variables called principal components; each principal component captures in a descending order as much of the variation of the initial variables (i.e. spectral wavenumbers) as possible. First and second principal component explained 71.09% and 16.01% of total spectral variation, respectively. The scores plot of first versus second principal component did not highlight any specific spectrum outlier, and this was also confirmed by computing the robust Mahalanobis distance for each sample. Therefore, all spectra were retained for further statistical analysis.

**Prediction models.** In order to develop and to externally validate the prediction models, for each trait of interest the dataset was divided randomly into two subsets, namely a calibration set (80% of the observations) and a validation set (20% of the observations). The prediction models included each milk mineral and MCP as dependent variable and the edited milk spectra wavenumbers as predictor variables. The calibration set was used to develop the MIRS models through PLS and UVE

combined with PLS regression on the edited spectra, and these models were subsequently applied to the validation set. The validation set was used to assess the predictive ability of each prediction model, since the samples included were not considered to build the models. Leave-one-out cross validation was also performed in the calibration set. This process was repeated 1,000 times for the development of each prediction model, and it was performed both when equations were built through PLS regression on edited spectra and when equations were built using UVE combined with PLS regression (**UVE-PLS**) on edited spectra. Partial least squares regression was carried out using the package ‘Chemometrics with R’ (Wehrens, 2011), and UVE-PLS was tested using a homemade script in R software (R Development Core Team, 2015), following the approach of Gottardo et al. (2015). Moreover, PLS regression was carried out on spectra expressed in first derivative using a Savitzky-Golay filter with a linear polynomial and a window size of 3 points, but no improvement of predictive accuracy of equations was detected compared with PLS regression performed on untreated spectra. The optimal number of PLS factors (**#L**) to perform PLS regression was defined as the minimum number of factors to achieve the lowest root mean square error of cross validation (**RMSE<sub>CV</sub>**). Goodness-of-fit statistics considered were the coefficient of determination of cross validation (**R<sup>2</sup><sub>CV</sub>**) and of external validation (**R<sup>2</sup><sub>EV</sub>**), the **RMSE<sub>CV</sub>**, the root mean square error of external validation (**RMSE<sub>EV</sub>**), and the ratio of prediction to deviation (**RPD**), which was calculated as the ratio of the SD of the trait to the **RMSE<sub>EV</sub>**, and it was used to test the practical utility of the prediction models. In particular, RPD values greater than 2 are desired for practical application of the prediction model (Williams, 2007). Results represent the average goodness-of-fit statistics of the 1,000 analyses.

**Figure 1.** Plot of 923 spectra expressed in (A) transmittance, (B) absorbance with high noise regions included in box, and (C) absorbance after elimination of high noise regions.



## RESULTS

### *Summary Statistics of Calibration Dataset*

Descriptive statistics of milk yield, chemical composition predicted by MilkoScan FT+ (Foss Electric A/S, Hillerød, Denmark), reference mineral contents, and reference MCP of samples after removal of outlier reference values are reported in Table 1. Milk yield averaged 23.20 kg/d, mean percentages of fat, protein, and casein were 4.07, 3.60, and 2.81, respectively, and SCS averaged 2.82. The most abundant milk mineral was K (1,500.52 mg/kg), followed by Ca (1,348.22 mg/kg) and P (1,010.04 mg/kg). Magnesium content averaged 128.30 mg/kg, and it was the less concentrated in milk. Means of RCT,  $k_{20}$ , and  $a_{30}$  were 18.57 min, 5.20 min, and 28.07 mm, respectively. Minerals were characterized by appreciable variability, with CV ranging between 15.20% (K) and 23.05% (Na). Milk coagulation properties exhibited greater variability compared to milk chemical composition and mineral contents, with CV of 24.84%, 31.30%, and 44.07% for RCT,  $k_{20}$ , and  $a_{30}$ , respectively.

**Table 1.** Descriptive statistics of milk mineral composition and coagulation properties used for the development of prediction models.

Trait <sup>1</sup>	Mean	SD	Range	CV, %
Milk yield, kg/d	23.20	8.42	51.20	36.29
<b>Chemical composition</b>				
Fat, %	4.07	0.72	7.61	17.69
Protein, %	3.60	0.45	3.16	12.50
Casein, %	2.81	0.36	2.67	12.81
SCS	2.82	1.84	12.44	65.25
<b>Mineral composition, mg/kg</b>				
Ca	1,348.22	229.60	1,281.52	17.03
K	1,500.52	228.04	1,108.58	15.20
Mg	128.30	22.10	112.71	17.23
Na	399.18	91.87	448.54	23.05
P	1,010.04	181.04	1,010.95	17.92
<b>Coagulation traits</b>				
RCT, min	18.57	4.61	24.70	24.84
k <sub>20</sub> , min	5.20	1.63	11.15	31.30
a <sub>30</sub> , mm	28.07	12.37	54.72	44.07

<sup>1</sup>SCS = somatic cell score; RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness 30 min after rennet addition.

### *Accuracy of Prediction Models*

Goodness-of-fit statistics of cross validation and external validation for MIRS prediction models are summarized in Table 2 and Table 3, respectively. The optimal #L selected by PLS regression was 15 for Ca, K, and Na, and it was 20 for Mg, P, RCT,  $k_{20}$ , and  $a_{30}$ . The  $R^2_{CV}$  (Table 2) of models for milk minerals developed using only PLS regression ranged from 0.29 (Na;  $RMSE_{CV}$  of 90.03 mg/kg) and 0.62 (P,  $RMSE_{CV}$  of 109.77 mg/kg). The  $R^2_{CV}$  ( $RMSE_{CV}$ ) of models for RCT,  $k_{20}$ , and  $a_{30}$  developed using only PLS regression was 0.46 (3.38 min), 0.46 (1.18 min), and 0.48 (9.26 mm), respectively. In external validation (Table 3),  $R^2_{EV}$  of milk minerals varied from 0.25 (Na) to 0.60 (P), and for MCP it ranged from 0.43 (RCT) to 0.46 ( $a_{30}$ ).

Uninformative variable elimination reduced the number of wavenumbers used by the software to compute PLS regression and both  $RMSE_{CV}$  and  $RMSE_{EV}$ , and increased  $R^2_{CV}$  and  $R^2_{EV}$  for all prediction models (Table 2 and Table 3). The greatest increment of explained variance in external validation was observed for Na prediction, with  $R^2_{EV}$  that improved from 0.25 to 0.40 (+60%; Table 3), followed by  $k_{20}$  prediction, whose explained variance in external validation increased by 27.3% ( $R^2_{EV}$  from 0.44 to 0.56). For the other traits, the increment of explained variance in external validation was between 13.0 ( $a_{30}$ ) and 25.6% (RCT) (Table 3). The UVE procedure reduced by at least 80% the number of wavenumbers used in PLS regression, with the only exception of Na (60.9%) and P (75.9%) prediction models (Table 2 and Table 3).

The RPD was calculated only when models were generated by UVE-PLS, and in external validation it ranged from 1.31 (Na) to 2.05 (P) for milk minerals, and from 1.34 ( $k_{20}$ ) to 1.59 (RCT) for MCP (Table 3).



**Table 2.** Average fitting statistics<sup>1</sup> of prediction models for milk mineral composition and milk coagulation properties in leave-one-out cross validation obtained from partial least squares (PLS) regression only and from PLS after uninformative variable elimination (UVE-PLS) procedure.

Trait <sup>2</sup>	#L	PLS			UVE-PLS			RPD
		N <sub>1</sub>	RMSE <sub>CV</sub>	R <sup>2</sup> <sub>CV</sub> (SD)	N <sub>2</sub>	RMSE <sub>CV</sub>	R <sup>2</sup> <sub>CV</sub> (SD)	
Mineral composition, mg/kg								
Ca	15	873	146.20	0.60 (0.08)	113	120.00	0.68 (0.06)	1.91
K	15	873	139.11	0.60 (0.05)	173	120.00	0.69 (0.05)	1.90
Mg	20	873	15.62	0.61 (0.05)	93	12.30	0.65 (0.05)	1.80
Na	15	873	90.03	0.29 (0.06)	341	68.80	0.42 (0.08)	1.34
P	20	873	109.77	0.62 (0.07)	210	83.90	0.71 (0.08)	2.16
Coagulation traits								
RCT, min	20	873	3.38	0.46 (0.03)	163	2.86	0.55 (0.03)	1.61
k <sub>20</sub> , min	20	873	1.18	0.46 (0.03)	144	1.00	0.59 (0.03)	1.63
a <sub>30</sub> , mm	20	873	9.26	0.48 (0.05)	110	8.43	0.56 (0.05)	1.47

<sup>1</sup>#L = number of model PLS factors; N<sub>1</sub> = number of wavenumbers in PLS regression; RMSE<sub>CV</sub> = root mean square error in cross validation; R<sup>2</sup><sub>CV</sub> = coefficient of determination in cross validation; N<sub>2</sub> = number of wavenumbers in PLS regression after UVE procedure; RPD = ratio of prediction to deviation.

<sup>2</sup>RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness 30 min after rennet addition.

**Table 3.** Average fitting statistics<sup>1</sup> of prediction models for milk mineral composition and milk coagulation properties in external validation obtained from partial least squares (PLS) regression only and from PLS after uninformative variable elimination (UVE-PLS) procedure.

Trait <sup>2</sup>	#L	PLS			UVE-PLS			RPD
		N <sub>1</sub>	RMSE <sub>EV</sub>	R <sup>2</sup> <sub>EV</sub> (SD)	N <sub>2</sub>	RMSE <sub>EV</sub>	R <sup>2</sup> <sub>EV</sub> (SD)	
Mineral composition, mg/kg								
Ca	15	873	153.02	0.58 (0.08)	113	122.00	0.67 (0.06)	1.88
K	15	873	144.23	0.57 (0.08)	173	120.00	0.69 (0.08)	1.90
Mg	20	873	18.22	0.57 (0.06)	93	12.50	0.65 (0.05)	1.77
Na	15	873	91.76	0.25 (0.10)	341	70.00	0.40 (0.08)	1.31
P	20	873	115.43	0.60 (0.08)	210	88.12	0.68 (0.05)	2.05
Coagulation traits								
RCT, min	20	873	4.00	0.43 (0.05)	163	2.90	0.54 (0.05)	1.59
k <sub>20</sub> , min	20	873	1.30	0.44 (0.06)	144	1.22	0.56 (0.06)	1.34
a <sub>30</sub> , mm	20	873	9.30	0.46 (0.03)	110	9.00	0.52 (0.03)	1.37

<sup>1</sup>#L = number of model PLS factors; N<sub>1</sub> = number of wavenumbers in PLS regression; RMSE<sub>EV</sub> = root mean square error in external validation; R<sup>2</sup><sub>EV</sub> = coefficient of determination in external validation; N<sub>2</sub> = number of wavenumbers in PLS regression after UVE procedure; RPD = ratio of prediction to deviation.

<sup>2</sup>RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness 30 min after rennet addition.

## DISCUSSION

### *Data Variation*

Mid-infrared spectroscopy prediction models require representative variability in the calibration dataset. Regarding milk minerals variation, CV for Ca and P of the present study (17.03 and 17.92%, respectively) was greater than CV reported by Toffanin et al. (2015) for the same minerals (11.28 and 11.74%, respectively). However, Toffanin et al. (2015) focused their study only on Italian HF cows sampled in a short period (3 mo). On the other hand, Soyeurt et al. (2009) developed MIRS prediction models on a multi-breed dataset (n = 100) of HF, Jersey, Belgian Blue, Montbéliarde, and Normande cattle breeds over a 1-year sampling, and CV for Ca, K, Mg, Na, and P were comparable to those reported in the current research. Concerning MCP, data variability was similar to that reported by Cassandro et al. (2008) on a large-scale study of 1,071 Holstein-Friesian cows (CV of 27 and 35% for RCT and  $a_{30}$ , respectively).

### *Variable Selection and Accuracy of Prediction Models*

Spectral data are characterized by multicollinearity: indeed, some wavenumbers can be rewritten as a linear function of others. Partial least squares regression compresses the initial spectral matrix by creating another matrix containing a smaller amount of variables called PLS factors. Subsequently, these factors are used in the regression equation, and the less relevant and unstable information of the initial spectral matrix is discarded (Næs et al., 2002). Theoretically, there would be no need for variables selection, since PLS regression already ignores non-informative wavenumbers. However, a large number of wavenumbers and a small number of

samples could still have a negative impact on the final accuracy of the developed prediction model, and this is the main reason to use variable selection techniques. There are several variable selection methods in the literature, and they have been recently reviewed by Mehmood et al. (2012). Uninformative variable elimination was firstly introduced by Centner et al. (1996), and it basically creates a stability criterion for each variable; if the criterion is below a specific threshold, this indicates that the specific wavenumber is not essential for the development of the prediction model. By discarding these wavenumbers, the accuracy of prediction models is expected to increase. Recently, UVE has been applied by Gottardo et al. (2015) to 208 milk spectra to develop MIRS prediction models for Ca and titratable acidity. Those authors reported a substantial improvement of the  $R^2_{EV}$ , from 0.46 to 0.55 and from 0.72 to 0.80 for Ca and titratable acidity, respectively. Moreover, Niero et al. (2016) applied UVE method for the prediction of casein and whey fractions, and they obtained an increment of  $R^2_{CV}$  from 0.83 to 0.88, 0.36 to 0.60, and 0.66 to 0.74 for  $\alpha$ -CN,  $\beta$ -CN, and  $\kappa$ -CN models, respectively. The  $R^2_{CV}$  of whey fractions models increased from 0.31 to 0.37 and from 0.31 to 0.47 for  $\alpha$ -LA and  $\beta$ -LG, respectively.

To our knowledge only two studies have investigated the potential use of MIRS to predict milk mineral content, but none of them used UVE-PLS procedure for the development of calibration models. Soyeurt et al. (2009) developed calibration models for the same milk minerals investigated in the present study, and they reported greater  $R^2_{CV}$  for Ca (0.87), Na (0.65), and P (0.85), similar  $R^2_{CV}$  for Mg (0.65), and lower  $R^2_{CV}$  for K (0.36), compared with findings of the current study. Toffanin et al. (2015) developed calibration equations for Ca and P using 208 milk samples of HF cows through PLS regression, and they obtained  $R^2_{CV}$  of 0.56 and 0.70 for Ca and P, respectively, which were lower than those obtained in the present study. Only Soyeurt

et al. (2009) quantified the predictive ability of MIRS models on an independent dataset, and they reported  $R^2_{EV}$  of 0.97, 0.14, and 0.88 for Ca, Na, and P, respectively.

The prediction of MCP by MIRS has been reported in both Italian BS (De Marchi et al., 2009) and HF (De Marchi et al., 2013) cows, and by Visentin et al. (2015) in a multi-breed dataset of Irish cattle (HF, Jersey, and Norwegian Red) reared in a grazing dairy system. Results from the present research were comparable with those of Visentin et al. (2015), in terms of fitting statistics of both internal and external validation, but less accurate than those of De Marchi et al. (2013), who obtained  $R^2_{CV}$  of 0.76, 0.72, and 0.70 for RCT,  $k_{20}$ , and  $a_{30}$ , respectively. Nevertheless, the study of De Marchi et al. (2013) was carried out using a quite different reference analysis; indeed, even if they used a Formagraph instrument, they extent the time of the analysis until 60 min to allow the detection of non-coagulating milk samples (i.e. samples that did not coagulate within 30 min). The wide range of variation in the reference dataset improved the overall accuracy of the prediction models.

## CONCLUSIONS

Mid-infrared spectroscopy has been used to develop calibration models for milk minerals and coagulation properties. Models explained moderate variance of studied traits and thus they were not accurate enough to be proposed for analytic purposes. However, these models could represent a valid tool for a quick and cost-effective screening and/or acquirement of phenotypes at population level, and they could be applied for research purposes to spectral data for analysis. Finally, the use of UVE-PLS approach has validated to be an interesting approach to improve the accuracy of prediction models. Future research will investigate the feasibility of using

mid-infrared predictions as indicator traits to genetically improve milk minerals and MCP.

### **ACKNOWLEDGEMENTS**

The authors would like to thank Luis Kerschbaumer and Thomas Kerschbamer (Sennereiverband Südtirol, Bolzano, Italy), Christian Plitzner (Vereinigung der Südtiroler Tierzuchtverbände, Bolzano, Italy), Claudio Stecchi (CTS informatica srl, Bolzano, Italy), and Luciano Magro, Massimo Cagnin, and Martina Isaia (Department of Agronomy, Food, Natural Resources, Animals and Environment, University of Padova) for technical support.

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**Factors associated with milk processing characteristics predicted by mid-infrared spectroscopy in a large database of dairy cows**

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## ABSTRACT

Despite milk processing characteristics being important quality traits, little is known about the factors underlying their variability, due primarily to the resources required to measure these characteristics in a sufficiently large population. Cow milk coagulation properties, namely rennet coagulation time, curd-firming time, and curd firmness 30 and 60 min after rennet addition, heat coagulation time, casein micelle size, and pH were generated from available mid-infrared spectroscopy prediction models. The prediction models were applied to 136,807 spectra collected from 9,824 Irish dairy cows from research and commercial herds. Sources of variation were investigated using linear mixed models which included the fixed effects of calendar month of test, milking time in the day, linear regressions on the proportion of Friesian, Jersey, Montbeliarde, Norwegian Red, and “Other” breeds in the cow, coefficient of heterosis, coefficient of recombination loss, parity, stage of lactation, and the two-way interaction parity-by-stage of lactation. Within and across parity cow effects, contemporary group, and a residual term were also included as random effects in the model. Supplementary analyses considered the inclusion of either test-day milk yield or milk protein concentration as fixed effects covariates in the multiple regression models. Milk coagulation properties were most favorable (i.e. short rennet coagulation time and strong curd firmness) for cheese manufacturing in early lactation, concurrent with the lowest values of both pH and casein micelle size. Milk coagulation properties and pH deteriorated in mid-lactation but improved towards the end of lactation. In direct contrast, heat coagulation time was more favorable in mid-lactation and less suitable (i.e. short heat coagulation time) for high temperature treatments in both early and late lactation. Relative to multiparous cows, primiparous cows, on average, yielded milk with shorter rennet coagulation time and higher heat coagulation time.

Milk from the evening milking session had shorter rennet coagulation time and greater curd firmness, as well as lower heat coagulation time and lower pH compared to milk from the morning session. Jersey cows, on average, yielded milk more suitable for cheese production rather than for milk powder production. When protein concentration was included in the model, the improvement of milk coagulation properties toward the end of lactation was no longer apparent. Results from the present study may aid in decision-making for milk manufacturing, especially in countries characterized by a seasonal supply of fresh milk.

**Key words:** milk processability, seasonal variation, milk quality, lactation

## INTRODUCTION

Milk processing characteristics are indicators of both the potential and ease of transforming raw milk into dairy products, including value-added products (i.e., cheese and butter) as well as products with extended shelf-life (UHT milk and milk powder). Several quality traits contribute to milk processing characteristics, such as rennet coagulation time (**RCT**), curd-firming time (**k<sub>20</sub>**), curd firmness 30 or 60 min after rennet addition to milk (**a<sub>30</sub>** and **a<sub>60</sub>**, respectively), heat coagulation time (**HCT**), casein micelle size (**CMS**), and pH. Rennet coagulation time, **k<sub>20</sub>**, **a<sub>30</sub>**, and **a<sub>60</sub>** are generally referred to as milk coagulation properties. Short to medium milk rennet reactivity, as well as greater curd firming capacity, are desirable attributes for cheese-making and are associated with greater cheese yield (Aleandri et al., 1989; Formaggioni et al., 2008; Pretto et al., 2013). Heat coagulation time, which depicts milk protein stability when subjected to heat treatment, has a fundamental role in extending milk shelf-life (Sikand et al., 2010). Smaller casein micelles are preferable

when transforming milk into both cheese (Glantz et al., 2010) and milk powder (Chen et al., 2014), and lower pH is generally desired for processing milk into cheese (Pretto et al., 2013) but not into milk powder (Singh, 2004).

Within and across lactation variability among dairy cows has been documented for both milk coagulation properties and pH (Ikonen et al., 2004; Vallas et al., 2010; Penasa et al., 2014). Milk coagulation properties and pH in mid-lactation tend to be least favorable for cheese-making (Ikonen et al., 2004; Vallas et al., 2010), and both milk coagulation properties and pH also deteriorate with cow parity (Penasa et al., 2014). Breed differences for milk coagulation properties have been documented in dairy cows (De Marchi et al., 2007; Poulsen et al., 2013). Both De Marchi et al. (2007) and Poulsen et al. (2013) reported that milk produced from higher-yielding dairy breeds had, on average, reduced coagulation ability. Few studies have quantified the association between dairy cow breed and both HCT (McLean et al., 1987; Barłowska et al., 2014) and CMS (Devold et al., 2000; Auldist et al., 2002) and these studies have generally been limited in population size. Because of the high resource requirements to accurately quantify milk processing characteristics, only a few studies (Ikonen et al., 2004; Vallas et al., 2010; Poulsen et al., 2013), which have generally been limited to milk coagulation properties, have attempted to identify contributing factors to the variability in milk coagulation properties using a large population of cows. Contributing factors to variability in milk coagulating characteristics has been investigated in 4,664 Finnish Ayrshire dairy cows (Ikonen et al., 2004), in 4,191 primiparous Estonian Holstein (Vallas et al., 2010), and in 456 Danish Holstein, 436 Danish Jersey and 407 Swedish Red lactating cows (Poulsen et al., 2013).

The objective of the present study was to identify the factors associated with a range of milk processing characteristics, predicted by mid-infrared spectroscopy

analysis, in a large database of seasonal calving grass-based dairy cows. Factors identified in the present study will be useful in a decision support tool for the dairy industry to predict and manage their product portfolio influenced by milk processability.

## **MATERIALS AND METHODS**

### ***Data***

A total of 174,062 milk samples spectra collected between January 2013 and December 2015 from 10,394 dairy cows in 76 Irish herds were available. Of the 76 farms, seven were research farms operated by Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork, Ireland). Cows (n = 1,661) in these research herds participated in various experimental treatments based on the evaluation of different sward varieties and feeding strategies, different stocking rates, different calving periods, and different lengths of grazing period. A total of 2,956 lactation records were available from these cows. Almost all the animals were fed a basal diet of grazed pasture and were sporadically supplemented with a small quantity of concentrates as per the experimental treatment. Animals were milked twice a day, at 0700 hr (**AM**) and 1500 hr (**PM**), and milk yield was recorded at each milking. Individual milk samples were taken separately, once weekly, on consecutive PM and AM milking. On average, 78 milk samples per cow were available from the research herds.

The remaining 69 herds in the dataset were commercial herds operating in South-West Ireland. Animals (n = 8,733) were milked twice a day, and a combined individual cow AM+PM milk composite sample was collected (approximately 1,249 samples per month) and sent for mid-infrared spectroscopy analysis as part of an on-

going research study. Milk yield of the commercial herds represented the entire daily yield obtained from the day of milk testing. The average number of collected milk samples per cow was 5.15 and a total of 14,873 lactation records were available.

Samples were collected from cows generally calving between January and April, inclusive; however a small proportion (1.81%) of samples originated from a research farm where calving between September and November, inclusive, was adopted. Milk samples from all 76 herds were kept at refrigerating temperature and analyzed within a week of collection using the same MilkoScan FT6000 (Foss Electronic A/S, Hillerød, Denmark) in the laboratory of Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork, Ireland). Milk chemical composition (concentration of protein, casein, fat, lactose, total solids, and urea) was derived for all milk samples and SCC was determined using a Fossomatic (Foss Electronic A/S, Hillerød, Denmark). Moreover, for each milk sample, spectral information containing 1,060 mid-infrared transmittance data in the region between 900 and 5,000  $\text{cm}^{-1}$  was captured and stored.

### ***Development of Prediction Models for Milk Technological Traits***

Details of the selection of milk samples, measurement of the reference values, and the development of the prediction equations for the milk processing characteristics are described by Visentin et al. (2015). Briefly, during the years 2013 and 2014, a calibration dataset was generated using individual bovine milk samples collected from the seven Irish research herds also contributing to the data in the present study. Milk coagulation properties were determined on 560 samples using a Formagraph (Foss Electronic A/S, Hillerød, Denmark). The output of the Formagraph is a graph of curd firmness against time where RCT represents the time required to



induce milk coagulation after rennet addition,  $k_{20}$  is the time between the gel development and the achievement of a width of 20 mm in the graph, and  $a_{30}$  and  $a_{60}$  are the width of the graph after 30 and 60 min from rennet addition, respectively.

Milk HCT was measured on 509 samples using a hot oil bath (Elbanton BV, Kerkdriel, The Netherlands), by setting the temperature to 140 °C at an oscillating speed of 8 rpm. Heat coagulation time was recorded when, at visual inspection, milk started to flocculate.

Casein micelle size, which represents the average diameter of casein micelles of a milk sample, was measured on 688 samples using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurement was carried out at 25 °C using the noninvasive backscatter optics at 173°. Milk pH was recorded on 708 samples with a SevenCompact pH meter S220 (Mettler Toledo AG, Greifensee, Switzerland).

Prediction models were developed for each trait separately using partial least squares regression (PROC PLS, SAS Institute Inc., Cary, NC) as detailed by Visentin et al. (2015). The accuracy of the prediction models is summarized in Table 1.

### ***Generation of Predictions from Spectral Data***

For all 174,062 milk samples collected in the present study, spectral data were transformed from transmittance to absorbance values by taking the logarithm to the base 10 of the reciprocal of the transmittance value. Two spectral wavelength regions (1,580-1,710  $\text{cm}^{-1}$  and 2,990-3,690  $\text{cm}^{-1}$ ), characterized by low signal-to-noise ratio and associated with water absorption (Hewavitharana and Brakel, 1997), were discarded prior to statistical analysis. Subsequently, principal component analysis

(PROC PRINCOMP, SAS Institute Inc., Cary, NC, USA) was undertaken on the milk spectra for outlier detection.

To identify only the spectra similar to those used to develop the prediction models, the Mahalanobis distance from the centroid (i.e., the mean of first, second, third, and fourth principal component) of the cluster of samples included in the calibration dataset was calculated as the sum of squares of the centered and scaled scores of the first 4 principal components (Brereton, 2015). Outlier spectra from the entire dataset was subsequently defined as samples with a Mahalanobis distance from the centroid of the calibration dataset >97.5% percentile of a  $\chi^2$  distribution with  $p$  degrees of freedom. Following the removal of outliers, 157,192 spectra (i.e., 90% of the initial dataset) from 10,112 dairy cows were retained (Figure S1 and Figure S2). The prediction models were applied to these spectra to predict RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, and pH.

### ***Data Editing***

Obvious data errors for predicted RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, and pH and the milk production traits (i.e., milk yield, SCC, and concentrations of protein, fat, casein, lactose, total solids, and urea) were discarded. Somatic cell score was calculated as  $\log_{10}$  of SCC to normalize the distribution. Only data between 5 and 305 DIM for the first ten parities were retained. Records were discarded from the database if greater than 3 standard deviations from the mean of the respective trait.

Cow breed composition was recorded in the national database and, for the purpose of the present study, was defined as the proportion of Holstein, Friesian, Jersey, Montbeliarde, Norwegian Red, and “Other”. Contemporary group of experimental treatment by test-date was defined for milk samples from cows in

research herds, whereas contemporary group in the commercial herds was defined as herd-test-date. Contemporary groups with less than 10 observations were discarded. Following editing of the data, 136,807 milk samples from 16,543 lactations from 9,824 dairy cows were available for further analyses.

Coefficients of heterosis and recombination loss were calculated for each cow using the following formulae (VanRaden and Sanders, 2003):

$$\text{Heterosis} = 1 - \sum_{i=1}^n \text{sire}_i * \text{dam}_i$$

and

$$\text{Recombination loss} = 1 - \sum_{i=1}^n \frac{\text{sire}_i^2 + \text{dam}_i^2}{2},$$

where  $\text{sire}_i$  and  $\text{dam}_i$  are the proportion of genes of the breed  $i$  in the sire and the dam, respectively.

### ***Statistical Analyses***

Pearson correlation coefficients (PROC CORR, SAS Institute Inc., Cary, NC, USA) were computed between the milk composition and milk processing traits. Factors associated with each milk processing trait were determined using linear mixed animal models in ASREML (Gilmour et al., 2011). Fixed effects treated as categorical variables were parity (5 classes: first, second, third, fourth, and fifth and later parities), stage of lactation (10 classes: 5-30, 31-60, ..., 241-270, and 271-305 days-post-calving), milking session (3 classes: AM, PM for the research herds; combined AM+PM for the commercial herds), month of test (12 classes: January, ..., December), coefficient of heterosis (12 classes: 0, >0 and  $\leq 0.1$ , ..., >0.9 and <1, 1),

coefficient of recombination loss (12 classes: 0, >0 and  $\leq 0.1$ , ..., >0.9 and <1, 1), and the two-way interaction of parity-by-stage of lactation. The proportion of each breed (Friesian, Jersey, Montbeliarde, Norwegian Red, and other; Holstein was not included to avoid linear dependency in the model) were each treated as a continuous variable. Random terms included the effect of test-day within lactation, within- and across-lactation effects, and the contemporary group. Preliminary analyses revealed that stage of lactation and month of test were not confounded owing to the presence of both autumn calving (identified by a specific experimental treatment) as well as spring calving cows; therefore, the reported least squares means for month of test were independent of the seasonality of the data. Supplementary analyses were undertaken where either test-day milk yield or milk protein concentration was also included in the model as a covariate. In all instances, presented least squares means were for a reference animal representing a third parity, 100% Holstein, milked in the morning, averaged across all classes of stage of lactation as well as all calendar months of the year.

## RESULTS

### *Descriptive Statistics and Phenotypic Correlations*

The coefficient of variation (CV) of milk yield was 57.14%, while lower variability was evident for milk composition. Indeed, the CV for concentrations of protein, fat, casein, lactose, and total solids were 10.51%, 23.26%, 11.74%, 4.41%, and 8.99%, respectively. A greater CV was nevertheless observed for both urea concentration (40.95%) and SCS (27.37%). Regarding milk processing characteristics, the CV of milk coagulation properties was 30.54% (RCT), 29.43% ( $k_{20}$ ), 24.09% ( $a_{30}$ ), and 12.33% ( $a_{60}$ ). The CV was 64.80%, 7.32%, and 1.20% for

HCT, CMS, and pH, respectively. The summary statistics relative to the reference animal used to derive the least squares means in the current study are presented in Table 1.

Pearson correlation coefficients between milk yield, milk composition, SCS, and milk processing characteristics are in Table 2. The correlations between milk yield and the milk technological traits were generally weak ranging from -0.37 (between milk yield and  $a_{30}$ ) to 0.33 (between milk yield and  $k_{20}$ ). Regarding the nitrogen fraction of the milk, the strongest correlations were between protein concentration and  $k_{20}$  (-0.59) and between protein concentration and  $a_{30}$  (0.54). A moderate negative correlation was evident between RCT and  $a_{30}$  (-0.54), whereas a strong negative correlation existed between  $k_{20}$  and  $a_{30}$  (-0.89). Heat coagulation time was positively correlated with both milk urea (0.24) and CMS (0.17), but was negatively correlated (-0.19) with pH. A strong positive correlation existed between RCT and pH (0.66), whereas only a weak correlation was evident between CMS and RCT (0.22).

**Table 1.** Number of test-day records (n) and least squares means (LSM) of milk yield, predicted milk composition, somatic cell score (SCS), and predicted milk technological traits for the reference animal<sup>1</sup>. The accuracy<sup>2</sup> of the prediction models are from Visentin et al. (2015).

Trait <sup>3</sup>	n	LSM	R <sup>2</sup> <sub>C</sub>	SEP <sub>C</sub>
Milk, kg	134,155	15.92		
Protein, %	128,561	3.61		
Casein, %	128,615	2.71		
Fat, %	128,647	3.90		
Lactose, %	128,510	4.70		
Total solids, %	128,747	12.87		
Urea, mg/dL	127,996	30.06		
SCS, units	76,595	1.69		
RCT, min	136,102	21.84	0.61	5.64
k <sub>20</sub> , min	136,340	5.94	0.59	1.47
a <sub>30</sub> , mm	126,799	27.42	0.50	10.33
a <sub>60</sub> , mm	135,605	32.10	0.26	11.32
HCT, min	134,185	6.80	0.55	1.58
CMS, nm	136,165	170.54	0.23	28.11
pH	136,126	6.71	0.73	0.06

<sup>1</sup>Third parity cow, 100% Holstein, milked in the morning, averaged across all classes of stage of lactation as well as all calendar months of test.

<sup>2</sup>R<sup>2</sup><sub>C</sub> = coefficient of determination in cross validation; SEP<sub>C</sub> = standard error of prediction in cross validation.

<sup>3</sup>SCS = log<sub>10</sub>(SCC); RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness 30 min after rennet addition to milk; a<sub>60</sub> = curd firmness 60 min after rennet addition to milk; HCT = heat coagulation time; CMS = casein micelle size.

**Table 2.** Pearson correlation coefficients between milk yield, milk composition, somatic cell score (SCS), and milk technological traits.

Trait <sup>1</sup>	Milk yield	Protein	Casein	Fat	Total solids	Urea	SCS	RCT	k <sub>20</sub>	a <sub>30</sub>	a <sub>60</sub>	HCT	CMS
Protein	-0.32												
Casein	-0.32	0.97											
Fat	-0.54	0.42	0.43										
Total Solids	-0.50	0.62	0.65	0.93									
Urea	-0.02	0.11	0.05	0.06	0.02								
SCS	0.05	0.15	0.11	0.14	0.09	0.05							
RCT	0.17	0.00*	0.04	-0.19	-0.09	-0.08	0.11						
k <sub>20</sub>	0.33	-0.59	-0.55	-0.43	-0.43	-0.32	-0.06	0.53					
a <sub>30</sub>	-0.37	0.54	0.51	0.42	0.38	0.27	0.13	-0.54	-0.89				
a <sub>60</sub>	-0.08	0.51	0.46	-0.12	-0.06	0.13	0.12	-0.15	-0.54	0.60			
HCT	-0.05	-0.02	-0.04	-0.12	-0.21	0.24	0.05	-0.22	-0.29	0.47	0.48		
CMS	-0.15	0.30	0.31	0.06	0.07	-0.23	0.21	0.22	0.08	0.15	0.33	0.17	
pH	0.24	-0.06	-0.03	-0.22	-0.14	0.02	0.03	0.66	0.32	-0.37	-0.14	-0.19	-0.04

<sup>1</sup>SCS = log<sub>10</sub>(SCC); RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness 30 min after rennet addition to milk; a<sub>60</sub> = curd firmness 60 min after rennet addition to milk; HCT = heat coagulation time; CMS = casein micelle size.

\*Correlation not different from zero ( $P > 0.05$ ).

### ***Factors Associated with Milk Processing Characteristics***

Categorical fixed effects associated with all the milk processing characteristics included month of test ( $P < 0.001$ ), milking session ( $P < 0.001$ ), parity ( $P < 0.001$ ), stage of lactation ( $P < 0.001$ ), as well as a two-way interaction parity-by-stage of lactation ( $P < 0.001$ ). Heterosis was associated ( $P < 0.05$ ) with HCT, CMS, and pH, while recombination loss was associated ( $P < 0.05$ ) with RCT,  $k_{20}$ ,  $a_{30}$ , and pH. Milk processing characteristics were associated ( $P < 0.01$ ) with the proportion of Norwegian Red (with the exception of CMS), Friesian, and Jersey. The proportion of Montbeliarde was associated only with CMS and pH ( $P < 0.05$ ).

***Milk Coagulation Properties.*** The least squares means for RCT,  $k_{20}$ ,  $a_{30}$ , and  $a_{60}$  indicated poor coagulation ability (i.e., long RCT and weak  $a_{30}$ ) in January, followed by an improvement in February persisting until June ( $P < 0.001$ ; Figure 1); all milk coagulation-related traits subsequently deteriorated until September ( $P < 0.001$ ), but improved again to the end of the calendar year ( $P < 0.001$ ; Figure 1). Milk collected from the PM milking session, relative to the AM session, was characterized by superior coagulation ability, as evidenced by stronger  $a_{30}$  and shorter RCT ( $P < 0.001$ ; data not shown). Relative to a Holstein cow, a greater proportion of Friesian, Jersey, and Norwegian Red was associated with improved coagulation ability ( $P < 0.001$ ; Table 3). Jersey cows had the best milk coagulation-related traits ( $P < 0.001$ ) but, when average protein percentage was included as a covariate in the model, the previously detected superiority in either  $k_{20}$  or  $a_{30}$  of the Jersey relative to both Norwegian Red and Friesian no longer existed ( $P > 0.05$ ; data not shown). Milk coagulation properties were most favorable in animals with a recombination rate between 40% and 50% ( $P < 0.05$ ).

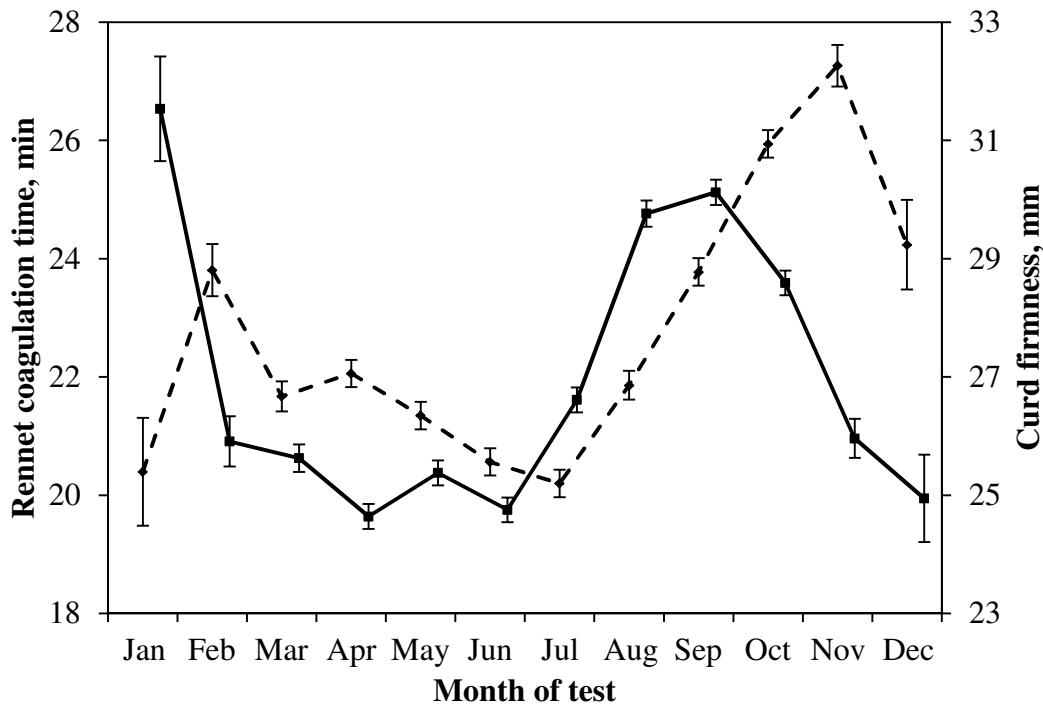


Milk coagulation properties were more favorable, on average, for primiparous animals ( $P < 0.001$ ; Figure 2). Irrespective of cow parity, at the very start of lactation, RCT (Figure 2) and  $k_{20}$  were both short, while  $a_{30}$  was strong (Figure 2). Rennet coagulation time deteriorated ( $P < 0.001$ ) until mid-lactation, with the two-way interaction between parity and stage of lactation manifesting itself as an earlier peak in RCT for primiparae (121-150 DIM) relative to multiparae (91-120 DIM) (Figure 2). Overall,  $a_{30}$  deteriorated ( $P < 0.001$ ) to the peak of lactation (31-60 DIM), but subsequently improved to the end of lactation for all cow parities ( $P < 0.001$ ; Figure 2). However, a two-way interaction parity-by-stage of lactation existed, resulting in a lesser nadir  $a_{30}$  in 31-60 DIM for multiparae compared with primiparae ( $P < 0.001$ ; Figure 2). Irrespective of cow parity, when models were adjusted for protein percentage, the recovery in either  $k_{20}$  or  $a_{30}$  after peak of lactation was no longer obvious, while adjustment for milk yield in the multiple regression models did not impact the lactation profile or the significance of the multiple regression coefficients for breed proportions of any coagulation-related trait.

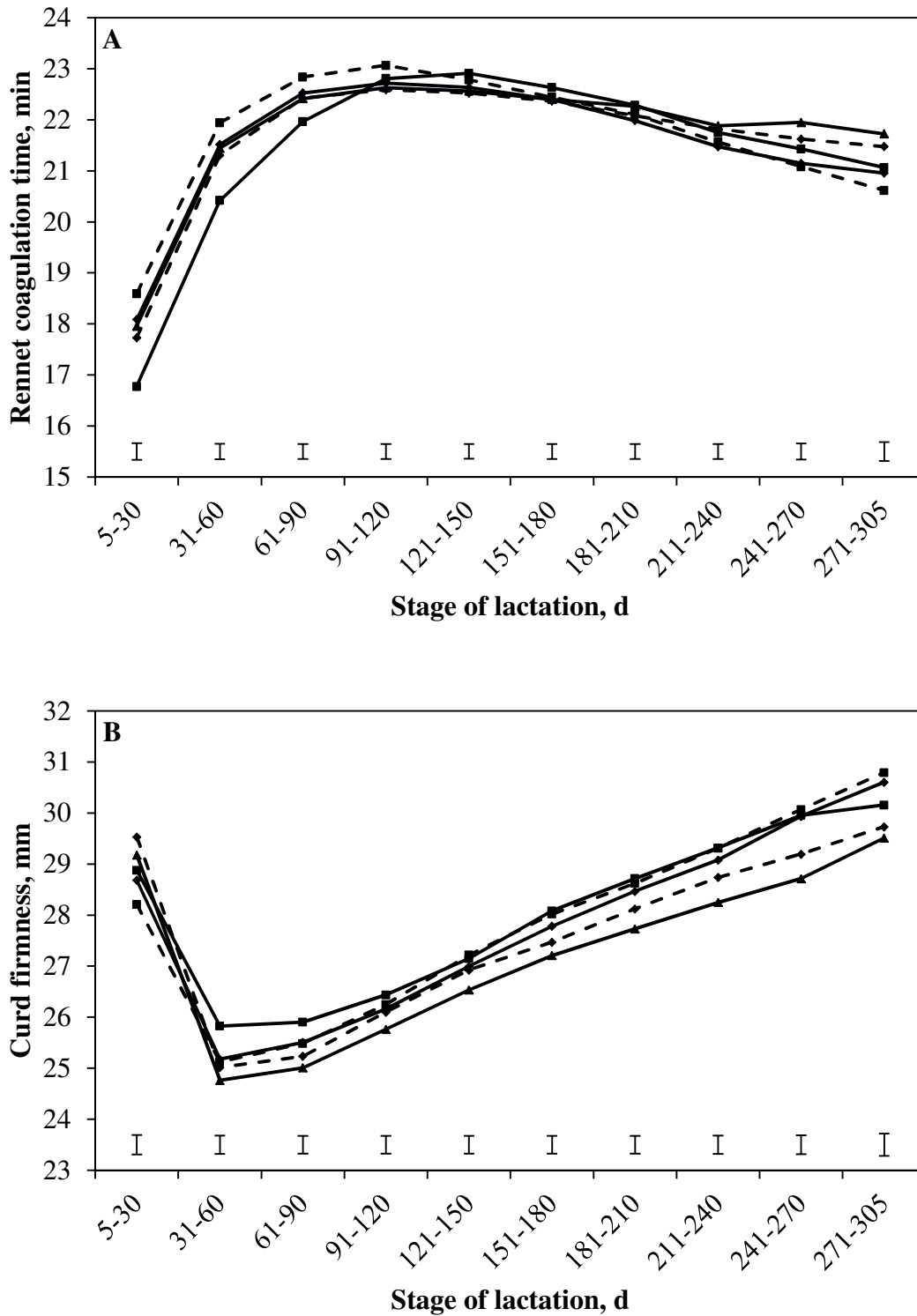
**Table 3.** Regression coefficients (SE in parentheses) of rennet coagulation time (RCT), curd-firming time ( $k_{20}$ ), curd firmness 30 and 60 min after rennet addition to milk ( $a_{30}$  and  $a_{60}$ , respectively), heat coagulation time (HCT), casein micelle size (CMS), and pH on the proportion of each breed estimated from the multiple regression model. Holstein was considered the reference breed.

	Holstein	Friesian	Jersey	Montbeliarde	Norwegian Red
RCT, min	0 <sup>a</sup>	-2.10 <sup>b</sup> (0.28)	-4.59 <sup>c</sup> (0.24)	0.51 <sup>a</sup> (0.42)	-1.77 <sup>b</sup> (0.36)
$k_{20}$ , min	0 <sup>a</sup>	-0.60 <sup>b</sup> (0.10)	-1.85 <sup>c</sup> (0.08)	0.14 <sup>a</sup> (0.14)	-0.52 <sup>b</sup> (0.12)
$a_{30}$ , mm	0 <sup>a</sup>	2.23 <sup>b</sup> (0.35)	6.21 <sup>c</sup> (0.31)	-0.90 <sup>a</sup> (0.52)	2.23 <sup>b</sup> (0.45)
$a_{60}$ , mm	0 <sup>a</sup>	0.41 <sup>b</sup> (0.17)	1.97 <sup>c</sup> (0.14)	-0.34 <sup>a</sup> (0.25)	1.09 <sup>d</sup> (0.21)
HCT, min	0 <sup>a</sup>	0.44 <sup>b</sup> (0.16)	-0.37 <sup>c</sup> (0.14)	0.20 <sup>abd</sup> (0.24)	0.80 <sup>bd</sup> (0.21)
CMS, nm	0 <sup>a</sup>	-1.88 <sup>b</sup> (0.61)	-4.54 <sup>c</sup> (0.53)	-1.99 <sup>b</sup> (0.90)	0.99 <sup>a</sup> (0.79)
pH	0 <sup>a</sup>	-0.02 <sup>bd</sup> (0.003)	-0.01 <sup>b</sup> (0.003)	0.01 <sup>c</sup> (0.005)	-0.03 <sup>d</sup> (0.004)

<sup>a-d</sup>Values within rows differing in superscript are different ( $P < 0.05$ ).



**Figure 1.** Least squares means (with standard error bars) for rennet coagulation time (—■—) and curd firmness 30 min after rennet addition to milk (---◆---) across month of test.



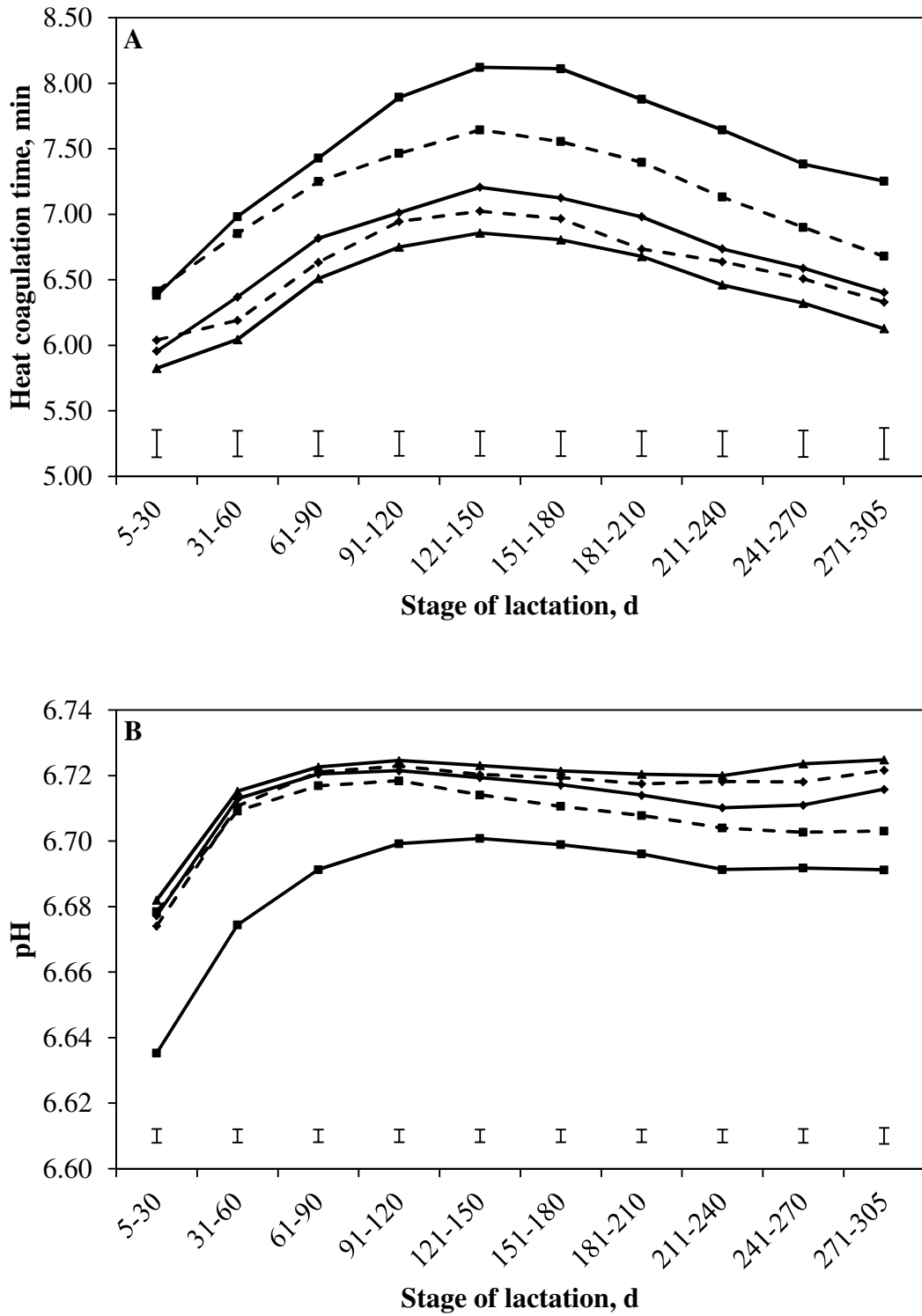
**Figure 2.** Least squares means for A) rennet coagulation time, and B) curd firmness 30 min after rennet addition to milk across stage of lactation for parity 1 (—■—), parity 2 (---■---), parity 3 (—◆—), parity 4 (---◆---), and parity ≥ 5 (—▲—). The error bars represent the mean SE per stage of lactation across parities.

**Heat Coagulation Time.** Milk HCT was, on average, the lowest in January (5.29 min, SE=0.78) but remained relatively constant ( $P > 0.05$ ) until September ( $P < 0.01$ ; 6.85 min, SE=0.17), after which HCT increased until the end of the calendar year ( $P < 0.001$ ; data not shown). Milk HCT was greater ( $P < 0.001$ ) in the AM milking (6.81 min, SE=0.09) compared with the PM milking session (5.99 min, SE=0.09; data not shown). Relative to the Holstein, a greater proportion of Jersey was associated with reduced HCT ( $P < 0.01$ ; Table 3). Milk HCT increased with increasing Friesian or Norwegian Red breed proportion ( $P < 0.01$ ; Table 3). The effect of heterosis coefficient was of little biological impact. Primiparous cows had the greatest HCT ( $P < 0.001$ ; 7.61 min, SE=0.09; Figure 3). On average, HCT peaked around mid-lactation (121-150 DIM), irrespective of animal parity, while at both the start and end of lactation, milk was less suitable for heat treatment ( $P < 0.001$ ; Figure 3). The two-way interaction between parity and stage of lactation manifested itself as a less evident reduction in HCT in late lactation for primiparae compared with multiparae ( $P < 0.001$ ; Figure 3).

**Casein Micelle Size.** Least squares means for CMS decreased ( $P < 0.001$ ) from January (172.60 nm, SE=1.83) to March (166.03 nm, SE=0.49) and subsequently increased to June ( $P < 0.001$ ; 170.82 nm, SE=0.43). Casein micelle size declined again in July ( $P < 0.001$ ; 165.82 nm, SE=0.44) but subsequently peaked in September ( $P < 0.001$ ; 176.74 nm, SE=0.44), after which it declined ( $P < 0.001$ ; data not shown). Linear regression coefficients from the multiple regression models were negative for Jersey, Friesian, and Montbeliarde ( $P < 0.05$ ; Table 3). Although significant ( $P < 0.05$ ), the contribution of heterosis coefficient was biologically small. The lowest CMS was evident for primiparous cows ( $P < 0.001$ ; 169.39 nm, SE=0.29) increasing with parity number although the mean CMS remained relatively constant

after parity 3 ( $P > 0.05$ ; data not shown). Within lactation, milk CMS increased consistently ( $P < 0.001$ ) with stage of lactation but CMS of primiparae plateaued in late lactation (211-305 DIM) resulting in a two-way interaction parity and stage of lactation (data not shown).

**Milk pH.** Least squares means for milk pH reduced ( $P < 0.001$ ) from January (6.81, SE=0.01) to July (6.68, SE=0.003), but remained relatively constant for the remainder of the calendar year, varying between 6.70 (SE=0.004) and 6.73 (SE=0.003) in November and September, respectively (data not shown). Milk pH from the PM milking session was lower (pH=6.68, SE=0.002) than milk pH from the AM milking session ( $P < 0.001$ ; pH=6.71, SE=0.002; data not shown). Increased proportion of Friesian, Jersey, and Norwegian Red was associated with lower pH relative to the Holstein ( $P < 0.001$ ; Table 3). The contribution of both heterosis and recombination loss coefficient was small. Milk pH was lowest for primiparous cows ( $P < 0.001$ ; 6.69, SE=0.002; Figure 3). On average, milk pH peaked between 91 and 120 DIM, which persisted to the end of lactation irrespective of cow parity (Figure 3). The observed impact of the two-way interaction parity-by-stage of lactation, although significant ( $P < 0.001$ ), was biologically small (Figure 3).



**Figure 3.** Least squares means for A) heat coagulation time and B) milk pH across stage of lactation for parity 1 (—■—), parity 2 (---■---), parity 3 (—◆—), parity 4 (---◆---), and parity  $\geq 5$  (—▲—). The error bars represent the mean SE per stage of lactation across parities.

## DISCUSSION

The objective of the present study was to identify factors associated with the variability in milk processing traits, predicted by mid-infrared spectroscopy, on a large population of dairy cows in a seasonal calving grass-based dairy production system. Results clearly demonstrated that variability existed among animals due to animal breed composition, parity, and stage of lactation; the differences among cow characteristics even persisted after adjustment for differences in cow milk yield. Milk processability also varied across the calendar months of test as well as between morning and evening milking.

The correlations between the milk coagulation properties in the present study were all consistent with previous reports which investigated milk coagulation properties both at the phenotypic and genetic level in dairy cows (Ikonen et al., 2004; Cassandro et al., 2008; Vallas et al., 2010). The positive correlation between RCT and CMS in the present study corroborates Chen et al. (2014) based on 550 individual milk samples from UK Holstein cows, and the positive correlation between urea concentration and HCT in the present study was similar to findings of Singh (2004) and Reid et al. (2015) in dairy cows.

### *Effect of National Herd Breed Composition on the National Milk Pool*

The contribution of breed differences to variability in milk composition have already been reported in both grazed grass-fed (Auld et al., 2002; Sneddon et al., 2016) and TMR-fed (Barłowska et al., 2014) dairy cows, demonstrating that milk yielded by Jersey cows had greater protein concentration than milk of Holstein cows. Evidence from the present study indicated that the superior milk coagulation properties of Jersey cows relative to the Holstein contemporaries were not only due to

the higher milk protein concentration typical of Jerseys, which in the present study was 4.10%. Therefore, it appears that Holsteins may have a genetic predisposition to inferior milk clotting characteristics over and above their genetic merit for protein concentration. This hypothesis was supported by Caroli et al. (2009) who identified a polymorphism in genes coding for  $\kappa$ -casein (*CSN3\*E*) in Holstein-Friesian cows with an unfavorable association with milk coagulation ability. Geary et al. (2010) suggested that Jersey cows could potentially yield more cheese per unit of milk compared with Holsteins. However, the greater milk yield of Holstein cows (5,217 kg) compared with Jersey contemporaries (4,230 kg) reported by Coffey et al. (2016) could potentially offset the lower cheese production per unit of milk from the Jerseys, a conclusion also supported by Sneddon et al. (2016). A possible short-term strategy to improve coagulation-related traits at the industry level could be to blend milk produced by Holstein-Friesians with milk yielded by Jerseys. Such a strategy was also suggested by Bland et al. (2015), who demonstrated a positive (quadratic) relationship between curd firmness and the proportion of Jersey milk.

Milk HCT of the different cow breeds studied in the present study was similar to results reported by Barłowska et al. (2014) in Polish Holstein-Friesian, Jersey, and Simmental cows, despite the Polish cows being fed TMR. At an industry level, the majority of heat stability problems occurs when milk is fortified with additives, such as minerals (Ca, Mg, and Zn), or cocoa and tea extracts (Singh, 2004), which normally have an unfavorable impact on resistance to heat treatments. Given the lower HCT of Jersey milk as observed in the present study, blending Jersey milk with milk from other breeds may have implications on the heat stability of milk processed. To our knowledge, no evidence exists on the effect of blending milk produced by different cow breeds on milk heat stability.



### ***The Increasing Need for Dairy Products and the Implications for the Dairy Processing Industry***

Expansion in herd size, coupled with increased productivity, is required to satisfy the increasing world demand for milk and dairy products (Alexandratos and Bruinsma, 2012). In Irish spring calving dairy herds, rate of herd expansion has been demonstrated to be associated with a reduction in herd average parity number (Jago and Berry, 2011); moreover, given the abolition of milk quota in the European Union, it is anticipated that herd size in Ireland will increase at an annual rate of 3% (Dillon, 2011). Therefore, in the medium term, the contribution of milk from younger parity cows to the national milk pool is likely to increase. Results from the present study indicated that, irrespective on the final output of milk processing, milk produced by young cows had improved processability both for cheese manufacturing (i.e., shorter RCT and greater  $a_{30}$ ) as well as for milk powder production (i.e., greater HCT). Therefore, a general improvement of milk processability may be expected in the following years, which potentially may improve the profitability of the entire dairy sector. This is important when interpreting national trends in milk quality so as not to attribute the improvement in milk quality solely to improvements in management and genetic merit over that period; improvements in milk processing characteristics are expected therefore to slow down as the rate of expansion slows or comes to a halt.

### ***Seasonal Variability in Milk Processing Characteristics and Implications for the Dairy Processing Sector***

The variability in milk coagulation properties across lactation documented in the present study corroborate previous international studies by Penasa et al. (2014) in

Italian Holstein-Friesian, Brown Swiss, and Simmental dairy cows, by Ikonen et al. (2004) in Finnish Ayrshires, and by Vallas et al. (2010) in Estonian Holsteins. Such variability has implications in countries where seasonal calving predominates, such as in Ireland and New Zealand but also to a lesser extent in other countries such as Australia and the UK. Indeed, seasonal calving implies seasonality of milk supply and quality. For instance, in Ireland the greatest proportion (80%; Berry et al., 2013) of dairy farms concentrate calvings between January and April, inclusive, of each calendar year. The aim of this production system is to synchronize the onset of the lactation with the initiation of grass growth and to maximize the utilization of low-cost grazed pasture in the diet (Berry et al., 2013). The multiple regression model proposed in the present study accounted for both stage of lactation and calendar month implying that model solutions for both effects could be considered additive; disentangling of the effects was possible by the inclusion of autumn calving cows (6.90% of the data) in the analysis in the present study. The observed variability in milk quality across calendar months might therefore reflect changes in quantity and quality of the diet fed; in the start and end of the calendar year in Ireland cows are generally indoors and fed mainly grass silage (O'Brien et al., 1999). Changes in diet and housing system are known to be associated with changes in milk SCC, total bacteria count, and laboratory pasteurization count (O'Connell et al., 2015), which in turn could potentially impact the respective milk processability. The consequence of seasonal milk supply and quality is less relevant in countries with year-round calving systems and therefore the supply (and quality) of the herd milk pool fluctuates less throughout the year. The variability in milk processing attributes across the year in seasonal calving herds, nonetheless, represents important challenges for dairy processors in their attempts to ensure product consistency.

In a scenario when the final output of milk processing is cheese, short reactivity to rennet addition and strong curd firming capacity are desired characteristics. Indeed, a positive association exists between curd firmness and cheddar cheese yield (Bynum and Olson, 1982; Johnson et al. 2001). Strong curd firmness is associated with improved cheese yield also for cheese intended for long maturation before human consumption, as evidenced by Aleandri et al. (1989) in the production of Parmigiano Reggiano and Pretto et al. (2013) in the production of Grana Padano. To our knowledge, only one formula considers  $a_{30}$  as a predictor of cheese yield, and this was implemented by Aleandri et al. (1989) for the prediction of Parmigiano Reggiano cheese yield, and therefore not directly applicable to the national production system due to differences in the cheesemaking processes. However, the linear regression coefficient of  $a_{30}$  on casein content, which is a predictor of cheese yield (Van Slyke and Price, 1949), in the multiple regression model developed in the present study, was 0.04 units. Therefore, assuming a milk fat level of 3.90% and a water content of full fat Cheddar cheese of 37.7% (Fenelon and Guinee, 1999), milk produced by early lactation cows (5-30 DIM) would yield 2.20% more cheese than milk yielded by cows at peak lactation (31-60 DIM), when  $a_{30}$  was the lowest.

In direct contrast, in a scenario where the main objective of the milk processor is to extend milk shelf-life through heat treatments, resistance to high temperature is a desired characteristic. Results from the present study indicated that a higher HCT was achieved in mid-lactation, suggesting that processors can potentially reduce propensity of milk to fouling and thus increase production times for manufacture of skim and whole milk powder in this lactation period, when coagulation-related traits are the less favorable for cheese production. This trend was also documented by

Sneddon et al. (2016), modeling supply lactation curves for yields of dairy products of 1,073 Holstein, 726 Jersey, and 2,534 Holstein x Jersey crossbred first-lactation seasonal calving New Zealand cows.

Finally, animal breeding may also contribute to enhancing the manufacturing characteristics of milk (Cassandro et al., 2008; Tiezzi et al., 2013). Therefore access to routinely measurable (cost-effective, quick and accurate) phenotypes is becoming more and more important to achieve genetic gain and to provide processors with a more detailed knowledge of the trait(s) of interest (Berry, 2015). Infrared spectroscopy, through the exploitation of interactions between infrared radiation and sample molecules, is the most commonly used technique to provide quick and low-cost measurements of milk composition, and has recently been highlighted as an indicator of milk quality (De Marchi et al., 2014; McDermott et al., 2016a,b) and animal production traits (McParland and Berry, 2016), including dairy cow health and fertility (Bastin et al., 2016).

## **CONCLUSIONS**

A particular aspect that emerged from the present study was that, in general, characteristics associated with more favorable milk heat stability were associated with those less favorable for cheese production. Furthermore, breeds yielding milk with more favorable heat stability were least suitable for cheese-making. These results could be useful for processors with managing their processing systems especially in countries characterized by seasonal supply of fresh milk, in order to manage their product portfolio accounting for variation in processing characteristics. Finally, the inclusion of milk processability traits into the national breeding goal may be of interest to stakeholders. However, genetic variation of milk processability and

correlations with milk yield, fat, and protein yield and concentration, as well as the economic value attributable to milk processability require further quantification.

### **ACKNOWLEDGEMENTS**

Funding for the current study was received from the Irish Department of Agriculture, Food and the Marine, Research Stimulus Fund project 11/SF/311, Breed Quality. Laboratory technical support by Bernard Corrigan, Jim Flynn, Owen Kenny, and Paula O'Connor (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland) is gratefully acknowledged.

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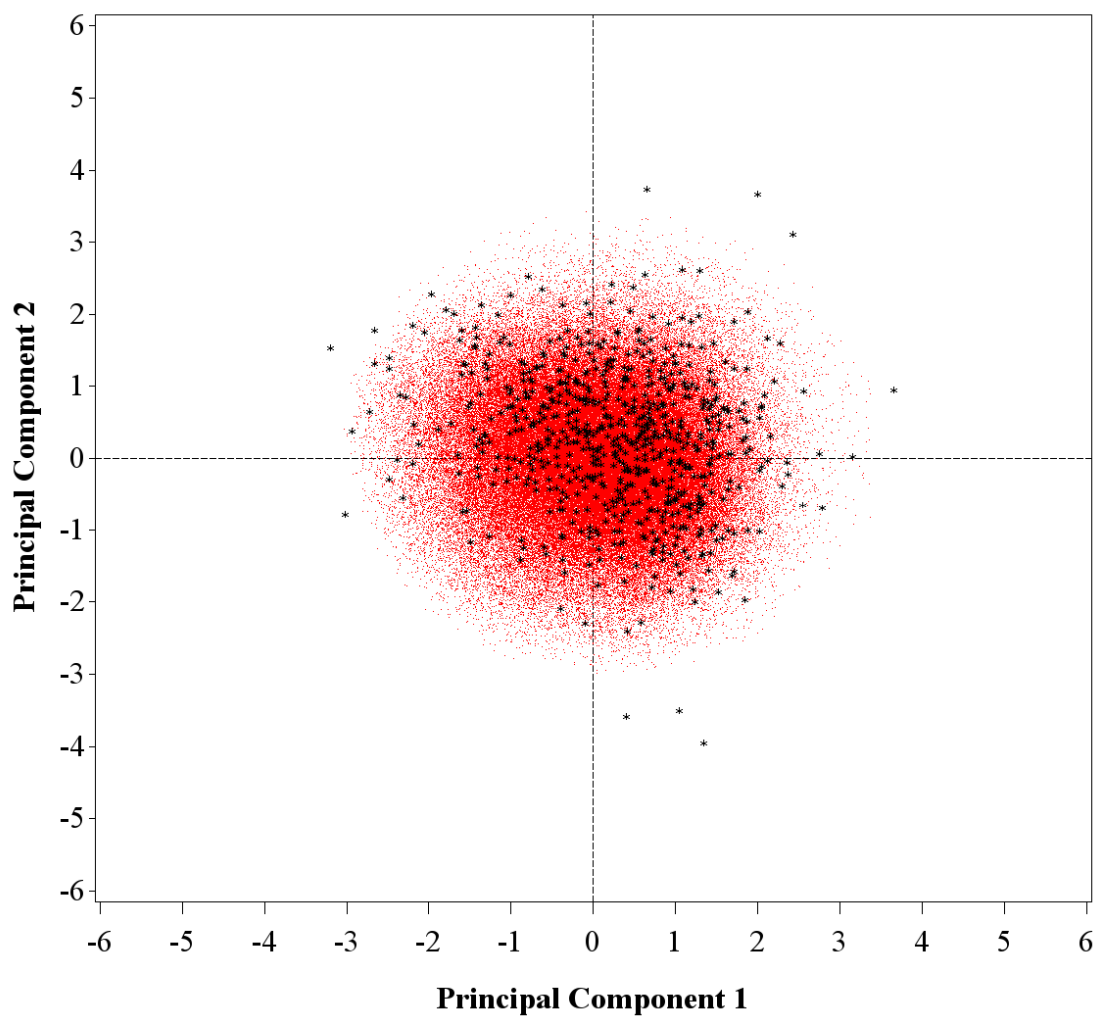
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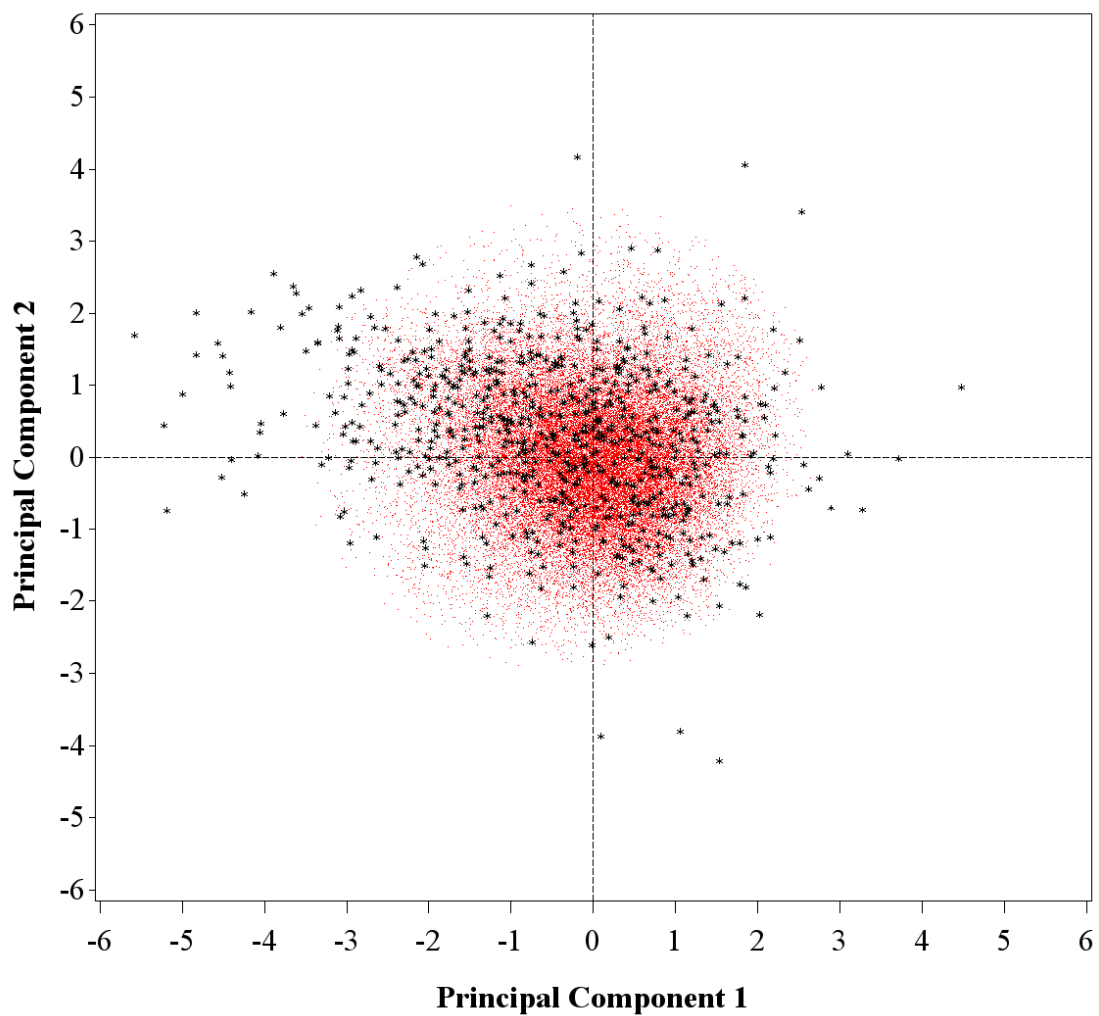
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## SUPPLEMENTARY MATERIAL



**Figure S1.** Scatter plot of principal component 1 (x-axis) versus principal component 2 (y-axis) of milk spectra collected from research herds with unknown reference values (red dots) and of milk spectra of the calibration dataset (i.e., with known reference values; black stars).



**Figure S2.** Scatter plot of principal component 1 (x-axis) versus principal component 2 (y-axis) of milk spectra collected from commercial herds with unknown reference values (red dots) and of milk spectra of the calibration dataset (i.e., with known reference values; black stars).

**Phenotypic characterization of major mineral composition predicted by mid-infrared spectroscopy in cow milk**

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## ABSTRACT

Population-level phenotyping is, for some traits, hardly achievable due to limitation of reference analyses. Mid-infrared spectroscopy (**MIRS**) is a quick and cost-effective laboratory technique allowing the prediction of innovative milk quality features on a large-scale. The present study aimed at investigating factors associated with milk Ca, K, Mg, Na and P contents predicted by MIRS models on a large multi-breed spectral dataset of 123,240 test-day records. Two dairy (Holstein-Friesian and Brown Swiss) and two dual-purpose (Simmental and Alpine Grey) cattle breeds were considered. Sources of variation of predicted milk mineral contents were studied using linear mixed models, including the fixed effects of breed, month of sampling, stage of lactation, parity and interactions between the main effects. Random factors were herd nested within breed, cow nested within breed and the residual. Calcium content was greater in milk of dual-purpose than dairy cattle breeds. Simmental cows produced milk with the greatest content of Ca and Na, and milk of Holstein-Friesian had the lowest P content. Variation of content of Ca, Mg, Na and P throughout lactations exhibited an opposite trend to that of milk yield, with the lowest values around the peak of lactation. On the other hand, K content mirrored the trend of milk yield. Multiparous cows had lower content of milk minerals, with the exception of Na, compared with first parity animals. Environmental factors identified in the present study can be considered for within-breed genetic evaluation to adjust records of milk mineral contents for these effects.

**Keywords:** dairy cattle, mid-infrared spectroscopy, milk mineral content, phenomics

## INTRODUCTION

The acquisition of individual-level phenotypic information using rapid and cost-effective methodologies is becoming an extremely relevant challenge, since genomic information is nowadays easily available at low-cost. However, large-scale collection of economically important traits is often hampered by high recording costs, thus preventing their inclusion in breeding programs and in quality-based payment systems. Mid-infrared spectroscopy (**MIRS**) is worldwide recognized as one of the most accessible and efficient technologies to collect phenotypes at individual level (De Marchi et al., 2014). In national milk recording systems, individual cow milk samples are routinely collected and analysed through MIRS to determine their chemical composition (fat, protein, casein and lactose percentages). The output of milk MIRS analysis (i.e. spectrum) is a representation of the energy absorbed by the sample itself, when hit by the light, at a specific wavenumber in the region between 900 and 5000  $\text{cm}^{-1}$ . Storage of this information is feasible and once new MIRS prediction models are developed, spectra can be used to predict new phenotypes at no additional costs. Mid-infrared spectroscopy prediction models for milk mineral content (Ca, K, Mg, Na and P) have been developed by Visentin et al. (2016) using an algorithm of variable selection (Gottardo et al., 2015) to improve the accuracy of prediction. The use of variable selection algorithms, coupled with partial least squares (**PLS**) regression, is fundamental for data reduction and for an improvement of data interpretability (Mehmood et al., 2012).

An adequate daily intake of minerals is important to maintain both infant and adult people at a good health (Cashman, 2006; Haug et al., 2007). Moreover, milk minerals, particularly Ca and P, are important indicators of milk quality at the dairy industry level because they play a central role in the coagulation process of milk and



thus on cheese making efficiency (Lucey and Fox, 1993). There is a paucity of studies that have investigated phenotypic variation of milk mineral content at population level, mainly because its determination through reference analysis is costly and time-demanding. Therefore, the objective of the present research was to investigate factors associated with milk minerals predicted by MIRS in a large database of dairy and dual-purposes cattle breeds.

## MATERIALS AND METHODS

### *Data*

The initial dataset comprised 132,380 spectra of individual milk samples from 15,173 cows collected during the monthly test-day recording in Bolzano province (Italy) between January 2012 and December 2013 (Visentin et al., 2015). The study area is mostly characterized by small farms with traditional feeding (forage or hay and concentrates), and usually during the summer season cows are moved to highland pastures. The dataset included records of 4 cattle breeds reared in single-breed herds: Holstein-Friesian (**HF**, n = 2,020 cows), Brown Swiss (**BS**, n = 5,475 cows), Alpine Grey (**AG**, n = 3,154 cows) and Simmental (**SI**, n = 4,524 cows). Animals were from 6 to 450 days in milk and from parity 1 to 15. Preservative was added to milk samples (Bronysolv; ANA.LI.TIK Austria, Vienna, Austria) immediately after collection and processed according to International Committee for Animal Recording (**ICAR**) recommendations at the milk laboratory of the South Tyrol Dairy Association (Sennereiverband Südtirol, Bolzano, Italy). For each sample, protein (%), casein (%) and fat (%) were determined, and spectral information, containing 1060 infrared transmittance data in the region between 900 and 5000  $\text{cm}^{-1}$ , were stored using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Values of somatic cell

count (SCC) were assessed by Fossomatic (Foss Electric A/S, Hillerød, Denmark) and transformed to somatic cell score (SCS) through the formula  $SCS = 3 + \log_2(SCC/100,000)$ .

### ***Reference analyses and MIRS prediction models***

Detailed information about samples collection, reference analyses and development of MIRS prediction models is available from Visentin et al. (2016). Briefly, in 2014 a total of 251 individual bovine milk samples were collected in the same province and herds contributing to the data of the present study. Reference analysis for the determination of Ca, K, Mg, Na and P was conducted in the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) using Inductively Coupled Plasma Optical Emission Spectrometry, Ciros Vision EOP (SPECTRO Analytical Instruments GmbH, Kleve, Germany) after mineralization.

Prediction models were developed using PLS regression after uninformative variable elimination procedure as described by Visentin et al. (2016). Coefficients of determination (root mean square errors) in validation were 0.67 (122.00 mg/kg), 0.69 (120.00 mg/kg), 0.65 (12.50 mg/kg), 0.40 (70.00 mg/kg) and 0.68 (88.12 mg/kg) for Ca, K, Mg, Na and P, respectively.

### ***Phenotypic characterization***

Statistical analyses were performed using SAS software ver. 9.3 (SAS Institute Inc., Cary, NC, USA). Spectral information expressed in transmittance was transformed into absorbance by taking the  $\log_{10}$  of the reciprocal of the transmittance. Prior to multivariate analysis, two high noise level regions (1600 to 1700  $\text{cm}^{-1}$  and

3040 to 3660  $\text{cm}^{-1}$ ), which are known to be related to water absorption (Hewavitharana and van Brakel, 1997) were discarded. Principal component analysis was carried out in order to identify the spectra ( $n = 132,380$ ) similar to those used to develop the MIRS prediction models ( $n = 251$ ). The first 6 principal components were retained as explaining the 97.02% of the total variance. The Mahalanobis distance from the centroid of the cluster of the spectra used to develop the MIRS models was calculated to identify the outlier spectra. Instrumental or temporal drifts did not represent an issue given that the laboratory equipment was the same for the whole duration of the study. Moreover, the principal component analysis did not identify two evident clusters based on the different years of data collection (2012-2013). Following this approach, 9140 spectra were discarded from further analysis. Mid-infrared spectroscopy prediction models were then applied on the remaining 123,240 spectra from 14,389 cows to predict major milk minerals. For each milk composition trait (i.e. protein, casein and fat percentages, and SCS), as well as for each predicted milk mineral, values that deviated more than 3 standard deviations from the respective mean were discarded.

Sources of variation of milk mineral content were investigated using the following mixed linear model:

$$y_{ijklmno} = \mu + B_i + M_j + Y_k + S_l + \text{Parity}_m + (B \times M)_{ij} + (B \times S)_{il} + (B \times \text{Parity})_{im} \\ + (S \times \text{Parity})_{lm} + H_n(B_i) + \text{Cow}_o(B_i) + e_{ijklmno},$$

where  $y_{ijklmno}$  is MIRS-predicted content of Ca, K, Mg, Na or P,  $\mu$  is the overall intercept of the model,  $B_i$  is the fixed effect of the  $i$ th breed ( $i = \text{HF, BS, AG, SI}$ ),  $M_j$  is the fixed effect of the  $j$ th month of sampling ( $j = 1$  to 12),  $Y_k$  is the fixed effect of the  $k$ th year of sampling ( $k = 2012, 2013$ ),  $S_l$  is the fixed effect of the  $l$ th class of stage of lactation of the cow ( $l = 1$  to 45, with the first being a class from 6 to 10 d,

followed by 10-d classes),  $\text{Parity}_m$  is the fixed effect of the  $m$ th parity of the cow ( $m = 1$  to 5, with class 5 including cows from parity 5 to 15),  $(\text{B} \times \text{M})_{ij}$  is the fixed interaction effect between breed and month of sampling,  $(\text{B} \times \text{S})_{il}$  is the fixed interaction effect between breed and stage of lactation,  $(\text{B} \times \text{Parity})_{im}$  is the fixed interaction effect between breed and parity,  $(\text{S} \times \text{Parity})_{lm}$  is the fixed interaction effect between stage of lactation and parity,  $\text{H}_n(\text{B}_i)$  is the random effect of the  $n$ th herd nested within the  $i$ th breed  $\sim N(0, \sigma_{\text{H}(\text{B})}^2)$ ,  $\text{Cow}_o$  is the random effect of the  $o$ th cow nested within the  $i$ th breed  $\sim N(0, \sigma_{\text{COW}(\text{B})}^2)$ , and  $e_{ijklmno}$  is the random residual  $\sim N(0, \sigma_e^2)$ . Significance of breed effect was tested on the cow within breed variance. A multiple comparison of means was performed for breed effect using Bonferroni's test ( $P < 0.05$ ).

The same approach of data selection, application of MIRS prediction models and generation of MIRS-predicted phenotypes has been applied by Visentin et al. (2015) to predict milk coagulation properties (**MCP**) of the same milk samples considered in the present study. Milk coagulation properties investigated by Visentin et al. (2015) were rennet coagulation time (**RCT**, min; the time taken from rennet addition to the development of the gel), curd-firming time (**k<sub>20</sub>**, min; the time taken from gel development to the achievement of a 20 mm-strong curd) and curd firmness at 30 min (**a<sub>30</sub>**, mm; the strength of the curd after 30 min from rennet addition). Pearson correlations between milk minerals were assessed, and also between such traits and MCP from Visentin et al. (2015).

## RESULTS

### *Descriptive statistics and phenotypic correlations*

Table 1 summarizes the descriptive statistics for milk yield, chemical composition, SCS and minerals of samples retained after removal of spectral outliers. Milk yield averaged 22.74 kg/d, and means for fat, protein and casein were 4.03%, 3.53% and 2.76%, respectively. Traits predicted by MIRS were characterized by moderate to large coefficients of variation, which ranged from 13.13% (P) to 23.36% (Mg). The proportion of phenotypic variance of milk minerals accounted by the cow effect ranged from 2.01% (K) to 32.45% (P), and the ratio between herd and phenotypic variance was between 4.80% (Mg) and 12.92% (Ca).

Pearson correlations ( $r$ ) between milk minerals (Table 2) varied from -0.20 (Ca and K, and K and Na) to 0.66 (K and Mg). Moderate to low relationships were assessed between Ca and P ( $r = 0.53$ ), Na and P ( $r = 0.32$ ), Ca and Na ( $r = 0.29$ ) and Mg and P ( $r = 0.24$ ) ( $P < 0.05$ ). Calcium and P were the minerals with the strongest association with milk protein and casein percentages, ranging from 0.31 (Ca with protein) to 0.37 (P with casein) ( $P < 0.05$ ). Strong and unfavourable correlations were estimated between RCT and  $a_{30}$  ( $r = -0.87$ ), and between  $k_{20}$  and  $a_{30}$  ( $r = -0.64$ ) ( $P < 0.05$ ). Curd-firming time was unfavourably associated with protein ( $r = -0.58$ ) and casein percentages ( $r = -0.61$ ) ( $P < 0.05$ ), whereas  $a_{30}$  was favourably correlated with these constituents ( $r = 0.21$  with protein and  $r = 0.23$  with casein percentages;  $P < 0.05$ ). Rennet coagulation time was weakly correlated with milk composition. Regarding correlations between MCP and minerals, Ca and P were the elements with the greatest association with milk coagulation ability, and correlations ranged from -0.35 and -0.39 with  $k_{20}$  to 0.37 and 0.28 with  $a_{30}$ , respectively ( $P < 0.05$ ). Finally, SCS was positively and moderately associated with Na (0.22;  $P < 0.05$ ).

**Table 1.** Descriptive statistics and variance accounted by cow ( $\sigma^2_C$ ) and herd ( $\sigma^2_H$ ) effects for milk yield, composition, somatic cell score (SCS), mineral content, and coagulation properties.

Trait	Mean	SD	Range	CV, %	$\sigma^2_C$ , %	$\sigma^2_H$ , %
Milk yield, kg/d	22.74	7.56	60.50	33.25	25.92	35.51
Chemical composition, %						
Fat	4.03	0.61	4.43	15.13	25.26	8.83
Protein	3.53	0.41	3.56	11.61	36.67	18.18
Casein	2.76	0.30	2.54	10.87	39.44	18.71
SCS, units	2.44	1.85	9.96	75.82	24.14	7.32
Mineral content, mg/kg						
Ca	1,364.56	182.26	1,597.53	13.36	31.87	12.92
K	1,668.84	297.05	1,699.42	17.80	2.01	7.62
Mg	140.63	32.85	198.90	23.36	8.18	4.80
Na	442.83	71.37	690.50	16.12	26.86	7.60
P	1,035.57	135.99	1,131.07	13.13	32.45	12.02
Milk coagulation properties <sup>1</sup>						
RCT, min	18.57	4.61	24.7	24.80	39.41	10.14
k <sub>20</sub> , min	5.20	1.63	11.15	31.30	40.44	9.03
a <sub>30</sub> , mm	28.07	12.37	54.72	44.10	39.23	10.58

CV: coefficient of variation.

**Table 2.** Pearson correlations between milk chemical composition, somatic cell score (SCS), mineral content and coagulation traits predicted by mid-infrared spectroscopy.

	Fat	Protein	Casein	SCS	Ca	K	Mg	Na	P	RCT	k <sub>20</sub>
Protein	0.43										
Casein	0.43	0.98									
SCS	0.10	0.21	0.20								
Ca	0.18	0.31	0.32	0.05							
K	0.00 <sup>a</sup>	-0.09	-0.09	-0.02	-0.20						
Mg	0.24	0.23	0.25	0.05	-0.02	0.66					
Na	0.04	0.21	0.17	0.22	0.29	-0.20	-0.03				
P	0.21	0.36	0.37	0.00 <sup>a</sup>	0.53	-0.06	0.24	0.32			
RCT	0.03	0.11	0.10	0.13	-0.26	-0.06	-0.06	0.18	-0.17		
k <sub>20</sub>	-0.29	-0.58	-0.61	-0.04	-0.35	-0.01	-0.36	0.06	-0.39	0.39	
a <sub>30</sub>	0.11	0.21	0.23	-0.04	0.37	0.03	0.14	-0.10	0.28	-0.87	-0.64

RCT: rennet coagulation time (min); k<sub>20</sub>: curd-firming time (min); a<sub>30</sub>: curd firmness 30 min after rennet addition. Data of RCT, k<sub>20</sub> and a<sub>30</sub> are from Visentin et al. (2015b).

<sup>a</sup>Correlations not different from zero ( $P > 0.05$ ).

### ***Breed effect***

Least squares means of milk yield, chemical composition and mineral content for HF, BS, AG and SI breeds are in Table 3. Milk production ranged from 14.67 kg/d (AG) to 25.28 kg/d (HF). Fat, protein and casein percentages of milk from BS cows were greater ( $P < 0.05$ ) than those of the other breeds, and AG exhibited the poorest fat percentage (3.93%;  $P < 0.05$ ). Regarding the nitrogen fraction, AG and SI breeds had intermediate levels of both protein (3.57% and 3.58%, respectively) and casein (2.79% and 2.80%, respectively), and HF cows had the poorest percentage of both these constituents ( $P < 0.05$ ). The greatest (2.77) and lowest (2.50) values of SCS were estimated for BS and SI, respectively.

Overall, mineral content varied significantly across breeds. In particular, milk of SI cows had the greatest content of Ca and Na (1426.44 mg/kg and 460.69 mg/kg, respectively;  $P < 0.05$ ) and that of HF exhibited the lowest content of Ca and P (1306.60 mg/kg and 972.68 mg/kg, respectively;  $P < 0.05$ ). Milk of AG breed had significantly ( $P < 0.05$ ) greater Ca (1370.06 mg/kg) than that of HF (1306.60 mg/kg) and BS (1350.42 mg/kg).



**Table 3.** Least squares means (with standard errors) of milk yield, composition, somatic cell score (SCS) and mineral content of cows of different breeds.

Trait	Holstein- Friesian	Brown Swiss	Alpine Grey	Simmental
Milk yield, kg/d	25.28 (0.37) <sup>a</sup>	20.92 (0.17) <sup>b</sup>	14.67 (0.22) <sup>c</sup>	21.08 (0.20) <sup>b</sup>
Chemical composition, %				
Fat	4.06 (0.02) <sup>a</sup>	4.24 (0.01) <sup>b</sup>	3.93 (0.01) <sup>c</sup>	4.11 (0.01) <sup>a</sup>
Protein	3.38 (0.02) <sup>a</sup>	3.68 (0.01) <sup>b</sup>	3.57 (0.01) <sup>c</sup>	3.58 (0.01) <sup>c</sup>
Casein	2.65 (0.01) <sup>a</sup>	2.88 (0.01) <sup>b</sup>	2.79 (0.01) <sup>c</sup>	2.80 (0.01) <sup>c</sup>
SCS, units	2.61 (0.06) <sup>ab</sup>	2.77 (0.03) <sup>a</sup>	2.61 (0.04) <sup>b</sup>	2.50 (0.04) <sup>b</sup>
Mineral content, mg/kg				
Ca	1,306.60 (6.54) <sup>a</sup>	1,350.42 (3.08) <sup>b</sup>	1,370.06 (3.99) <sup>c</sup>	1,426.44 (3.60) <sup>d</sup>
K	1,686.26 (8.47) <sup>a</sup>	1,660.07 (3.98) <sup>b</sup>	1,665.40 (5.32) <sup>ab</sup>	1,675.73 (4.73) <sup>ab</sup>
Mg	138.52 (0.80) <sup>a</sup>	143.13 (0.38) <sup>b</sup>	137.24 (0.52) <sup>a</sup>	144.69 (0.46) <sup>b</sup>
Na	454.71 (1.97) <sup>a</sup>	452.52 (0.94) <sup>a</sup>	454.79 (1.24) <sup>a</sup>	460.69 (1.10) <sup>b</sup>
P	972.68 (4.74) <sup>a</sup>	1,034.79 (2.24) <sup>b</sup>	1,025.78 (2.91) <sup>b</sup>	1,030.70 (2.61) <sup>b</sup>

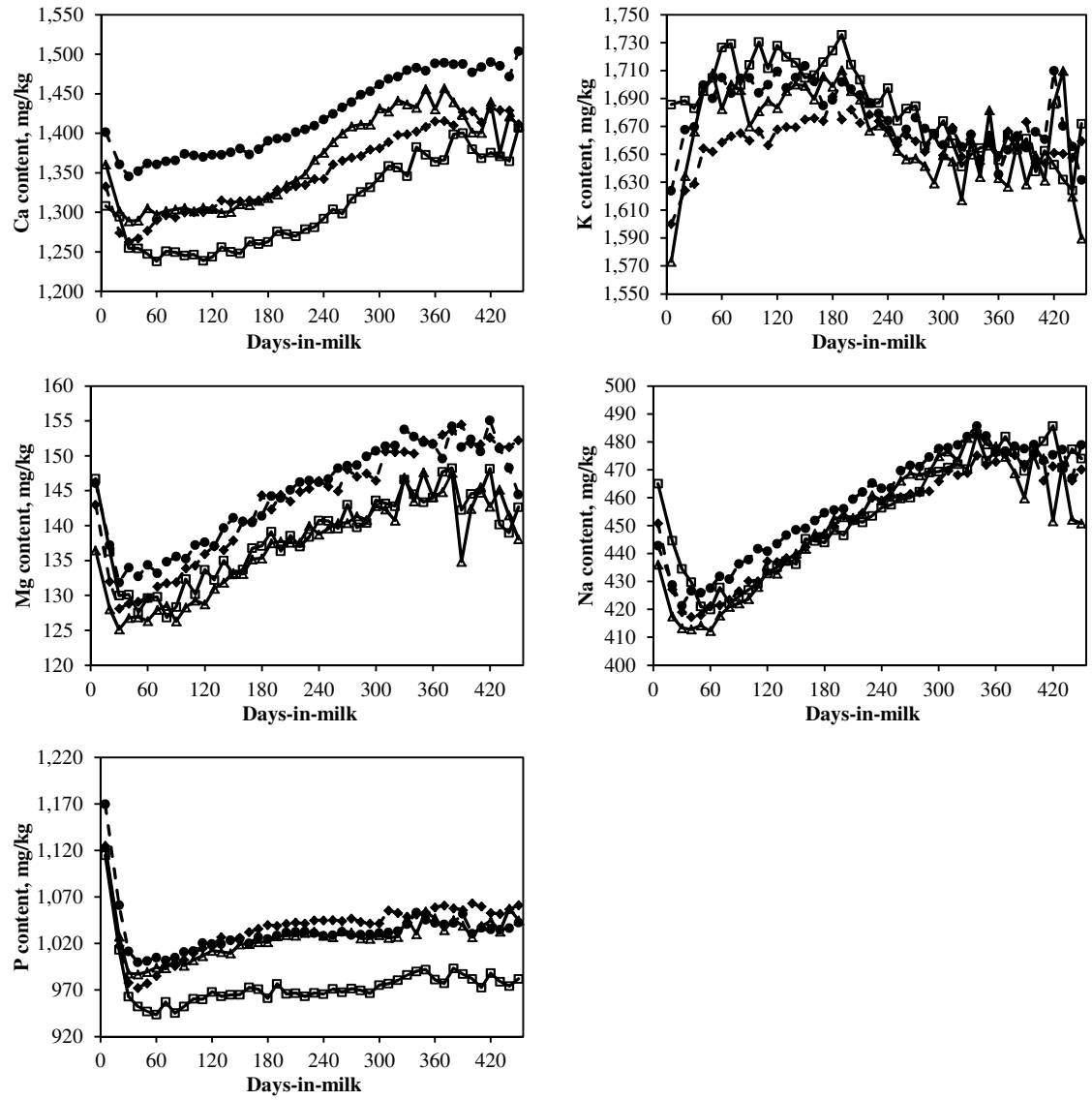
Least squares means with different superscript letters within a row are significantly different ( $P < 0.05$ ).

### *Lactation, parity, and temporal effects*

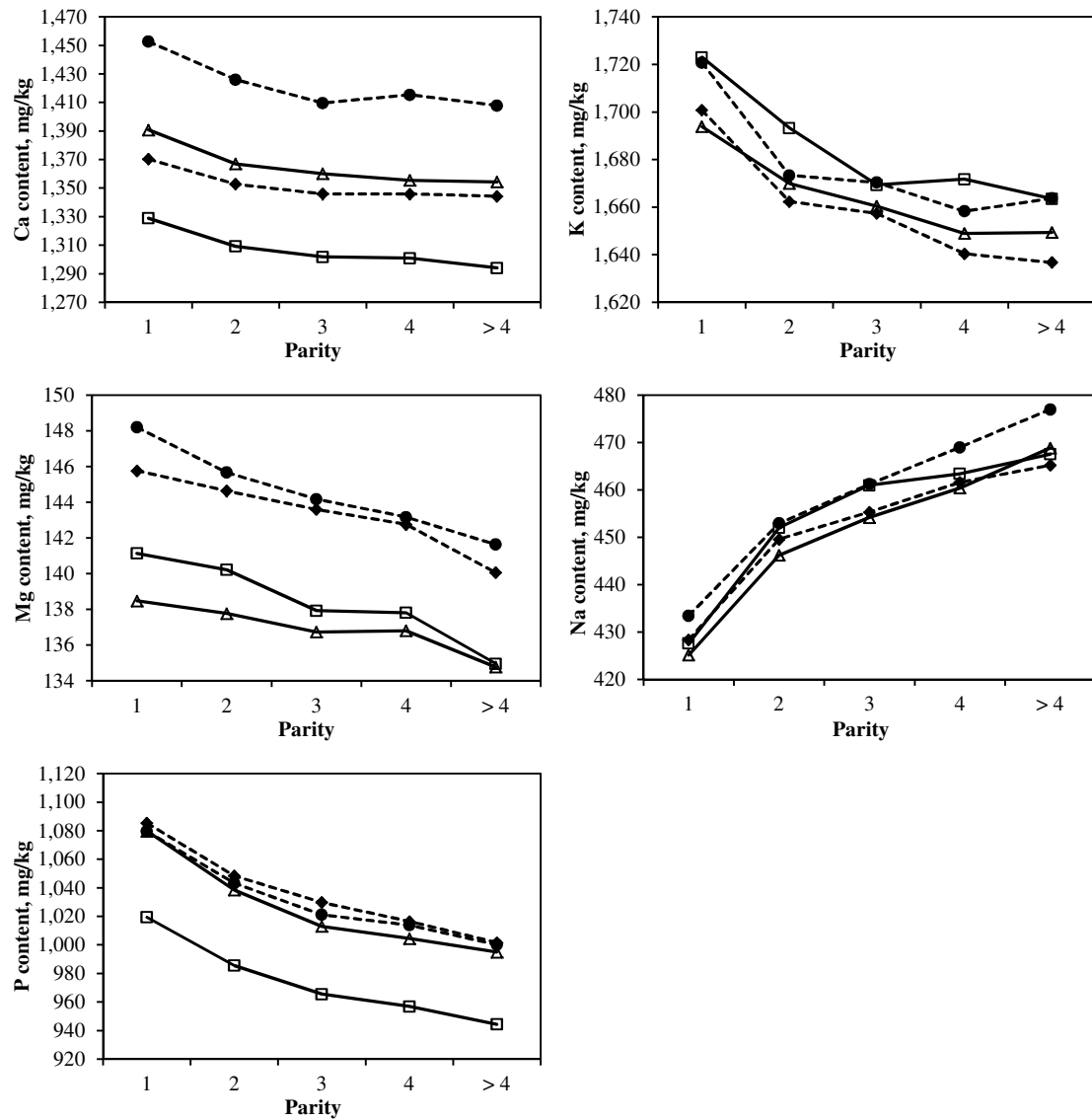
Figure 1 depicts the least squares means of predicted milk mineral composition across lactation for HF, BS, AG and SI cows. Calcium, Mg, Na and P contents exhibited an opposite trend to that of milk yield across days in milk, having the minimum peak of content at the maximum peak for milk yield, whereas K was the only mineral which resembled the trend of milk yield across lactation.

Trend of predicted mineral composition across parity is depicted in Figure 2. With the exception of Na, minerals contents were greater in milk of primiparous than multiparous cows, and the lowest values were observed for cows at fifth and later parity. Milk minerals content also varied across the calendar month of the year with similar trends for all the four cattle breeds considered in the present study (data not shown). The decrease of all minerals content which occurred in the first part of the year halted during the summer season (June to September).

**Figure 1.** Least squares means of (A) Ca, (B) K, (C) Mg, (D) Na and (E) P content across lactation for Holstein-Friesian (—□—), Brown Swiss (- -♦- -), Alpine Grey (—△—) and Simmental (- -●- -) cows.



**Figure 2.** Least squares means of (A) Ca, (B) K, (C) Mg, (D) Na and (E) P content across parity for Holstein-Friesian (—□—), Brown Swiss (-♦-), Alpine Grey (—△—) and Simmental (-●-) cows.



## DISCUSSION

In order to generate robust prediction models, large variability of both spectral and trait(s) of interest is desired in the calibration dataset. Mean mineral contents were consistent with findings of Soyeurt et al. (2009) on a dataset (n = 100) of HF, Jersey, Belgian Blue, Montbéliarde and Normande breeds, and Toffanin et al. (2015) on 208 HF cows. Moreover, coefficients of variation of milk minerals calculated in the present study were in accordance with the results of Soyeurt et al. (2009). In the present study, the variability of the spectral data used to develop the MIRS prediction models was sufficient to represent the 93% of the total spectra variation. This was somewhat expected given that the dataset used to develop the prediction models by Visentin et al. (2016) included samples of the same province and breeds considered in the present study.

The importance of milk minerals on MCP has been reported by Malacarne et al. (2014) and Toffanin et al. (2015), who demonstrated that milk samples with optimal coagulation profile were those with the greatest content of Ca (and P), particularly colloidal, and total Mg, and the lowest values of chloride. The correlations between milk minerals, and between milk minerals and milk composition were expected since about two third of Ca and P, and one third of Mg measured in milk, are bonded to form calcium phosphate, which plays an essential role for the casein micelle structure (Holt et al., 2013). Finally, the positive association between Na and SCS was probably due to physiological reason; indeed, an infection to the mammary gland, which results in higher SCS, increases the permeability of blood capillaries. Therefore, Na and chloride, which are more concentrated into the extracellular liquid, are poured into the mammary lumen (Summer et al., 2009).

### ***Effects of cow breed on predicted traits***

The differences of milk chemical composition across cow breeds have been well documented in literature (Barlowska et al., 2006; Carroll et al., 2006; Malacarne et al., 2006; De Marchi et al., 2007; Penasa et al., 2014) and such differences were consistent with the results of the present study. Milk mineral characterization of HF was investigated by Carroll et al. (2006) and van Hulzen et al. (2009). Also, milk mineral profile of SI and BS breeds was studied by Barlowska et al. (2006) and Carroll et al. (2006), respectively. To our knowledge, the present study is the first dealing with the determination of milk minerals content of AG cows, a local cattle breed reared in the Alps. Alpine local breeds are part of cultural and social traditions and, although characterized by lower productivity compared to HF cows, they are more adapted to marginal areas. Moreover, De Marchi et al. (2007), in agreement with results of the present study, demonstrated that local breeds, including AG, produced milk with more favourable processing characteristics (i.e. shorter RCT and greater  $a_{30}$ ) than HF and BS cows. Recently Pretto et al. (2013) estimated a positive association between  $a_{30}$  and cheese yield, and such a conclusion could have serious implications for the valorisation of local Alpine breeds.

### ***Effects of stage of lactation and parity on predicted traits***

Variation of Ca, Mg, Na and P contents throughout lactation (Figure 1) was consistent with Carroll et al. (2006) and van Hulzen et al. (2009), following an opposite trend than milk yield. Therefore, a dilution effect could be assumed to explain this variation. Moreover, the increase of the aforementioned elements could be associated with the higher casein level of milk from cows in late lactation, as reported by Holt et al. (2013). Particularly at the onset of lactation, an excess of

dietary cations is a risk factor for milk fever, and forages normally offered to dry cows have high K content (Goff and Horst, 1997). Therefore, the increment of milk K content observed in the present study in early lactation might be due to a dietary excess of this mineral, although no data on feeding rations were available to justify this hypothesis. Van Hulzen et al. (2009) estimated that herd variance for K was, on average, 50% greater than herd variance for other minerals, such as Ca, Mg, P and Zn, and suggested that management strategies (such as feeding) could have a relatively high impact on K variation. Such a statement on K content was also supported by the results of the present study, as the proportion of phenotypic variance accounted by the herd effect was larger than the variance accounted by the cow effect. The variation across calendar month for milk minerals might be due to the change in the diet of animals reared in the Alpine area, since a quite common practice in Alpine regions is to move cows to highland pastures during summer (June to September).

Least squares means of Ca and P contents across parity (Figure 2) were consistent with the findings of Kume et al. (1998), who reported the same decreasing trend in Holstein dairy cows. Indeed, it has been hypothesized that the utilization of these minerals at the mammary gland level is lower in multiparous cows (Kume and Tanabe, 1993). In particular, the mobilization of Ca from bones to blood is less efficient due to a lower activity of parathyroid hormone, which is involved in osteoplastic activities. In the present study, a reduction of casein percentage in older cows was observed, and this evidence could explain the decreasing trend of Ca, P and Mg contents, which are known to be mainly bonded to casein micelle. The lowest K and the highest Na contents were observed in multiparous cows, and this was in association with the increasing trend of SCS across parity. Great Na and low K levels are associated with high SCS (Summer et al., 2009), which is an indicator of

(sub)clinical mastitis. Overall, the decreasing trend for Ca, K, Mg and P might be also explained by a dilution effect of these minerals due to higher milk yield across parity observed in the present study.

## **CONCLUSIONS**

Mid-infrared spectroscopy prediction models can be successfully applied to large spectral datasets for phenotypic characterization of major minerals in milk. Breed, stage of lactation and parity were important sources of variation of major milk mineral contents at the population level, and significant differences among breeds were detected for all traits. Dual-purpose breeds were characterized by greater mineral contents compared to dairy breeds. Factors identified in the present study will be useful for within-breed genetic analyses to adjust records for significant environmental effects.

## **ACKNOWLEDGMENTS**

The authors would like to thank Luis Kerschbaumer and Thomas Kerschbamer (South Tyrol Dairy Association – Sennereiverband Südtirol, Bolzano, Italy), Christian Plitzner (Breeders Association of Bolzano province – Vereinigung der Südtiroler Tierzuchtverbände, Bolzano, Italy) and Claudio Stecchi (CTS informatica srl, Bolzano, Italy) for providing data used in this study and for technical support. This study was supported by the University of Padova (Ricerca Scientifica fondi quota EX 60% - 2014, project 60A08-7955/14, Italy).

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**Processing characteristics of dairy cow milk are moderately heritable**

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## ABSTRACT

Milk processing attributes represent a group of milk quality traits which are important to the dairy industry to inform product portfolio. Because, however, of the resources required to routinely measure such quality traits, there is a dearth of precise genetic parameter estimates from a large population of animals for these traits. Milk processing characteristics considered in the present study, namely rennet coagulation time, curd-firming time, curd firmness at 30 and 60 min after rennet addition, heat coagulation time, casein micelle size, and milk pH were all estimated using mid-infrared spectroscopy prediction equations. Variance components for these traits were estimated using 136,807 test-day records from 5 to 305 DIM from 9,824 cows using random regressions to model the additive genetic and within-lactation permanent environmental variances. Heritability estimates ranged from  $0.18 \pm 0.01$  (26 DIM) to  $0.38 \pm 0.02$  (180 DIM) for rennet coagulation time, from  $0.26 \pm 0.02$  (5 DIM) to  $0.57 \pm 0.02$  (174 DIM) for curd-firming time, from  $0.16 \pm 0.01$  (30 DIM) to  $0.56 \pm 0.02$  (271 DIM) for curd firmness at 30 min, from  $0.13 \pm 0.01$  (30 DIM) to  $0.48 \pm 0.02$  (271 DIM) for curd firmness at 60 min, from  $0.08 \pm 0.01$  (17 DIM) to  $0.24 \pm 0.01$  (180 DIM) for heat coagulation time, from  $0.23 \pm 0.02$  (30 DIM) to  $0.43 \pm 0.02$  (261 DIM) for casein micelle size, and from  $0.20 \pm 0.01$  (30 DIM) to  $0.36 \pm 0.02$  (151 DIM) for milk pH. Within-trait genetic correlations across DIM weakened as the number of days between compared intervals lengthened but were mostly  $>0.4$  except between the peripheries of the lactation. Eigenvalues and associated eigenfunctions of the additive genetic covariance matrix for all traits revealed that at least the 80% of the genetic variation among animals in lactation profiles was associated with the height of the lactation profile. Curd-firming time and curd firmness at 30 min were weakly to moderately genetically correlated with milk yield (from  $0.33 \pm 0.05$  to  $0.59$

$\pm 0.05$  for curd-firming time, and from  $-0.62 \pm 0.03$  to  $-0.21 \pm 0.06$  for curd firmness at 30 min). Milk protein concentration was strongly genetically correlated with curd firmness at 30 min ( $0.84 \pm 0.02$  to  $0.94 \pm 0.01$ ), but only weakly genetically correlated with milk heat coagulation time ( $0.19 \pm 0.06$  to  $-0.27 \pm 0.07$ ). Results from the present study indicate the existence of exploitable genetic variation for milk processing characteristics; because of possible indirect deterioration in milk processing characteristics due to selection for greater milk yield, emphasis on milk processing characteristics is advised.

**Key words:** milk coagulation; milk quality; milk technological; spectrometry; random regression

## INTRODUCTION

The importance of milk composition and udder health in the production of dairy products is generally well accepted (Williams, 2003; Murphy et al., 2016). Such importance underpins the inclusion of both milk composition and udder health in several dairy cow breeding objectives globally (Miglior et al., 2005). However, milk processability, which dictates the potential of transforming milk into different dairy products such as cheese and milk powder, is also an important characteristic of milk composition. Despite this, milk processability is not explicitly considered in national dairy cow breeding objectives.

Indicators of milk processability are commonly referred to milk coagulation properties and these include rennet coagulation time (**RCT**, min), curd-firming time (**k<sub>20</sub>**, min), curd firmness 30 (**a<sub>30</sub>**, mm) and 60 (**a<sub>60</sub>**, mm) min after rennet addition, and heat coagulation time (**HCT**, min), casein micelle size (**CMS**, nm), and milk pH.



Several factors are known to contribute to variability in milk processing characteristics such as cow breed (De Marchi et al., 2007; Poulsen et al., 2013; Chen et al., 2016), stage of lactation (Ikonen et al., 2004; Barłowska et al., 2014), parity number (Ikonen et al., 1999; Tyrisevä et al., 2004), and the diet ingested (Reid et al., 2015). The existence of genetic variation in milk coagulation properties has been documented in several Holstein-Friesian populations including populations from Italy (1,042 cows, Cassandro et al., 2008), Estonia (4,191 cows, Vallas et al., 2010; 5,216 cows, Pretto et al., 2014), Denmark (357 cows; Poulsen et al., 2015), and Finland (399 cows, Tyrisevä et al., 2004). These studies, however, have been limited in size contributing to large associated standard errors of the estimated (co)variance components, and these studies have estimated genetic parameters of milk coagulation properties using repeatability animal models; the exception to the latter are both Vallas et al. (2010) and Pretto et al. (2014) who used random regression animal models. To our knowledge, no study has attempted to quantify the existence of genetic variation in either HCT or CMS in dairy cows.

Large-scale recording of milk processing traits is often hampered by the associated resources necessary to undertake the reference methodologies. This therefore not only limits the dataset sizes to estimate precise genetic parameters, but also precludes the implementation of breeding strategies to improve these characteristics directly. Milk mid-infrared spectroscopy has been proposed as a phenotyping tool for a multitude of animal characteristics (Bastin et al., 2016; McParland and Berry, 2016) and milk quality traits (De Marchi et al., 2014), including milk processing attributes (Visentin et al., 2015). One of the main advantages of mid-infrared spectroscopy is that, once developed, new prediction

models can be applied to historical spectral data for the prediction of novel traits at no additional cost.

Therefore, the objective of the present study was to quantify the genetic variation in milk processing characteristics, and their correlations with milk-related performance traits, using random regression models fitted across lactation. Results of the present study will provide a greater and more precise knowledge of the extent of genetic variability that exists in milk processing characteristics and how this changes across lactation.

## **MATERIALS AND METHODS**

### ***Data***

Milk samples used in the present study originated from 76 Irish herds collected between January 2013 and December 2015, inclusive; a total of 174,062 milk samples from 10,394 dairy cows were collected.

Sixty-nine out of the aforementioned 76 farms were commercial herds located in the Munster region of Ireland; the average herd size was 126 cows. Animals in the commercial herds were milked twice a day, at approximately 0700 hr (**AM**) and again at approximately 1500 hr (**PM**), and a combined AM+PM individual milk sample was sporadically collected and sent for mid-infrared spectroscopy analysis to the laboratory of Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork, Ireland). On average, 1,249 samples were analyzed each month. Milk yield of the commercial farms represented the entire daily milk produced during the test-day recording. The average number of collected milk samples per cow was 5.15 and a total of 14,873 lactation records were available.

The remaining farms were operated by Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork). In these research herds, a total of 1,661 dairy cows were participating in various experimental treatments based on different feeding strategies and different management practices, including various stocking rates, calving periods, and length of grazing period. All cows were fed a basal diet of grazed pasture, but at times were offered a quantity of concentrates according to the experimental treatment. Animals were milked twice a day (AM and PM) and milk yield was recorded at each milking session. Milk yield of the two daily milking sessions was summed in order to obtain daily milk yield. Individual milk samples were taken separately, once weekly, on consecutive PM and AM milking. The average number of milk samples collected per cow was 78, while the total number of lactations available was 2,956.

Spectra information and milk chemical composition (i.e., concentration of protein, casein, fat, lactose, total solids, and urea) of all milk samples were generated using the same MilkoScan FT6000 (Foss Electronic A/S, Hillerød, Denmark) in the laboratory of Teagasc Animal and Grassland Research and Innovation Center (Moorepark, Fermoy, Co. Cork, Ireland), within a week of sample collection. The resulting milk spectra, containing 1,060 transmittance data points in the region between 900 and 5,000  $\text{cm}^{-1}$ , were stored. Somatic cell count (SCC) of all samples was determined using a Fossomatic (Foss Electronic A/S, Hillerød, Denmark).

### ***Milk Processing Phenotype Prediction***

Between the years 2013 and 2014, a calibration dataset was generated from individual bovine milk samples collected from the seven Irish research herds as described in detail by Visentin et al. (2015); data from cows milking in these 7

research farms also contributed to the larger dataset in the present study. Full details on the development of the mid-infrared spectroscopy prediction models for milk technological traits, including RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, and pH are reported by Visentin et al. (2015). RCT represents the time required to induce milk coagulation after rennet addition,  $k_{20}$  is the time between the gel development and the achievement of a width of 20 mm in the graph, and  $a_{30}$  and  $a_{60}$  are the width of the graph after 30 and 60 min from rennet addition, respectively. Milk HCT was the time taken for milk within a hot oil bath at 140 °C at an oscillating speed of 8 rpm to start to flocculate. Casein micelle size represents the average diameter of casein micelles of a milk sample.

The developed prediction models (Visentin et al., 2015) were applied to the larger spectral dataset of the current study to obtain the predicted milk processing phenotypes. Briefly, spectral data of the present study were converted from transmittance to absorbance by taking the  $\log_{10}$  of the reciprocal of the transmittance, and spectral wavelengths with low signal-to-noise ratio (1,580-1,710  $\text{cm}^{-1}$  and 2,990-3,690  $\text{cm}^{-1}$ ) were discarded. Principal component analysis (PROC PRINCOMP, SAS Institute Inc., Cary, NC, USA) was performed on the resulting edited spectra and the Mahalanobis distance from the centroid of the cluster of the samples included in the calibration dataset was calculated. Spectra whose Mahalanobis distance was greater than the 97.5% percentile of a  $\chi^2$  distribution with 4 (i.e. lowest number of principal component whose eigenvalues was greater than 1) degrees of freedom were considered as outliers and discarded. Following these edits, 157,192 spectra from 10,112 dairy cows remained, and the prediction models were applied to these spectra to generate predicted values for RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, and pH.

### ***Data Editing***

Only records between 5 and 305 DIM from parities  $\leq 10$  were retained. Obvious data errors for predicted RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, and pH and the milk production traits (i.e., milk yield, SCC, and concentrations of protein, fat, casein, lactose, total solids, and urea) were discarded. Somatic cell score (SCS) was calculated as  $\log_{10}(\text{SCC}/1,000)$ . Subsequently, records greater than 3 standard deviations from the mean of each trait were discarded. Cow breed composition was defined as the proportion of Holstein, Friesian, Jersey, Montbeliarde, Norwegian Red, and “Other”. Two contemporary groups were generated: 1) experimental treatment-test-date for milk samples from cows in the research herds and 2) herd-test-date for milk samples from cows in the commercial herds. Only contemporary groups with at least 10 observations were retained. Following all these edits, 136,807 milk samples from 16,543 lactations from 9,824 dairy cows were available for further analyses.

Coefficients of general heterosis and recombination loss were calculated for each cow as described by VanRaden and Sanders (2003):

$$\text{Heterosis} = 1 - \sum_{i=1}^n \text{sire}_i * \text{dam}_i$$

and

$$\text{Recombination loss} = 1 - \sum_{i=1}^n \frac{\text{sire}_i^2 + \text{dam}_i^2}{2},$$

where  $\text{sire}_i$  and  $\text{dam}_i$  are the proportion of genes of the breed  $i$  in the sire and the dam, respectively.

## Data Analysis

Variance components for milk processing characteristics, milk yield, and milk composition were estimated using random regression models fitted across lactation in ASREML (Gilmour et al., 2011). The pedigree of all animals was traced back at least four generations (where available), and comprised 41,232 animals. The data were divided into 10 groups based on days post-calving as 5 to 30 DIM, 31 to 60 DIM, 61 to 90 DIM, ..., 241 to 270 DIM, and 271 to 305 DIM. Within group, the estimated residual variance was assumed to be homogenous, whereas between groups the estimated residual variance could be heterogeneous. No residual covariance was assumed to exist among groups. The fitted model was:

$$y_{ijklmnopq} = Cont\_group_i + Session_j + \sum_{i=1}^k Breed_k + Het_l + Rec_m + \sum_{i=1}^n Par_o b_n DIM^n + \sum_{i=1}^n Cow_p b_n DIM^n + \sum_{i=1}^n PEwithin_p b_n DIM^n + PEacross_p + e_{ijklmnopq},$$

where  $y_{ijklmnopq}$  is RCT,  $k_{20}$ ,  $a_{30}$ ,  $a_{60}$ , HCT, CMS, pH, test-day milk yield, protein %, fat %, casein %, lactose %, urea mg/dL, and SCS for the animal  $p$ ;  $Cont\_group_i$  is the fixed effect of the  $i$ -th contemporary group (5,709 classes);  $Session_j$  is the fixed effect of the  $j$ -th class of milking session (3 classes: AM, PM, or combined AM+PM);  $Breed_k$  is the fixed effect of the  $k$ -th proportion of the  $k$ -th breed (5 breed proportions: Friesian, Jersey, Montbeliarde, Norwegian Red, and “Other”, all treated separately as continuous variables);  $Het_l$  is the fixed effect of the  $l$ -th class of coefficient of heterosis (12 classes: 0, >0 and  $\leq 0.1$ , ..., >0.9 and <1, 1);  $Rec_m$  is the fixed effect of the  $m$ -th class of coefficient of recombination loss (12 classes: 0, >0 and  $\leq 0.1$ , ..., >0.9 and <1, 1);  $Par_o$  is the fixed effect of the  $o$ -th class of parity (5 classes: 1, 2, 3, 4,  $\geq 5$ );  $b_n$  is the  $n$ -th order Legendre polynomial of DIM;  $Cow_p$  is the random effect for the additive genetic effect of the  $p$ -th animal;  $PEwithin_p$  is the random effect for the

within-lactation permanent environmental effect of the  $p$ -th animal;  $PE_{across_p}$  is the random effect for the across-lactation permanent environmental effect of the  $p$ -th animal;  $e_{ijklmnopq}$  is the residual term.

The most parsimonious order of the fixed Legendre polynomial was evaluated based on visual inspection of the resulting lactation profile for the different polynomial orders for each trait studied. In all instances, a third-order Legendre polynomial was the most appropriate, with minimal difference detected from the lactation profiles generated from higher order polynomials. To select the most parsimonious order for the random Legendre polynomial, different combinations of covariance functions were tested. The first model included a first order Legendre polynomial fitted only to the additive genetic effect. The second model considered a first order Legendre polynomial fitted also to the within-lactation permanent environmental effect. In the subsequent models, the polynomial order was increased incrementally by one unit with the order of the additive genetic effect being increased first, followed then by the within-lactation permanent environmental effect. The criteria set to select the most parsimonious order of the random Legendre polynomial were: 1) the log-likelihood ratio test of two nested models (Wilks, 1938), 2) the Akaike information criterion, and 3) the eigenvalues of the additive genetic (co)variance matrix to quantify the contribution of the highest polynomial order to the entire genetic variance. A third-order Legendre polynomial on the additive genetic effect and on the within-lactation permanent environmental effect minimized the Akaike information criterion and maximized model log-likelihoods.

Genetic covariance function coefficients from the random regression analyses were calculated as

$$\delta^2 = \Phi K \Phi',$$

where  $\delta^2$  is the 301 x 301 (co)variance matrix for the predicted milk technological trait, milk yield and milk chemical composition trait;  $\Phi$  is the 301 x  $n$  matrix of Legendre polynomial of DIM regression coefficients; and  $\mathbf{K}$  is the  $n$  x  $n$  estimated additive genetic (or within lactation permanent environmental effect) (co)variance matrix of the random polynomial coefficient. Standard errors of the heritability estimates were calculated using a Taylor series expansion as outlined in Fisher et al. (2004).

Genetic correlations between milk processing traits, milk yield, and milk chemical composition were calculated using a series of bivariate random regression models in ASREML (Gilmour et al., 2011) using the statistical model previously described for the univariate analyses. Residual groups were as defined in the univariate analyses, but residual covariances between traits within residual group were estimated. In order to achieve model convergence, the polynomial order of the random terms was reduced to a quadratic polynomial. Standard errors of the genetic correlations were estimated as outlined in Falconer and MacKay (1996).

For each studied trait, eigenvalues and eigenvectors were calculated from the additive genetic covariance matrix using PROC IML (SAS Institute Inc., Cary, NC, USA), and eigenfunctions were calculated as the product of the eigenvectors and the Legendre polynomial coefficients matrix.

In a supplementary analysis, the heritability and repeatability of milk yield and each of the milk processing and composition traits were also calculated using a univariate repeatability animal linear mixed model. Fixed effects fitted were the same as in the model previously described, but replacing the sets of fixed effects Legendre polynomial coefficients with stage of lactation (10 classes: 5-30 DIM, 31-60 DIM, ..., 241-270 DIM, and 271-305 DIM) as per Visentin et al. (2017).



The impact of genetic selection for milk processability on milk yield, and vice versa, was undertaken using selection index theory. An economic weight of one was applied to milk yield which was the only goal trait. (Co)variance components used in the selection index were those estimated in the present study averaged across all DIM from the bivariate random regression models; phenotypic correlations were those reported by Visentin et al. (2017). A restriction selection index was used to quantify the weighting required on the milk processing trait(s) of interest to halt any indirect deterioration due to selection on milk yield. The relative emphasis on an individual trait  $i$  was calculated as in Berry (2015):

$$\text{Emphasis}_i = \frac{|a_i * \sigma_i|}{\sum_{j=1}^n |a_j * \sigma_j|}$$

where  $a_i$  and  $a_j$  are the economic weight for the trait  $i$  and  $j$ , respectively, and  $\sigma_i$  and  $\sigma_j$  are the genetic standard deviations for trait  $i$  and  $j$ , respectively. Selection index theory was also used to quantify the number of progeny or number of lactations per cow required to achieve a given level of reliability for a milk processing trait of interest.

The proportion of genetic variation (i.e., coefficient of genetic variation) in each milk processing trait independent of genetic merit for milk protein percentage was estimated as:

$$CV_G = \frac{\sqrt{\sigma_{G_i}^2 * (1 - r_{i,prt}^2)}}{|\mu_i|} \%$$

where  $\sigma_{G_i}^2$  is the estimated additive genetic variance of the trait  $i$  in the present study,  $r_{i,prt}^2$  is the genetic correlation between the trait  $i$  and milk protein percentage estimated in the present study, and  $\mu_i$  is the mean of the trait  $i$  in the present study.

## RESULTS

Fewer records were available at the start and end of lactation (8,329 and 3,590 observations between 5-30 DIM and 271-305 DIM, respectively) relative to the other stages, in which the number of records ranged between 11,002 (241-270 DIM) and 17,954 (91-120 DIM). The descriptive statistics, heritability and repeatability (estimated using the repeatability animal model) of the milk processing traits, milk yield, and milk composition are in Table 1. The coefficient of genetic variation for the milk technological traits varied from 3.08% ( $a_{60}$ ) to 11.51% ( $k_{20}$ ), with the exception of CMS (2.06%) and milk pH (0.30%; Table 1). A large coefficient of genetic variation (10.79%) was evident for milk yield, whereas for milk composition, the coefficient of genetic variation ranged from 1.89% (lactose concentration) to 7.83% (fat concentration; Table 1). The heritability estimates for milk processing traits calculated using the repeatability animal model ranged from  $0.16 \pm 0.01$  (HCT) to  $0.43 \pm 0.02$  ( $k_{20}$ ), while the estimated heritability for the milk composition traits ranged from  $0.05 \pm 0.01$  (SCS) to  $0.46 \pm 0.02$  (protein and casein concentrations; Table 1).

**Table 1.** Number of samples (n), mean, phenotypic standard deviation (SD), heritability (standard error), repeatability (t; standard error), and coefficient of genetic variation (CV) for milk processing traits, milk yield, and milk composition post-editing.

Trait <sup>1</sup>	n	Mean	SD	h <sup>2</sup> (SE)	t (SE)	CV
RCT, min	136,102	20.37	6.22	0.28 (0.01)	0.36 (0.01)	8.10
k <sub>20</sub> , min	136,340	5.30	1.56	0.43 (0.02)	0.53 (0.01)	11.51
a <sub>30</sub> , mm	126,799	29.72	7.16	0.36 (0.02)	0.45 (0.01)	7.20
a <sub>60</sub> , mm	135,605	31.46	3.88	0.27 (0.01)	0.34 (0.01)	3.08
HCT, min	134,185	6.79	4.40	0.16 (0.01)	0.29 (0.01)	10.90
CMS, nm	136,165	169.57	12.41	0.31 (0.02)	0.43 (0.01)	2.06
pH, units	136,126	6.69	0.08	0.27 (0.01)	0.38 (0.01)	0.30
Milk yield, kg/d	117,279	20.87	6.35	0.18 (0.02)	0.52 (0.01)	10.79
Protein, %	128,561	3.71	0.39	0.46 (0.02)	0.59 (0.01)	4.58
Fat, %	128,647	4.60	1.07	0.29 (0.01)	0.31 (0.01)	7.83
Casein, %	128,615	2.81	0.33	0.46 (0.02)	0.59 (0.01)	4.98
Lactose, %	128,510	4.76	0.21	0.36 (0.02)	0.49 (0.01)	1.89
Urea, mg/dL	127,996	30.60	12.53	0.14 (0.01)	0.25 (0.01)	6.37
SCS, units	76,595	1.79	0.49	0.05 (0.01)	0.44 (0.01)	5.03

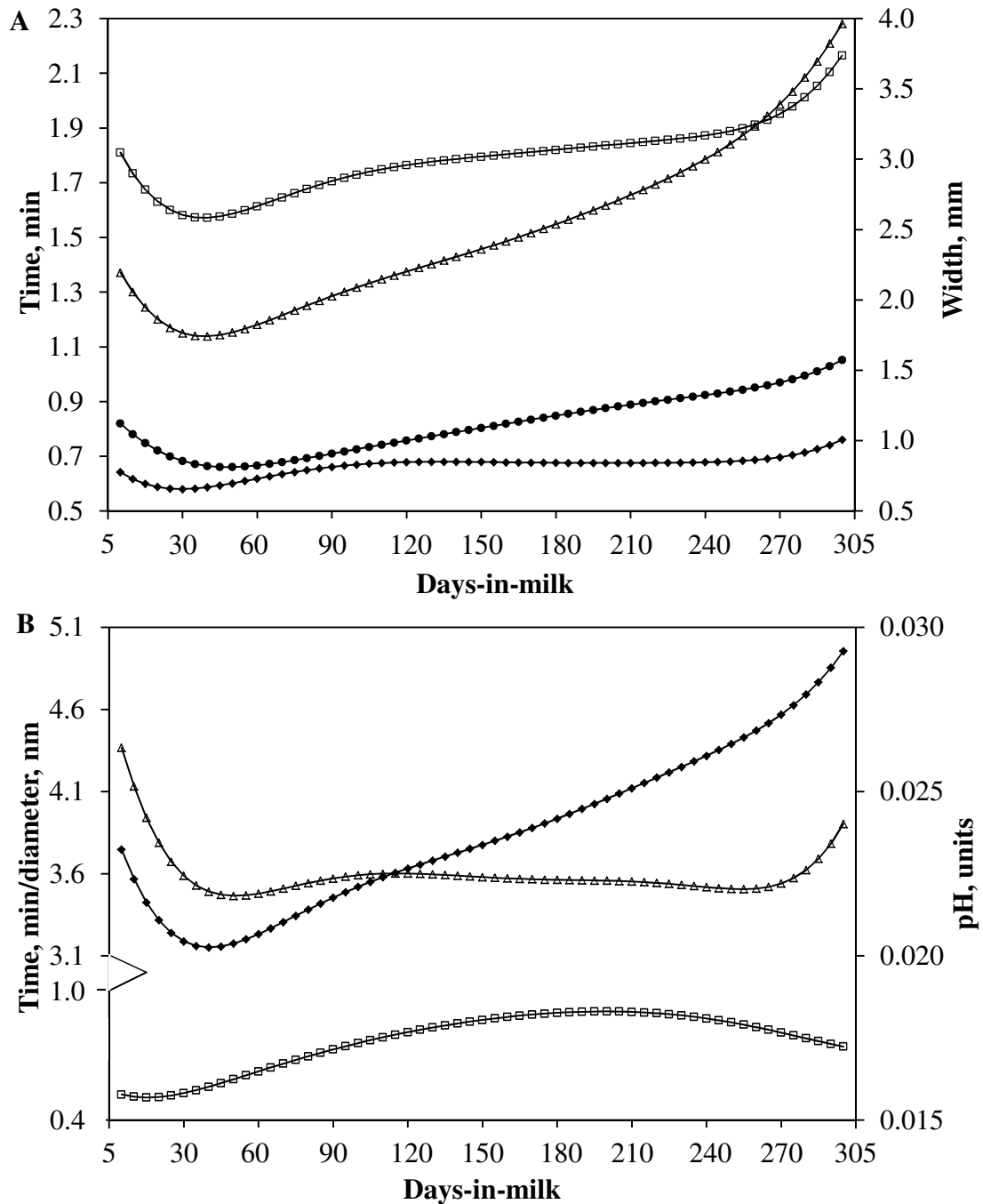
<sup>1</sup> RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness at 30 min; a<sub>60</sub> = curd firmness at 60 min; HCT = heat coagulation time; CMS = casein micelle size; SCS = log<sub>10</sub>(SCC).

### ***Genetic Variation and Heritability Estimates from the Random Regression Models***

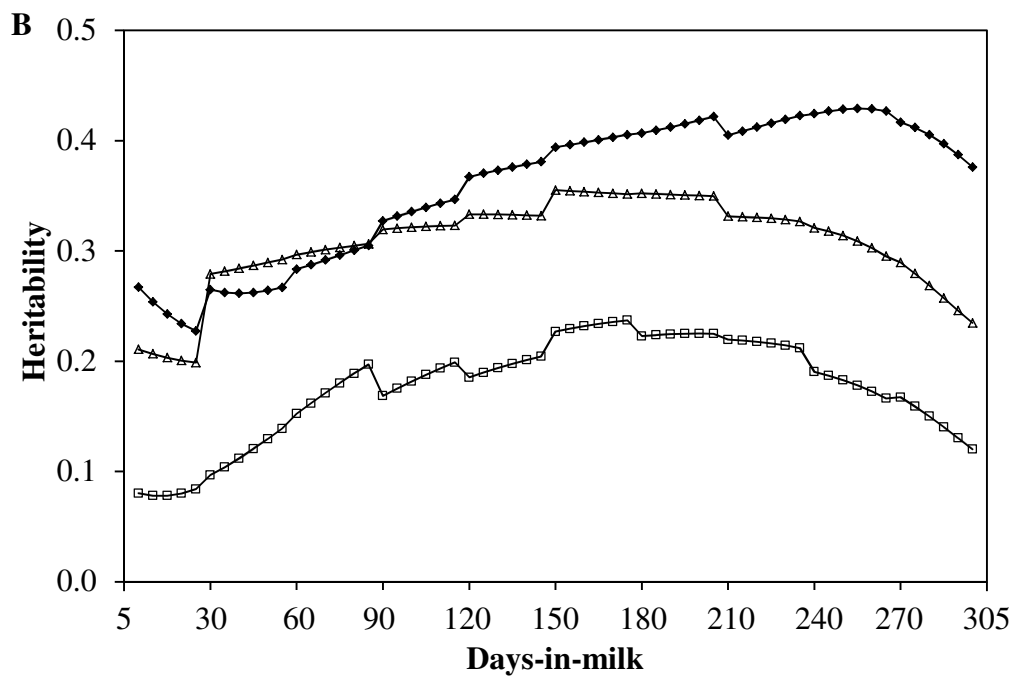
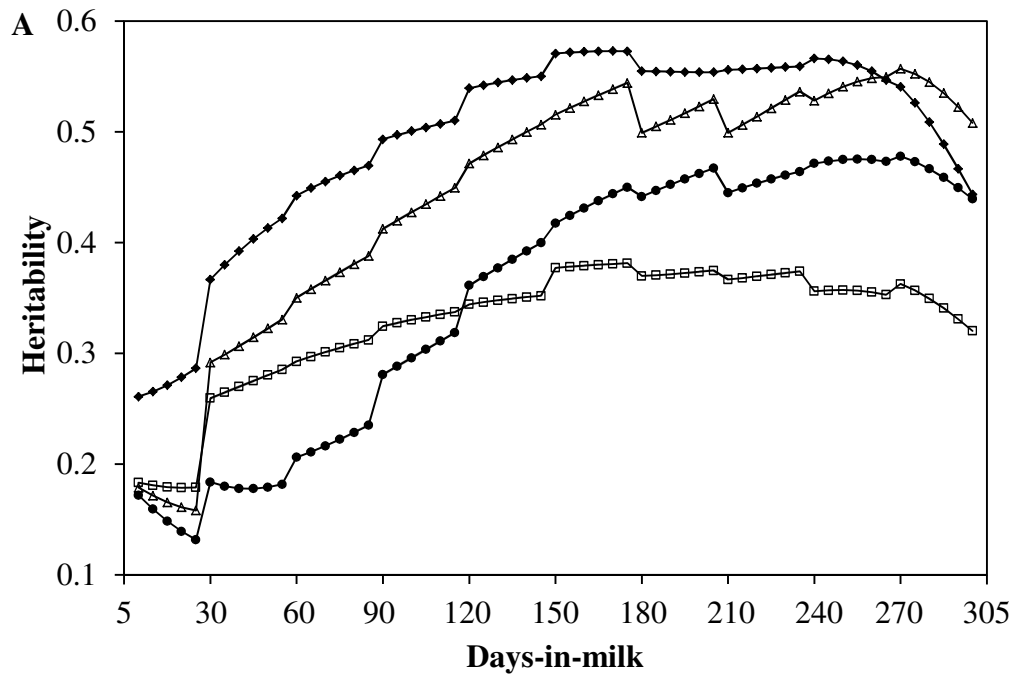
The genetic standard deviation across lactation of all milk coagulation properties and CMS followed a similar trend to each other (Figure 1), reducing from the beginning of lactation to 31-60 DIM, and increasing thereafter. The genetic standard deviation ranged from  $1.57 \pm 0.09$  min (43 DIM) to  $2.24 \pm 0.29$  min (305

DIM) for RCT, from  $0.58 \pm 0.01$  min (34 DIM) to  $0.78 \pm 0.03$  min (305 DIM) for  $k_{20}$ , from  $1.74 \pm 0.11$  mm (44 DIM) to  $4.12 \pm 0.62$  mm (305 DIM) for  $a_{30}$ , and from  $0.81 \pm 0.03$  mm (53 DIM) to  $1.62 \pm 0.12$  mm (305 DIM) for  $a_{60}$  (Figure 1). The genetic standard deviation for HCT decreased immediately after the onset of lactation ( $0.44 \pm 0.02$  min; 21 DIM), but increased thereafter until 210 DIM ( $0.96 \pm 0.03$  min), after which it decreased again (Figure 1). The genetic standard deviation for CMS was least at 45 DIM ( $3.15 \pm 0.36$  nm), but increased thereafter until the end of lactation ( $5.07 \pm 1.24$  nm; Figure 1). The genetic standard deviation of milk pH was small, ranging from  $0.022 \pm 0.001$  at 56 DIM to  $0.028 \pm 0.004$  at 5 DIM (Figure 1).

In general, heritability estimates for milk processing characteristics were the lowest in very early lactation (5-30 DIM), concurrent also with the greatest estimates of residual variances. The most heritable milk coagulation properties trait was  $k_{20}$  ( $0.26 \pm 0.02$  at 5 DIM to  $0.57 \pm 0.02$  at 174 DIM), followed by  $a_{30}$  ( $0.16 \pm 0.01$  at 30 DIM to  $0.56 \pm 0.02$  at 271 DIM),  $a_{60}$  ( $0.13 \pm 0.01$  at 30 DIM to  $0.48 \pm 0.02$  at 271 DIM), and RCT ( $0.18 \pm 0.01$  at 26 DIM to  $0.38 \pm 0.02$  at 180 DIM; Figure 2). Heritability estimates for the remaining processing attributes ranged from  $0.08 \pm 0.01$  (17 DIM) to  $0.24 \pm 0.01$  (180 DIM) for HCT,  $0.23 \pm 0.02$  (30 DIM) to  $0.43 \pm 0.02$  (261 DIM) for CMS, and  $0.20 \pm 0.01$  (30 DIM) to  $0.36 \pm 0.02$  (151 DIM) for pH (Figure 2). This therefore suggests that 8 to 56% of the adjusted phenotypic variability in the milk processing characteristics investigated in the present study were due to differences in additive genetic effects.



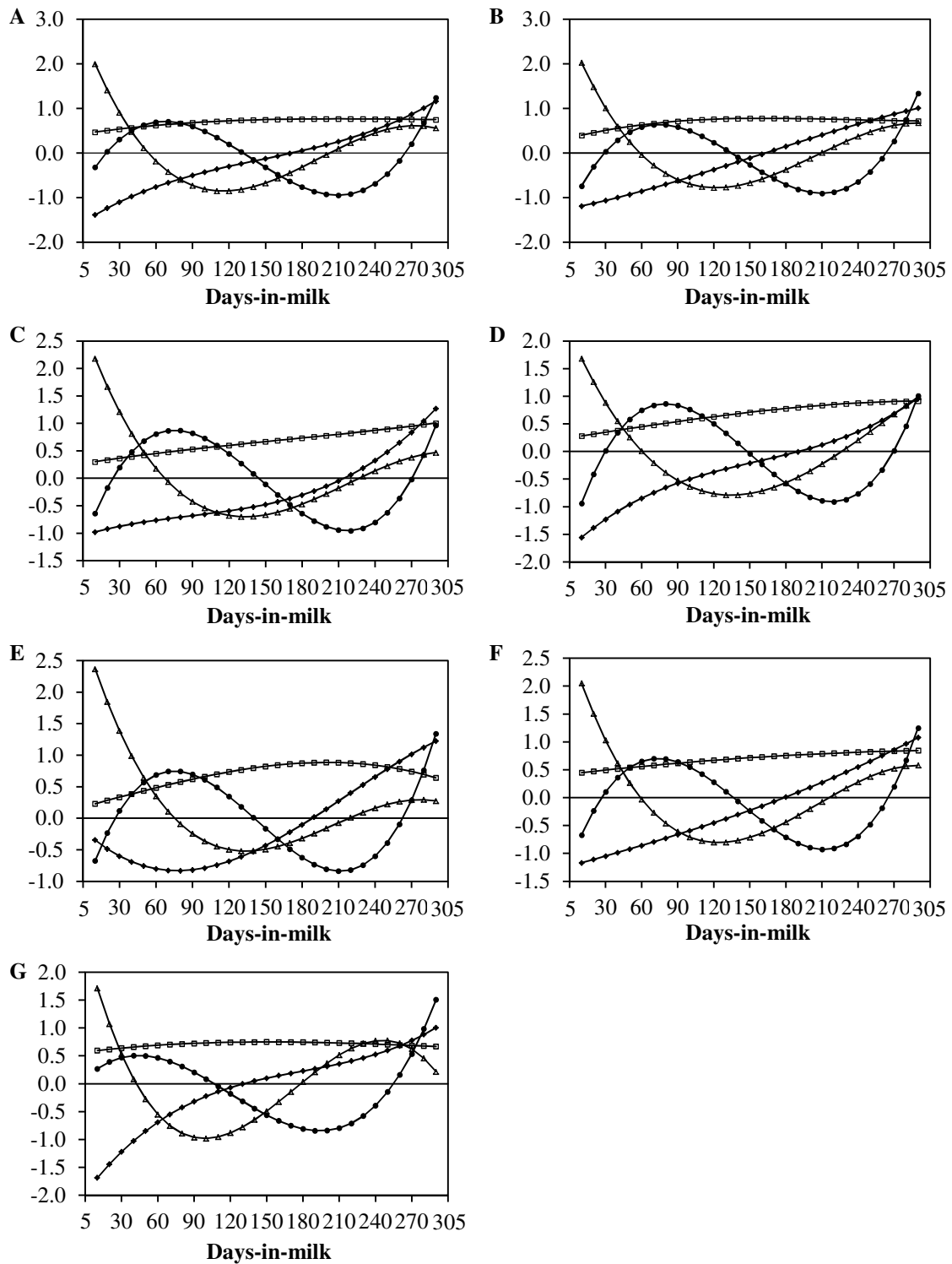
**Figure 1.** Genetic standard deviation (SE in parenthesis) for A) rennet coagulation time (—□—, min; 0.08 to 0.29), curd-firming time (—◆—, min; 0.01 to 0.03), curd firmness at 30 min (—△—, mm; 0.10 to 0.62), and curd firmness at 60 min (—●—, mm; 0.02 to 0.12), and B) heat coagulation time (—□—, min; 0.01 to 0.08), casein micelle size (—◆—, nm; 0.35 to 1.24), and pH (—△—, unitless; 0.001 to 0.004).



**Figure 2.** Heritability estimates for A) rennet coagulation time (—□—, min), curd-firming time (—◆—, min), curd firmness at 30 min (—△—, mm), and curd firmness at 60 min (—●—, mm), and B) heat coagulation time (—□—, min), casein micelle size (—◆—, nm), and pH (—△—, unitless). Standard error for the heritability estimates ranged between 0.01 and 0.03.

### *Eigenvalues and Eigenfunctions*

The eigenfunction associated with the largest eigenvalue was almost linear but positive across all DIM for all milk processing characteristics (Figure 3), as well as for both milk yield and milk composition. In all instances, the eigenfunction associated with the second largest eigenvalue changed from negative to positive after mid-lactation (163-215 DIM, with the exception of milk pH where the change in sign occurred at 132 DIM). For all the studied traits, including milk yield and milk composition, by far the greatest proportion of the genetic variance was explained by the intercept term; the genetic variance explained by the largest eigenvalue varied from 82% ( $a_{60}$ ) to 92% (HCT). The proportion of genetic variation explained by the smallest eigenvalue varied from 1% (HCT, CMS, and pH) to 2% (RCT,  $k_{20}$ ,  $a_{30}$ , and  $a_{60}$ ).

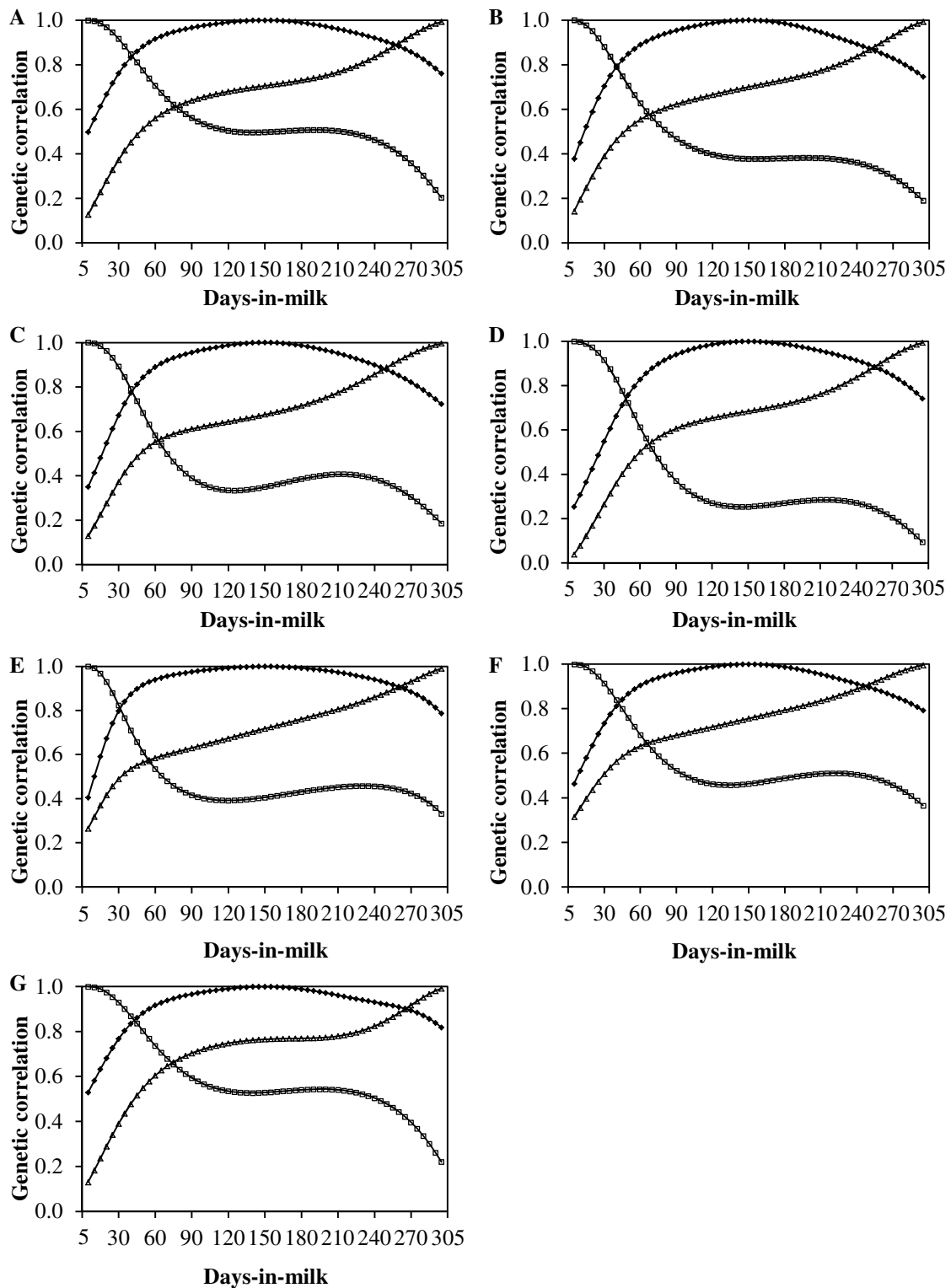


**Figure 3.** Eigenfunctions (y-axis, unitless) associated with the largest ( $-\square-$ ), first-middle ( $-\blacklozenge-$ ), second-middle ( $-\triangle-$ ), and smallest ( $-\bullet-$ ) eigenvalue for A) rennet coagulation time; B) curd-firming time; C) curd firmness at 30 min; D) curd firmness at 60 min; E) heat coagulation time; F) casein micelle size; G) pH.



### ***Within Trait Genetic Correlations***

In all instances, within trait genetic correlations weakened as the time between compared DIM lengthened but approached unity between adjacent DIM; nonetheless all within-trait pairwise DIM genetic correlations were positive. Within trait genetic correlations reached a minimum of  $0.12 \pm 0.01$  for RCT, a minimum of  $0.14 \pm 0.02$  for  $k_{20}$ , a minimum of  $0.13 \pm 0.03$  for  $a_{30}$ , a minimum of  $0.04 \pm 0.01$  for  $a_{60}$ , a minimum of  $0.26 \pm 0.01$  for HCT, a minimum of  $0.31 \pm 0.01$  for CMS, and a minimum of  $0.13 \pm 0.02$  for milk pH (Figure 4). Within trait genetic correlation for milk composition traits had a minimum of  $0.11 \pm 0.02$  and  $0.13 \pm 0.02$  for protein and fat concentrations, respectively (Figure S1).



**Figure 4.** Within trait genetic correlation between 5 DIM ( $-\square-$ ), 150 DIM ( $-\diamond-$ ), and 305 DIM ( $-\triangle-$ ) and the rest of lactation for A) rennet coagulation time; B) curd-firming time; C) curd firmness at 30 min; D) curd firmness at 60 min; E) heat coagulation time; F) casein micelle size; G) pH. Standard errors ranged between 0.00 and 0.02.

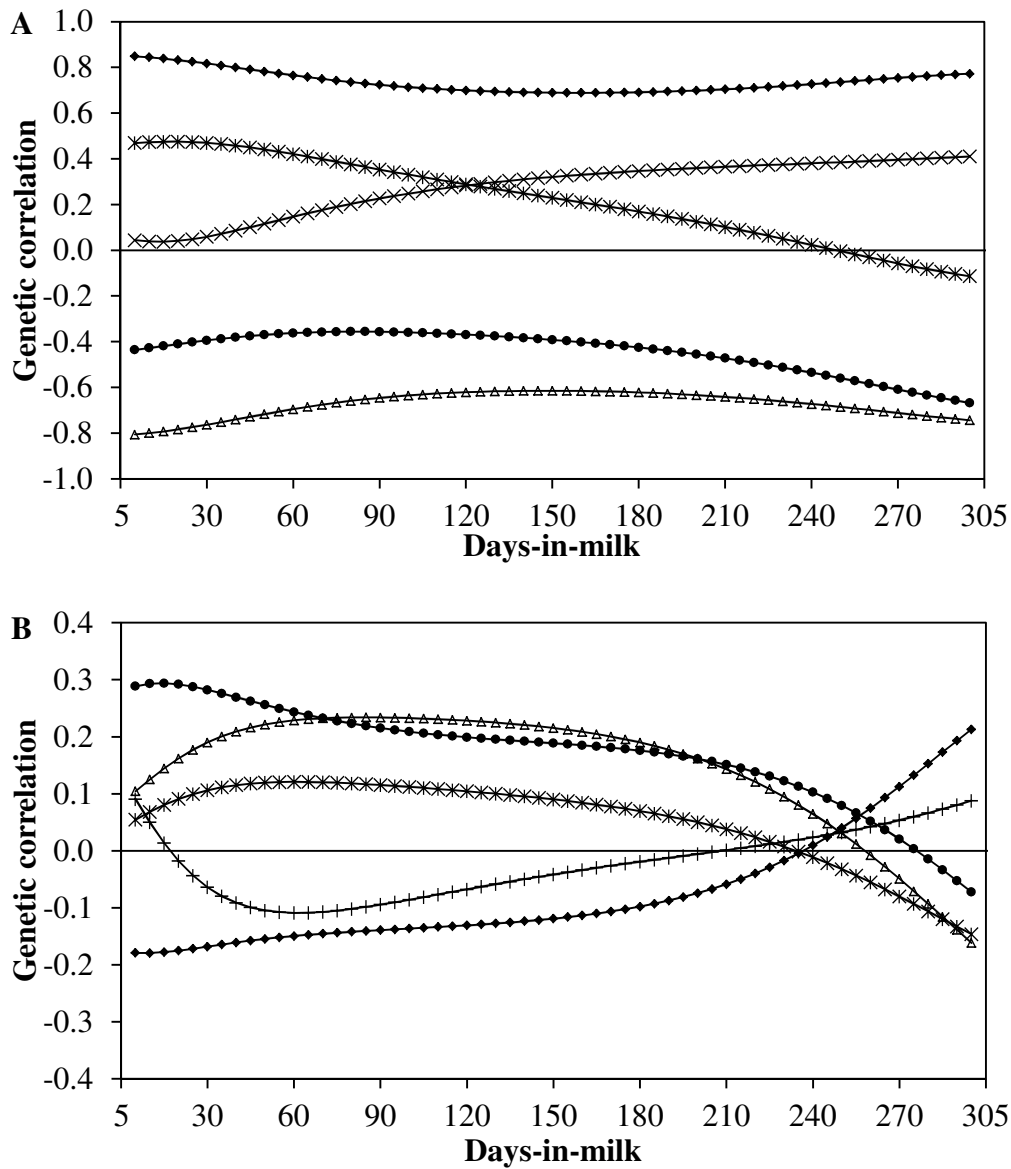
### ***Genetic Correlations among Traits***

The genetic correlations among traits averaged across all DIM are reported in Table 2 and those among traits for each DIM are in Figures 5 and 6. Genetic correlations among the milk coagulation properties were generally strong, ranging from  $0.69 \pm 0.01$  (160 DIM) to  $0.85 \pm 0.02$  (5 DIM) between RCT and  $k_{20}$ , and from  $-0.81 \pm 0.03$  (5 DIM) to  $-0.61 \pm 0.02$  (149 DIM) between RCT and  $a_{30}$  (Figure 5). Milk HCT and CMS were weakly correlated with all the other milk processing traits (Figure 5), while milk pH was strongly correlated only with RCT ( $0.73 \pm 0.04$  at 5 DIM to  $0.77 \pm 0.03$  at 305 DIM; data not shown). The correlations between milk yield and the milk processing traits were weak (from  $0.07 \pm 0.10$  at 305 DIM to  $0.21 \pm 0.04$  at 209 DIM with milk pH; Figure 6) to moderate (from  $0.33 \pm 0.05$  at 5 DIM to  $0.59 \pm 0.04$  at 303 DIM with  $k_{20}$ , and from  $-0.62 \pm 0.03$  at 283 DIM to  $-0.20 \pm 0.06$  at 5 DIM with  $a_{30}$ ). Milk protein concentration, similar to casein concentration, was strongly correlated with both  $k_{20}$  ( $-0.84 \pm 0.02$  at 5 DIM to  $-0.93 \pm 0.01$  at 305 DIM; Figure 6) and  $a_{30}$  ( $0.84 \pm 0.02$  at 21 DIM to  $0.94 \pm 0.01$  at 305 DIM; Figure 6), but weakly correlated with HCT ( $0.19 \pm 0.06$  at 36 DIM to  $-0.27 \pm 0.07$  at 305 DIM; Figure 6).

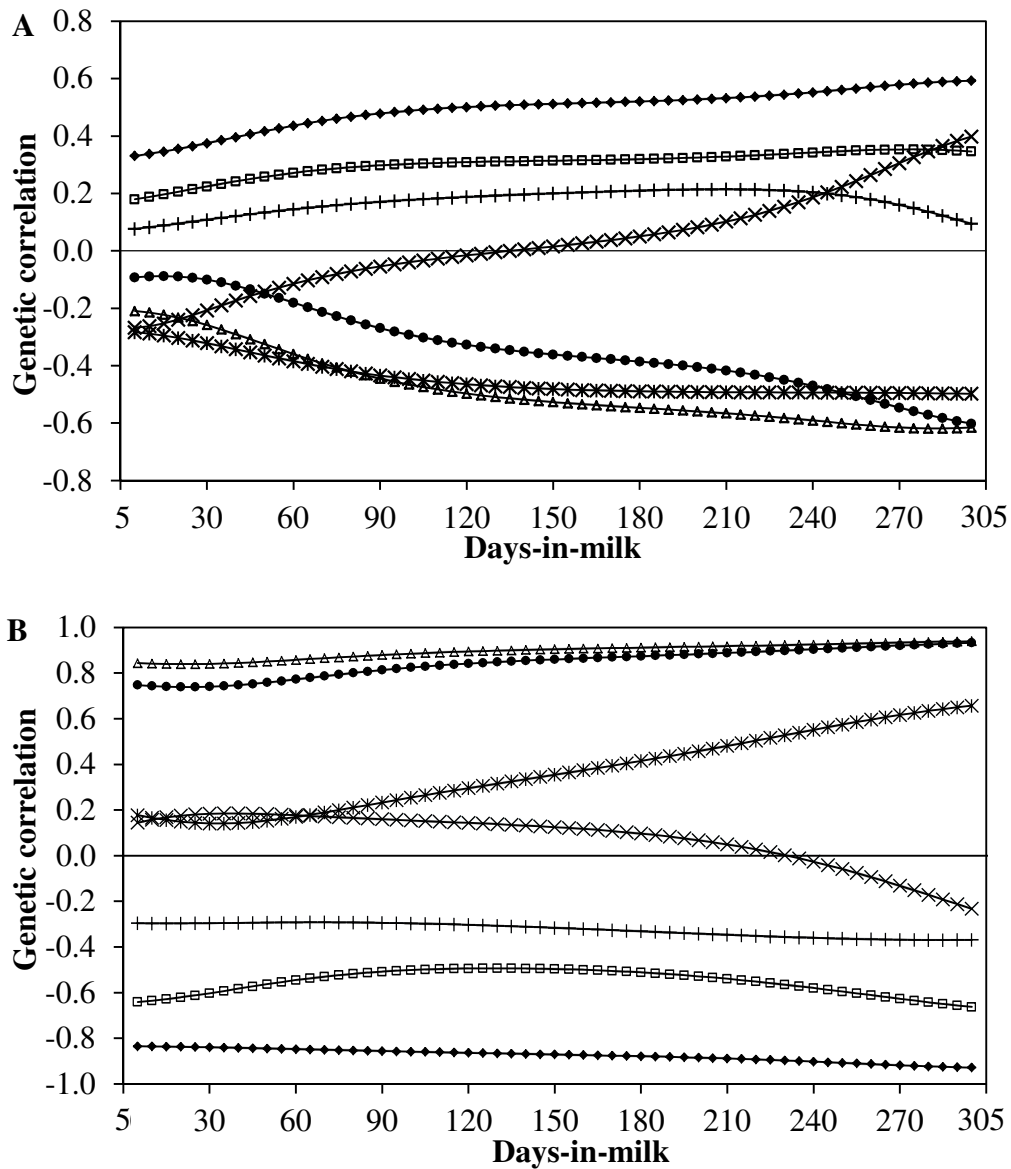
**Table 2.** Average genetic correlation coefficients calculated from the random regression models between milk yield, milk composition, somatic cell score (SCS), and milk technological traits.

Trait <sup>1</sup>	Milk yield	Protein	Casein	Fat	Lactose	Urea	SCS	RCT	k <sub>20</sub>	a <sub>30</sub>	a <sub>60</sub>	HCT	CMS
Protein	-0.58												
Casein	-0.58	0.98											
Fat	-0.60	0.70	0.70										
Lactose	0.07	-0.22	-0.13	-0.05									
Urea	0.03	-0.09	-0.10	0.05	-0.22								
SCS	-0.02	0.13	0.10	-0.01	-0.28	0.07							
RCT	0.31	-0.56	-0.57	-0.46	-0.05	0.30	0.13						
k <sub>20</sub>	0.50	-0.88	-0.87	-0.65	0.05	0.11	-0.02	0.74					
a <sub>30</sub>	-0.49	0.90	0.88	0.62	-0.23	-0.05	0.07	-0.68	-0.92				
a <sub>60</sub>	-0.35	0.85	0.84	0.40	-0.31	-0.03	0.12	-0.45	-0.74	0.81			
HCT	0.04	0.38	0.10	-0.03	0.16	0.40	0.02	0.28	-0.07	0.14	0.13		
CMS	-0.45	0.30	0.37	0.09	-0.45	-0.02	0.23	0.21	0.11	0.08	0.39	0.05	
pH	0.17	-0.33	-0.34	-0.24	0.05	0.05	0.05	0.75	0.43	-0.47	-0.20	-0.02	0.07

<sup>1</sup>SCS =  $\log_{10}(\text{SCC})$ ; RCT = rennet coagulation time; k<sub>20</sub> = curd-firming time; a<sub>30</sub> = curd firmness at 30 min; a<sub>60</sub> = curd firmness at 60 min; HCT = heat coagulation time; CMS = casein micelle size.



**Figure 5.** Genetic correlation (SE in parenthesis) between A) rennet coagulation time and curd-firming time ( $\blacklozenge$ ; 0.01 to 0.03), curd firmness at 30 min ( $\blacktriangle$ ; 0.01 to 0.03), curd firmness at 60 min ( $\bullet$ ; 0.02 to 0.07), heat coagulation time ( $\times$ ; 0.03 to 0.12), and casein micelle size ( $*$ ; 0.03 to 0.06), and B) heat coagulation time and curd-firming time ( $\blacklozenge$ ; 0.03 to 0.10), curd firmness at 30 min ( $\blacktriangle$ ; 0.03 to 0.11), curd firmness at 60 min ( $\bullet$ ; 0.03 to 0.11), casein micelle size ( $*$ ; 0.03 to 0.10), and pH ( $+$ ; 0.04 to 0.13).



**Figure 6.** Genetic correlation (SE in parenthesis) between A) milk yield and rennet coagulation time (–□–; 0.03 to 0.08), curd-firming time (–◆–; 0.02 to 0.05), curd firmness at 30 min (–△–; 0.02 to 0.06), curd firmness at 60 min (–●–; 0.03 to 0.07), heat coagulation time (–×–; 0.04 to 0.12), casein micelle size (–\*–; 0.03 to 0.06), and pH (–+–; 0.04 to 0.10), and B) milk protein concentration and rennet coagulation time (–□–; 0.01 to 0.04), curd-firming time (–◆–; 0.01 to 0.02), curd firmness at 30 min (–△–; 0.01 to 0.02), curd firmness at 60 min (–●–; 0.01 to 0.03), heat coagulation time (–×–; 0.02 to 0.10), casein micelle size (–\*–; 0.01 to 0.06), and pH (–+–; 0.02 to 0.06).

## DISCUSSION

The present study aimed to quantify the extent of genetic variability in milk processing characteristics predicted by mid-infrared spectroscopy from a large database of dairy cows reared mainly in a grazing dairy production system. Results indicated that genetic variation indeed exists for all milk processing traits, and that these milk quality features were only weakly to moderately genetically correlated with milk production and composition, with the exception of both  $k_{20}$  and  $a_{30}$  which were strongly (-0.88 and 0.90, respectively) correlated with the milk nitrogen constituents (i.e. protein and casein concentrations). Therefore, although genetic gain for milk processability could be achieved, the current selection objectives on production traits such as milk yield and fat and protein content (Miglior et al., 2005) are not fully exploiting the potential to genetically improve milk processability. Indeed, the coefficient of genetic variation in the milk processing features independent of genetic merit for protein concentration was 6.71% for RCT, 5.47% for  $k_{20}$ , 3.14% for  $a_{30}$ , 1.62% for  $a_{60}$ , 10.08% for HCT, and 1.97% for CMS.

The use of covariance functions in the present study facilitated the modeling of the genetic (co)variances of milk processing characteristics along the entire lactation. Covariance functions have been extensively applied to the analysis of longitudinal data (van der Werf et al., 1998; Nobre et al., 2003; Berry et al., 2007). In the present study, milk processing attributes were modeled using a third-order Legendre polynomial, similar to Preto et al. (2014) who used random regressions to model the genetic variation in RCT and  $a_{30}$  of dairy cows; Vallas et al. (2010), however, modeled both the additive genetic and permanent environmental variances using a second-order Legendre polynomial. The decomposition of the additive genetic (co)variance matrix into its eigenvectors and eigenvalues for all milk processing traits

in the present study revealed that the smallest eigenvalue was explaining only a small proportion of the additive genetic variance. Therefore, the addition of the third order polynomial did not improve dramatically the fit to the data and in fact may have over fitted the extremities of the lactation, as purported by Legarra et al. (2004) when modeling growth curves of beef cattle.

### ***Extent of Genetic Variability in Milk Processing Characteristics***

With the exception of CMS and milk pH, the coefficient of genetic variation in milk processing attributes, which in the present study ranged from 3.08% ( $a_{60}$ ) to 11.51% ( $k_{20}$ ), was comparable to the extent of genetic variation in milk yield (10.79%) and milk protein, fat, and casein concentration (4.58%, 7.83%, and 4.98%, respectively) also estimated in the present study. The existence of genetic variation in milk coagulation properties predicted by mid-infrared spectroscopy has previously been documented by Tiezzi et al. (2013) in a population of 16,089 Italian Holstein-Friesian dairy cows. The calculated coefficient of genetic variation based on the information provided by Tiezzi et al. (2013) was 8.43% and 19.33% for RCT and  $a_{30}$ , respectively. To our knowledge, however, the extent of genetic variation in HCT and CMS has never been previously quantified.

Based on normal distribution theory, the expected mean performance of the top 20% of individuals relative to the mean is 1.4 standard deviation units; this equates to a reduction in milk RCT of 2.31 min, 3 mm-stronger curd, greater milk ability to withstand high-temperature treatments (one extra min), and lower milk acidity (-0.03). Based on the positive phenotypic correlation between  $a_{30}$  and casein concentration estimated by Visentin et al. (2017) from the present dataset, these top 20% individuals could potentially yield 1.89% more cheese than the average



individuals, as casein concentration is a predictor of cheese yield (Van Slyke and Price, 1949). Assuming a milk fat concentration of 3.90% and a cheddar cheese water content of 37.7% (Visentin et al., 2017), such an improvement corresponds to 0.21 kg of extra cheese/100 kg milk processed. Therefore, over the entire 305-d lactation the potential extra revenue is of 35 €/cow, assuming the market price of Cheddar cheese is 3.20 €/kg (IFA, 2016) and individual milk yield per lactation of 5,217 kg (Coffey et al., 2016). The main consequence of low milk HCT is fouling at the dairy plant level, resulting in reduced production efficiency and greater associated costs for energy consumption as well as machinery maintenance which can account for up to 80% of the total production costs in processing (Bansal and Chen, 2006). Benefits therefore clearly exist to breeding programs for improved milk processing ability.

One obstacle to identifying animal divergent in genetic merit for milk processing characteristics (or most traits) is sufficient records to achieve a high accuracy of selection; this requires routine access to the relevant phenotypic data, which can be augmented by accompanying genomic data although the latter does still not preclude the necessity for phenotypic data. Based on the heritability and repeatability estimates from the present study, the number of progeny with one lactation record each required to achieve a reliability (i.e., squared accuracy of selection) of 0.70 is 31 for RCT, 19 for  $k_{20}$ , 24 for  $a_{30}$ , 31 for  $a_{60}$ , 56 for HCT, 28 for CMS, and 33 for pH. The average parity number of cows in the present study was 3, suggesting that the reliability of the prediction of animal genetic merit is 0.49 for RCT, 0.63 for  $k_{20}$ , 0.57 for  $a_{30}$ , 0.48 for  $a_{60}$ , 0.30 for HCT, 0.50 for CMS, and 0.46 for pH. Because all individual cow (and bulk tank) milk samples are routinely subjected to mid-infrared spectroscopy analysis, generating these quantities of phenotypic records using mid-infrared spectroscopy predicted milk processing characteristics

(Visentin et al., 2017) should be achievable, and importantly achievable at no marginal cost once the calibration equations are developed and validated.

### ***Breeding Strategies to Improve Product Consistency across Time***

Milk processing attributes, as well as output yield (i.e., cheese yield and whole and skimmed milk powder) have previously been documented to change with stage of lactation (Barłowska et al., 2014; Sneddon et al., 2016; Visentin et al., 2017). Because of the seasonal calving systems adopted by some countries such as Ireland (Berry et al., 2013), such temporal effects across lactation, compounded with seasonal effects (Visentin et al., 2017), manifest themselves as systematic temporal variability in milk quality across calendar months of the year. Such seasonal effects are minimized in many production systems through the adoption of year-round calving thereby ensuring product yield and consistency all year round. Nonetheless, the cyclic variability in milk processing characteristics in seasonal calving herds can represent a challenge for dairy processors (Downey and Doyle, 2007). Therefore, the possibility to alter the lactation profile for milk processing traits, through animal breeding, could be extremely advantageous in order to improve the efficiency of a manufacturing plant. The use of random regression models facilitates the estimation of breeding values for the trait under investigation across each DIM and thus facilitates the quantification of the potential to alter the lactation profile (Kirkpatrick et al., 1990). Because curd firmness is strong at the beginning (5-30 DIM) of lactation, but weakens to 31-60 DIM (Visentin et al., 2017), selection on the second eigenfunction for  $a_{30}$  could potentially reduce such a weakness with potential increased output losses (i.e., less cheese yield). Based on the evidence from the present study, however, the eigenfunction associated with the largest eigenvalue of all processing traits (as well as

milk composition and yield) did not change sign over the entire lactation. Because the genetic variance attributable to the intercept term of the covariance function explained more than 80% of the genetic variance, strategies to alter the shape of the lactation profile, although still possible, would require greater selection pressure on the DIM (or eigenfunction) of relevance. The conclusion of a similar (set of) genes affecting milk processing characteristics across the entire lactation was substantiated by the generally strong within-trait genetic correlations across DIM. Nonetheless, simultaneous genetic change in two traits can still be achieved in spite of moderate to strong genetic correlations as evidenced by the ability to simultaneously improve milk production and reproduction performance, even if antagonistically correlated (Berry et al., 2014). Therefore, being able to alter the lactation profile for milk processing characteristics, in tandem with altering the height of the lactation profile, could be extremely beneficial in seasonal calving production systems and help provide more consistent milk attributes across all seasons of the year.

### ***Consequences of Breeding for Milk Processability on Milk Production and Vice Versa***

Selection for increased milk production in dairy cows predominated historical breeding objectives globally (Miglior et al., 2005), and has contributed to more than double the lactation milk yield in 40 years (Oltenacu and Broom, 2010). Although production traits (milk, fat, and protein yield) still remain a large component of current breeding objectives, emphasis is also placed on non-production, functional traits such as health and reproduction (Miglior et al., 2005). While most countries have a negative weighting on milk yield in their national breeding goals, some countries like Latvia, South Africa, Switzerland, and USA still have a positive weight.

While most countries have a negative weight on milk yield, the generally large positive weight on fat and protein yield imply a positive expected response to selection for milk yield. Evidence from the present study indicates antagonistic genetic correlations between milk yield and milk coagulation properties (ranging from 0.31 to 0.50 in absolute value), and these antagonistic genetic correlations corroborate previous studies in Finnish Ayrshire (Ikonen et al., 2004) and Holstein dairy cows (Vallas et al., 2010). The results from the present study suggest that, based on selection index theory, each unit (e.g., kg) increase of daily milk yield is expected to lengthen RCT by 0.41 min, and to weaken  $a_{30}$  by 0.79 mm. As a consequence, emphasis should be given to at least one milk coagulation properties trait to halt such deterioration, although the consequence will be a reduced rate of genetic gain in milk production. For example, if the breeding goal consisted solely of milk yield, to halt any deterioration in  $a_{30}$  would require an emphasis of 31% (relative to the emphasis given to milk yield). In such a scenario, the genetic gain in milk yield would be only 87% of the genetic response when only milk yield was included in the breeding goal. This emphasis on  $a_{30}$ , however, would also be sufficient to halt any deterioration in RCT. Similarly, to achieve a genetic gain of 0.5 min in HCT, an emphasis of 36% (relative to the emphasis given to milk yield) would be required to be given to HCT, with negligible repercussion on the genetic response on milk yield. However, selection on both milk yield and HCT would still require emphasis also on at least one milk coagulation property to halt deterioration. The necessity of giving emphasis to a series of milk quality features, including technological characteristics, was also suggested by Henchion et al. (2016) based on stakeholder involvement in a Delphi study. Indeed, an emphasis of 16% on a milk quality sub-index was advised by Henchion et al. (2016) to halt any deterioration on milk quality traits.

## **CONCLUSIONS**

Results clearly indicate that exploitable genetic variation exists for all milk processing traits, suggesting that breeding for improved milk processability is indeed achievable. Although the fitted random regression models provide estimated breeding values for every day of lactation, with the exception of the two peripheries of the lactation, the within trait genetic correlations for all processing attributes were all moderate suggesting the trait measured at any stage of lactation could be assumed to be the same trait (Robertson, 1959). Therefore, unless there is interest in altering the lactation profile (e.g., in seasonal calving herds) for milk processing characteristics, a simple repeatability model may suffice to undertake genetic evaluations for these traits.

## **ACKNOWLEDGEMENTS**

Funding for the current study was received from the Irish Department of Agriculture, Food and the Marine, Research Stimulus Fund project 11/SF/311, Breed Quality. Laboratory and technical support by Bernard Corrigan, Jim Flynn, Owen Kenny, Paula O'Connor, and Alan Hurley (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland) is gratefully acknowledged.

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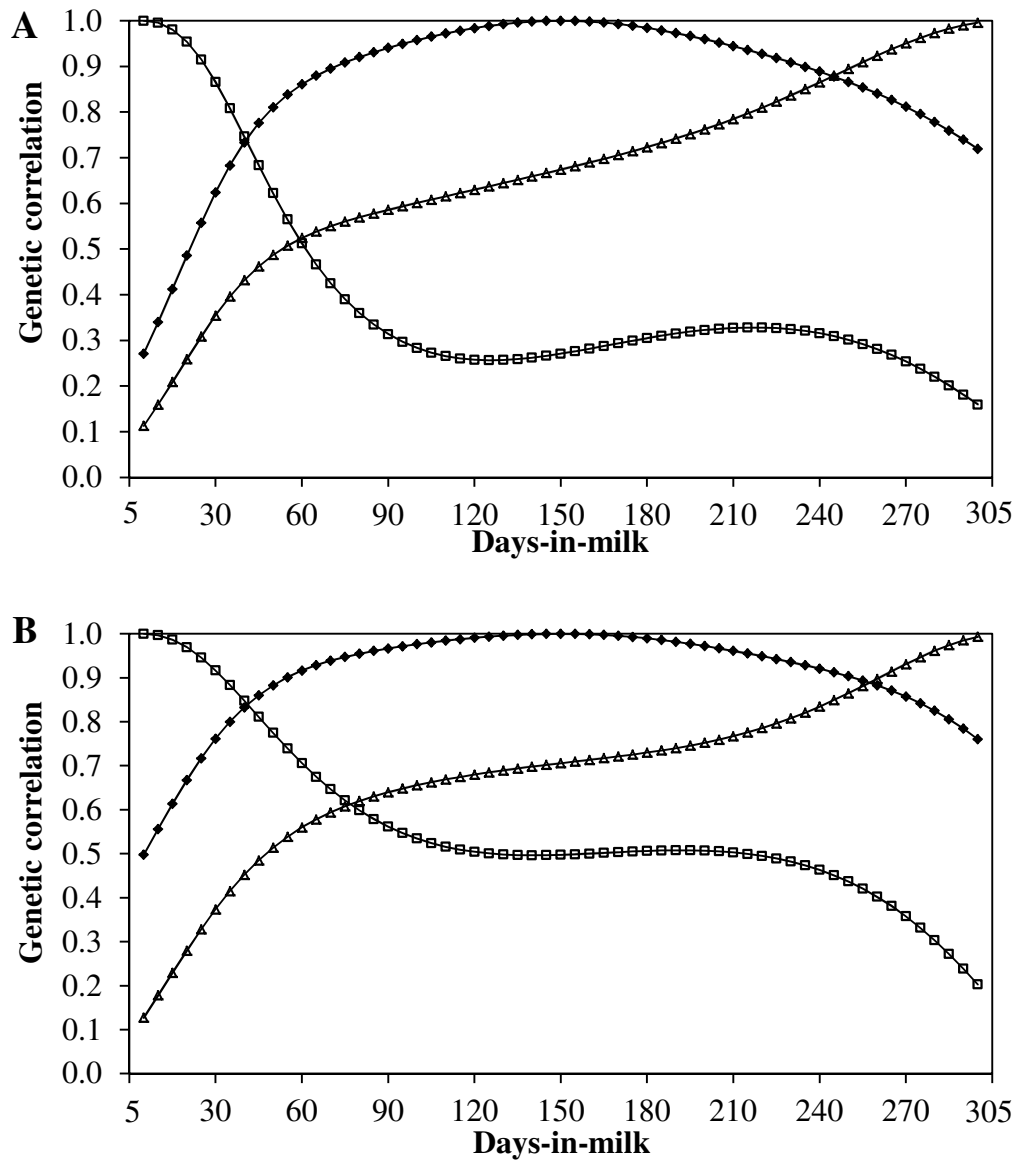


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## SUPPLEMENTARY MATERIAL



**Figure S1.** Within trait genetic correlation between 5 DIM ( $-\square-$ ), 150 DIM ( $-\diamond-$ ), and 305 DIM ( $-\triangle-$ ) and the rest of lactation for A) protein concentration; B) fat concentration. Standard errors ranged between 0.00 and 0.02.

**Genetic (co)variances between milk mineral content and chemical composition in  
Italian Holstein-Friesian dairy cattle**

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## ABSTRACT

Milk minerals are important from both processing and nutritional point of view. Little is known on the genetic parameters of such traits because of the resources required to collect these phenotypes on a large scale. The contents of Ca, K, Mg, Na, and P were quantified using mid-infrared spectroscopy on 12,223 test-day records from 1,171 Holstein cow between 5 and 305 days in milk (DIM). (Co)variance components were estimated using random regressions to model both the additive genetic and within lactation permanent environmental variances. Heritability estimates ranged from  $0.31 \pm 0.05$  (5 DIM) to  $0.67 \pm 0.04$  (181 DIM) for Ca, from  $0.18 \pm 0.03$  (60 DIM) to  $0.24 \pm 0.05$  (305 DIM) for K concentration, from  $0.08 \pm 0.03$  (15 DIM) to  $0.37 \pm 0.03$  (223 DIM) for Mg concentration, from  $0.16 \pm 0.03$  (30 DIM) to  $0.37 \pm 0.04$  (305 DIM) for Na concentration, and from  $0.21 \pm 0.04$  (12 DIM) to  $0.57 \pm 0.04$  (211 DIM) for P concentration. Within trait genetic correlations were almost unity between adjacent DIM to weaken as the time between pairwise interval increased, and were  $< 0.80$  only at the peripheries of the lactation. The analysis of the geometry of the additive genetic covariance matrix revealed that almost 90% of the additive genetic variation was accounted by the intercept term of the covariance functions. Averaged across DIM, the genetic correlations between milk minerals was positive, with the exception of the correlation between Ca and Na ( $-0.01$ ). Protein and minerals concentrations were positively correlated across DIM, while fat percentage was positively correlated throughout the entire lactation with Ca, K, and Mg, while turned from negative to positive with Na and P at 243 DIM and 50 DIM, respectively. The correlations between SCS and Na ranged from  $0.28 \pm 0.21$  (5 DIM) to  $0.79 \pm 0.18$  (305 DIM). Exploitable genetic variation exist for all milk minerals, although the

current national breeding objectives are partially contributing to an indirect positive response to selection of milk minerals.

**Keywords:** milk quality, milk processing, health, genetics

## INTRODUCTION

Although milk global demand is constantly increasing, meeting specific targets of product quality is still a crucial challenge for the dairy industry (FAO, 2017). A strategy to maximize the industry profit is to maximize milk added value; such strategy can be achieved through market segmentation so that the industry can differentiate its product portfolio, for example by providing the market with products with superior nutritional quality. Milk is an important source of functional molecules including long-chain fatty acids (Parodi, 1999), essential amino acids (McDermott et al., 2016), soluble thiols (Niero et al., 2015) and minerals (Gaucheron, 2005). Macro minerals in milk are generally referred to Ca, K, Mg, Na, and P (Cashman, 2006), and they are important for adults and infants, being involved in the homeostasis of the musculoskeletal and cardiovascular systems (Cashman, 2006; Haug et al., 2007; Uribarri and Calvo, 2014; Whelton and He, 2014).

Milk mineral content is known to vary due to several factors, such as bovine breed (Barlowska et al., 2006; Carroll et al., 2006), stage of lactation (Carroll et al., 2006; van Hulzen et al., 2009), parity number (Kume et al., 1998), and udder health status (Summer et al., 2009). Substantial additive genetic variation has been reported in Dutch Holstein-Friesians (1,860 cows; van Hulzen et al., 2009), and Danish Holsteins and Jerseys (456 and 436 cows, respectively; Buitenhuis et al., 2015). Because of the time and cost of reference laboratory analyses, measuring milk

minerals at a large-scale is hardly achievable. This challenge represents a limit when estimating genetic parameters of milk minerals, as estimates based on low number of records are normally associated to large standard errors and they may be biased if sampling methods do not cover completely the biological variability of the trait.

The use of mid-infrared spectroscopy to generate a high output of predicted phenotypes has been reported for several milk and animal factors (De Marchi et al., 2014; Bastin et al., 2016; McParland and Berry, 2016), including milk minerals (Soyeurt et al., 2009; Toffanin et al., 2015a; Visentin et al., 2016). Toffanin et al. (2015b) reported heritability estimates of  $0.10 \pm 0.04$  for Ca and  $0.12 \pm 0.04$  for P predicted by mid-infrared spectroscopy. However, to our knowledge, no studies have attempted to investigate the change of (co)variances for milk mineral composition throughout the lactation. Also, no estimates of repeatability of bovine milk minerals are available that the authors are aware of.

Therefore, the objective of the present study was to quantify the additive genetic variation of milk minerals predicted using mid-infrared spectroscopy, and the covariances between such traits and milk chemical composition, acidity, and udder health-related traits using random regressions fitted across lactation in Italian Holstein-Friesian dairy cattle.

## **MATERIALS AND METHODS**

### ***Data***

Between January 2012 and December 2013, a total of 132,380 milk samples from 15,173 dairy cows were collected in Bolzano Province (Italy) as described in Visentin et al. (2017a). In the present study, only milk samples collected from Holstein dairy cattle were retained; therefore, the dataset included 16,846 milk



samples from 2,020 Holstein cows and 2,838 lactations; animals were reared 95 single-breed commercial herds. All animals were milked twice daily, in the morning (AM) and again in the afternoon (PM). During the monthly test-day recording a milk sample was alternately collected during one milking session, immediately preserved with Bronysolv (ANA.LI.TIK Austria, Vienna, Austria) and brought to the milk laboratory of the South Tyrol Dairy Association (Sennereiverband Südtirol, Bolzano, Italy), where samples were processed according to International Committee for Animal Recording (ICAR) recommendation. The average number of milk samples collected per month was 702. Almost 55% of the cows participating to the present study calved between July and December. For each milk sample, milk chemical composition (protein, casein, fat, and lactose percentages, and urea content) and acidity (pH) were determined using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). The resulting milk spectrum, containing 1,060 transmittance data points in the region between 5,000 and 900  $\text{cm}^{-1}$ , was stored. Somatic cell count (SCC) was measured using Fossomatic (Foss Electric A/S, Hillerød, Denmark) and transformed to somatic cell score (SCS) through the formula  $\text{SCS} = 3 + \log_2(\text{SCC}/100,000)$ .

### ***Generation of predicted milk mineral composition and edits***

***Prediction models.*** In 2014 a total of 251 individual bovine milk samples were collected in the same area and herds contributing to the data of the present study. Full details on the dataset can be retrieved from Visentin et al. (2016). Milk samples were analyzed in the laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Italy) for Ca, K, Mg, Na, and P content (mg/kg) using Inductively Coupled Plasma Optical Emission Spectrometry, Ciros Vision EOP (SPECTRO Analytical Instruments GmbH, Kleve,

Germany) after mineralization. Prediction models for milk minerals were developed using partial least squares regression analysis. Models included the vector of each measured milk mineral as dependant variable, while spectra wavelengths (converted from transmittance to absorbance by taking the  $\log_{10}$  of the inverse of the transmittance) were considered as predictor variables. The coefficient of determination (root mean square error in parentheses) in validation were 0.67 (122.00 mg/kg), 0.69 (120.00 mg/kg), 0.65 (12.50 mg/kg), 0.40 (70.00 mg/kg), and 0.68 (88.12 mg/kg) for Ca, K, Mg, Na, and P, respectively. Full description of the reference methodology to measure milk minerals, as well as of the development of prediction models are in Visentin et al. (2016).

***Generation of predicted phenotypes.*** Prediction models were applied to the large spectral dataset previously described to generate predicted milk mineral composition. Firstly, principal component analysis (PROC PRINCOMP; SAS Institute Inc., Cary, NC) was applied to the large spectral dataset as well as to the dataset with reference values of milk mineral composition for the purpose of identifying the milk spectra (i.e. samples) of the larger dataset which were similar to those used to develop the prediction models. For this reason, the Mahalanobis distance from the centroid of the cluster of milk spectra with known mineral composition was computed for each milk spectrum of the larger spectral dataset as described by Brereton (2015); if a milk spectrum had a Mahalanobis distance greater than the 97.5% percentile of a  $\chi^2$  with 6 degrees of freedom (i.e. the lowest number of eigenvectors with eigenvalues > 1), then such spectrum was discarded from further statistical analysis. Predicted milk minerals were computed only for milk spectra (n=15,599) retained after the principal component analysis.

**Data editing.** Obvious data errors of milk chemical composition were discarded from the dataset. Records were retained if between 5 and 305 days in milk (DIM) for the first 10 parity. Lactations number  $\geq 5$  were grouped into a unique parity class. Contemporaries were defined as cows milking in the same herd test-date and contemporary groups with less than 3 observations were discarded from the data. Milk mineral composition was set to missing if the predicted value was  $> 3$  standard deviations from the mean of each milk mineral. Following all these edits, the final dataset included 12,223 milk samples from 1,717 Holstein cows and 2,549 lactations.

### **Statistical analysis**

(Co)variance components of predicted contents (mg/kg) of milk minerals (Ca, K, Mg, Na, and P), milk chemical composition (percentages of protein, casein, fat, and lactose), urea content (mg/dL), pH, and SCS were estimated in ASREML (Gilmour et al., 2011) using random regression models fitted across lactation. The pedigree was traced back 6 generations (where available) and included a total of 9,476 animals, including 1,485 sires and 6,476 dams. A total of 10 residual groups based on DIM were defined as 5-30 DIM, 31-60 DIM, 61-90 DIM, ..., 240-270 DIM, 271-305 DIM. Homogeneity of variance was assumed within residual group, while heterogeneity with no residual covariance was assumed among groups. The model fitted was as follow:

$$y_{lmnop} = HTD_l + \sum_{i=1}^n Par_m b_n DIM^n + \sum_{i=1}^n Cow_o b_n DIM^n + \sum_{i=1}^n PE_{within_o} b_n DIM^n + PE_{across_o} + e_{lmnop},$$

where  $y_{lmnop}$  is predicted milk mineral composition, milk chemical composition, urea, pH, and SCS;  $HTD_l$  is the fixed effect of the  $l$ th contemporary group (1,128 classes);  $Par_m$  is the fixed effect of the  $m$ th parity (5 classes: 1, 2, 3, 4,  $\geq 5$ );  $b_n$  is the  $n$ th-order of Legendre polynomial on DIM;  $Cow_o$  is the random effect for the additive genetic effect of the  $o$ th cow;  $PE_{within_o}$  is the random effect for the within lactation permanent environmental effect of the  $o$ th cow;  $PE_{across_o}$  is the random effect for the across lactation permanent environmental effect of the  $o$ th cow;  $e_{lmnop}$  is the residual term.

Based on the resulting lactation profile for each milk mineral, the most parsimonious order of the fixed Legendre polynomial was quadratic. The most parsimonious order of the random Legendre polynomial was chosen based on: i) the Log-likelihood ratio test (Wilks, 1938); ii) the Akaike information criterion; iii) the eigenvalue of the additive genetic covariance matrix, to quantify the contribution of the highest covariance function in explaining the additive genetic variance. In the first instance, a first-order covariance function was fitted only to the additive genetic effect; secondly a first-order Legendre polynomial was fitted also to the within lactation permanent environmental effect. Subsequently, the order of the random Legendre polynomial was increment of one unit firstly on the additive genetic effect and then on the within lactation permanent environmental effect. Based on the three criteria previously described, the order of the covariance function was chosen to be quadratic for the additive genetic effect and linear for the within lactation permanent environmental effect.

Covariance function coefficients were calculated as  $\delta^2 = \Phi\kappa\Phi'$ , where  $\delta^2$  is the  $301 \times 301$  covariance matrix for the traits studied,  $\Phi$  is the  $301 \times n$  Legendre polynomial matrix of DIM regression coefficient, and  $\kappa$  is the estimated  $n \times n$

covariance matrix of the random terms fitted with covariance functions (i.e., additive genetic and within lactation permanent environment). Standard errors of heritability estimates were calculated using a Taylor series expansion (Fischer et al., 2004).

Genetic correlations between milk mineral composition, milk chemical composition, urea, pH, and SCS at each DIM were calculated using a series of bivariate analyses using random regression models by fitting the same model previously described. Residual groups were as described for the univariate analysis, but residual covariance was estimated within each group. The order of Legendre polynomial was the same as for the univariate analysis. Standard errors of the genetic correlations were estimated as in Falconer and MacKay (1996):

$$\sigma(r_A) = \left[ \frac{(1-r_A^2)}{\sqrt{2}} \right] \left[ \sqrt{\frac{(\sigma_{h_x^2} \sigma_{h_y^2})}{(h_x^2 h_y^2)}} \right],$$

where  $\sigma$  denotes the standard error,  $r_A$  is the genetic correlation between trait  $x$  and trait  $y$ , and  $h^2$  denotes the heritability for trait  $x$  or trait  $y$ .

Eigenvalues and eigenvectors of the additive genetic covariance matrix for each trait studied were calculated using PROC IML (SAS Institute Inc., Cary, NC), while eigenfunctions were calculated as:

$$\psi_i(x) = \sum_{j=0}^{p-1} [k_{\psi_i}]_j \Phi_j(x)$$

where  $[k_{\psi_i}]_j$  is the  $j$ th element of the  $i$ th eigenvector of  $K$ ,  $\Phi_j$  is the  $j$ th element of the  $p-1$  order of fit of the Legendre polynomial matrix, and  $x$  is DIM.

A repeatability animal model was also used to estimate variance components for milk mineral composition, milk chemical composition, urea, pH, and SCS by fitting the same model previously described for the random regression analysis, but

excluding the sets of Legendre polynomial and by fitting the interaction between parity and DIM classes (10 classes: 5-30 DIM, 31-60 DIM, ..., 271-305 DIM).

For the purpose to evaluate the impact of including repeated measurement per individual on the response to selection for each milk mineral individually, the following formula was used (Walsh and Lynch, 2014):

$$R_x = i h_x \sigma_{g_x} \sqrt{\frac{n}{1 + t_x(n - 1)}}$$

where  $R_x$  is the response to selection for trait  $x$ ,  $i$  is the selection intensity (assumed 1),  $h_x$  is the square root of the heritability for trait  $x$ ,  $\sigma_{g_x}$  is the additive genetic standard deviation for trait  $x$ ,  $n$  is the number of samples collected for each individual, and  $t_x$  is the repeatability for trait  $x$ .

## RESULTS

The number of milk samples at the peripheries of the lactation was 960 (5-30 DIM) and 1,237 (271-305 DIM). In the remaining part of the lactation the number of samples ranged from 1,220 (241-270 DIM) to 1,303 (91-120 DIM). Table 1 reports the number of samples, the mean, and the results of variance components for milk mineral content, milk yield, and milk chemical composition estimated using the repeatability animal model. Estimates of heritability (repeatability in parentheses) for milk minerals ranged from  $0.19 \pm 0.03$  ( $0.25 \pm 0.01$ ) to  $0.54 \pm 0.04$  ( $0.66 \pm 0.01$ ) for K and Ca contents, respectively. Heritability estimates for milk chemical composition varied between  $0.25 \pm 0.04$  for fat percentage to  $0.39 \pm 0.05$  for protein and casein percentages (Table 1). Heritability estimates of  $0.26 \pm 0.04$ ,  $0.48 \pm 0.04$ , and  $0.11 \pm 0.03$  were calculated for urea content, pH, and SCS, respectively (Table 1). The coefficient of genetic variation ( $CV_g$ ) for minerals ranged from 3.33% (K content) to

6.61% (Ca content). Regarding the milk composition,  $CV_g$  varied between 1.99% (lactose percentage) and 6.89% (fat percentage). The largest  $CV_g$  were calculated for urea content and SCS (10.73% and 17.34%, respectively), whereas almost null genetic variation existed for pH (0.01%).

**Table 1.** Number of samples (n), mean, genetic standard deviation ( $\sigma_g$ ), heritability ( $h^2$ ), repeatability (t), and coefficient of genetic variation ( $CV_g$ ) estimated using the repeatability animal model for milk mineral content, milk yield, milk chemical composition, and somatic cell score (SCS). SE denotes the standard error.

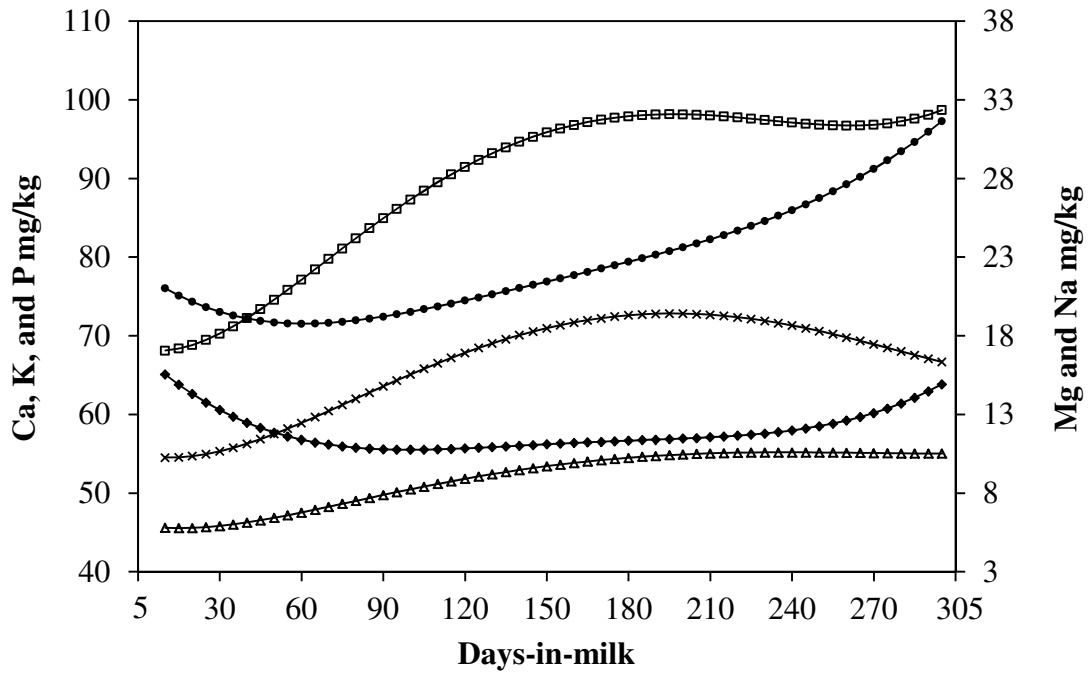
Trait	N	Mean	$\sigma_g$	$h^2$ (SE)	t (SE)	$CV_g$ , %
Ca, mg/kg	12,208	1295.29	85.58	0.54 (0.04)	0.66 (0.01)	6.61
K, mg/kg	12,165	1684.48	56.09	0.19 (0.03)	0.25 (0.01)	3.33
Mg, mg/kg	12,198	136.89	7.88	0.21 (0.03)	0.27 (0.01)	5.76
Na, mg/kg	12,223	439.06	21.56	0.24 (0.04)	0.39 (0.02)	4.91
P, mg/kg	12,220	995.04	62.25	0.42 (0.04)	0.53 (0.01)	6.23
Milk yield, kg/d	12,223	29.41	1.18	0.05 (0.03)	0.58 (0.01)	4.02
Protein, %	12,223	3.29	0.17	0.39 (0.05)	0.61 (0.01)	5.04
Casein, %	12,223	2.59	0.12	0.39 (0.05)	0.62 (0.01)	4.80
Fat, %	12,223	3.98	0.27	0.25 (0.04)	0.43 (0.01)	6.89
Lactose, %	12,223	4.76	0.09	0.37 (0.04)	0.59 (0.01)	1.99
Urea, mg/dL	12,223	20.25	2.17	0.26 (0.04)	0.42 (0.01)	10.73
pH, units	12,223	6.58	0.0001	0.48 (0.04)	0.62 (0.01)	0.01
SCS, units	11,318	2.72	0.47	0.11 (0.03)	0.55 (0.01)	17.34

### ***Random regression analysis***

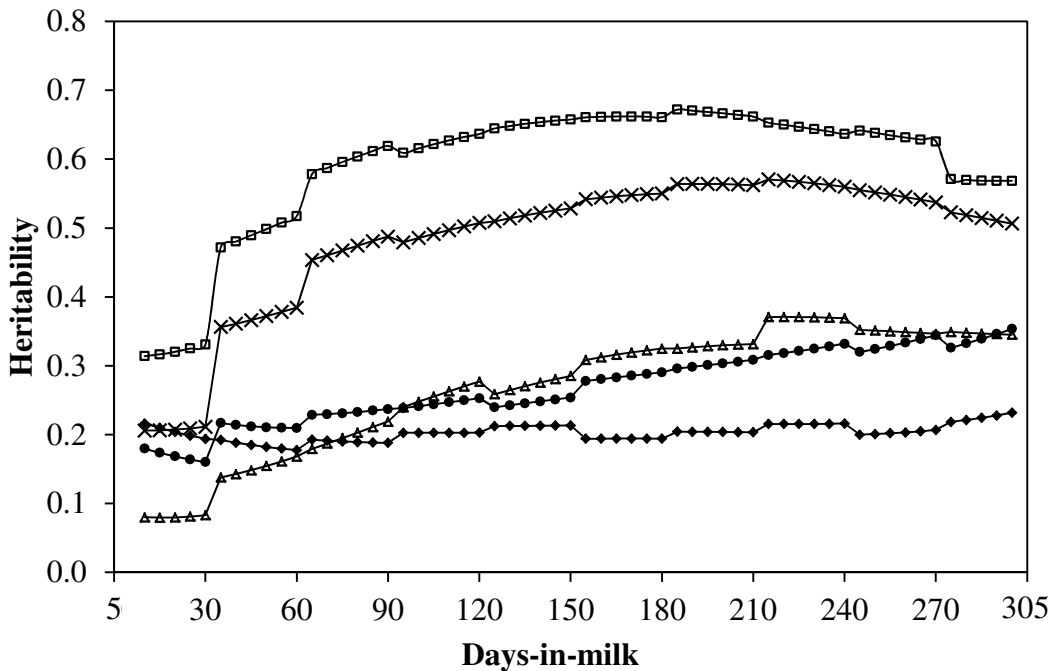
***Genetic variation.*** Changes of the genetic standard deviation of Ca, K, Mg, Na, and P contents across lactation are in Figure 1. The genetic standard deviation for Ca, Mg, and P increased until mid-lactation (151-180 DIM) and decreased thereafter. This trend was more evident for P than Ca and Mg contents. The lactation profile of the genetic standard deviation of K and Na contents was somewhat opposite to what observed for the aforementioned minerals. The genetic standard deviation ranged from  $68.09 \pm 5.57$  mg/kg (7 DIM) to  $100.17 \pm 5.23$  mg/kg (305 DIM) for Ca content,  $55.52 \pm 4.52$  mg/kg (99 DIM) to  $66.21 \pm 7.14$  mg/kg (5 DIM) for K content, and from  $5.78 \pm 1.03$  mg/kg (15 DIM) to  $10.59 \pm 0.58$  mg/kg (237 DIM) for Mg content (Figure 1). Regarding the remaining minerals, the genetic standard deviation varied between  $18.78 \pm 1.86$  mg/kg (57 DIM) and  $32.95 \pm 2.47$  mg/kg (305 DIM) for Na content, and between  $54.53 \pm 5.04$  mg/kg (12 DIM) and  $72.83 \pm 3.43$  mg/kg (196 DIM) for P content (Figure 1).

***Heritability estimates.*** Changes of the heritability of Ca, K, Mg, Na, and P contents estimated at each DIM are depicted in Figure 2. In all instances, heritability estimates were the lowest at the onset of lactation, where estimates of the residual variance were the highest (data not shown). Heritabilities ranged from  $0.31 \pm 0.05$  (5 DIM) to  $0.67 \pm 0.04$  (181 DIM) for Ca content,  $0.18 \pm 0.03$  (60 DIM) to  $0.24 \pm 0.05$  (305 DIM) for K content,  $0.08 \pm 0.03$  (15 DIM) to  $0.37 \pm 0.03$  (223 DIM) for Mg content,  $0.16 \pm 0.03$  (30 DIM) to  $0.37 \pm 0.04$  (305 DIM) for Na content, and  $0.21 \pm 0.04$  (12 DIM) to  $0.57 \pm 0.04$  (211 DIM) for P content (Figure 2).





**Figure 1.** Genetic standard deviation (SE in parentheses) for Ca (—□—, mg/kg; 4.63 to 5.23), K (—◆—, mg/kg; 4.51 to 7.14), Mg (—△—, mg/kg; 0.62 to 0.91), Na (—●—, mg/kg; 1.76 to 2.47), and P (—×—, mg/kg; 3.70 to 5.32) contents.



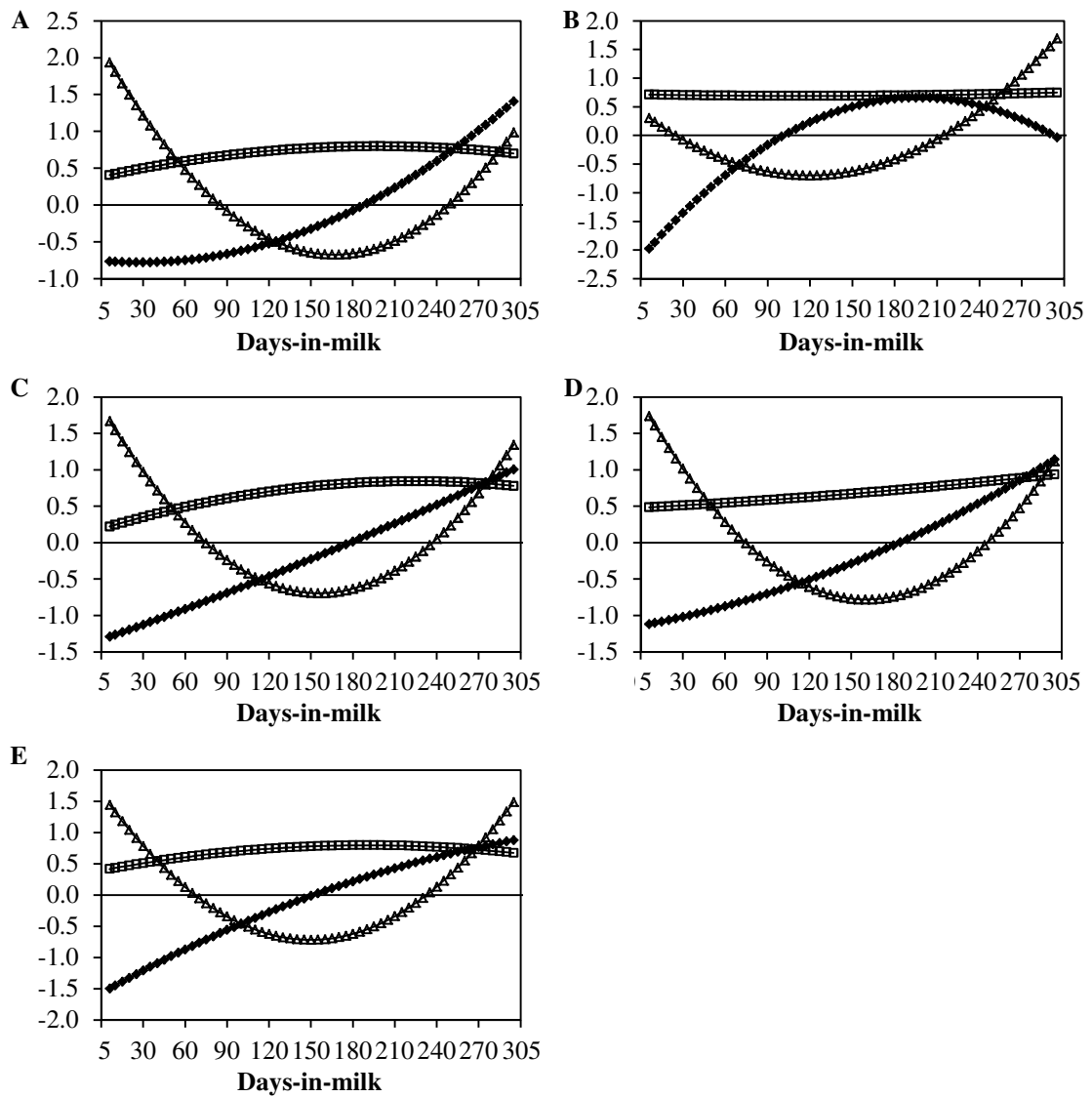
**Figure 2.** Heritability estimates (SE in parentheses) for Ca (—□—; 0.04 to 0.05), K (—◆—; 0.03 to 0.05), Mg (—△—; 0.02 to 0.05), Na (—●—; 0.03 to 0.05), and P (—×—; 0.03 to 0.05) contents.

***Decomposition of the additive genetic (co)variance matrix.*** The analysis of the geometry of the additive genetic covariance matrix of milk minerals revealed that the eigenvalue attached to the first eigenvector explained from 88.16% (Na content) to 92.78% (K content) of the additive genetic variance (Table 2). The eigenvalue attached to the last eigenvector explained from 1.27% (P content) to 3.92% (Na content) of the total additive genetic variance (Table 2). The eigenfunction associated to the largest eigenvalue did not change sign over the entire lactation in all instances (Figure 3). The eigenfunction associated to the middle eigenvalue changed sign after mid lactation, with the exception of K content in which such eigenfunction changed sign after 90 DIM. The eigenfunction associated to the smallest eigenvalue turned from positive to negative between 60 and 90 DIM, to subsequently turn from negative to positive between 241 and 270 DIM (Figure 3). The exception was K content, where the change of the sign (from positive to negative) occurred at 22 DIM, to turn to positive at 215 DIM (Figure 3).

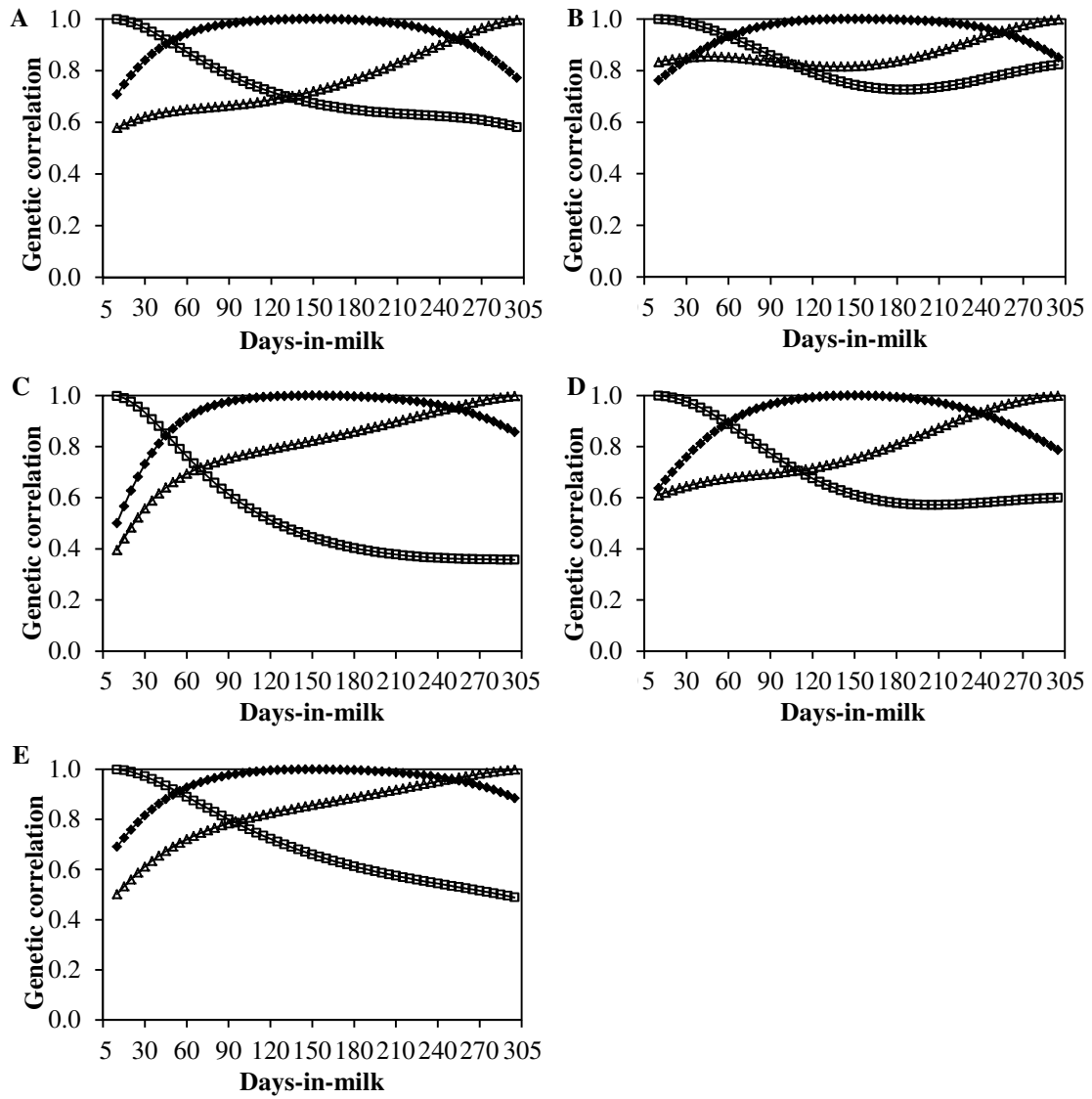
***Within-trait genetic correlations.*** The correlations of genetic variance estimates at 5 DIM, 150 DIM, and 305 DIM with genetic variance estimates of the rest of the lactation within each mineral are in Figure 4. The within-trait genetic correlations were almost unity between adjacent DIM to weaken while approaching the peripheries of the trajectory (Figure 4). The minimum of the within-trait genetic correlation was  $0.56 \pm 0.07$  (5 DIM vs 305 DIM),  $0.73 \pm 0.06$  (5 DIM vs 184 DIM),  $0.36 \pm 0.25$  (5 DIM vs 305 DIM),  $0.57 \pm 0.10$  (5 DIM vs 214 DIM), and  $0.48 \pm 0.10$  (5 DIM vs 305 DIM) for Ca, K, Mg, Na, and P contents, respectively (Figure 4).

**Table 2.** Percentage of genetic variation accounted by the largest, middle, and smallest eigenvalue for milk mineral content, milk yield, milk chemical composition, and somatic cell score (SCS).

Trait	Largest (%)	Middle (%)	Smallest (%)
Ca, mg/kg	91.07	6.36	2.57
K, mg/kg	92.78	4.41	2.81
Mg, mg/kg	92.00	5.88	2.12
Na, mg/kg	88.16	7.92	3.92
P, mg/kg	92.12	6.61	1.27
Milk yield, kg/d	55.07	34.79	10.14
Protein, %	89.13	6.76	4.11
Casein, %	89.09	6.92	3.99
Fat, %	91.26	7.33	1.40
Lactose, %	88.05	8.49	3.46
Urea, mg/dL	91.16	6.69	2.16
pH, units	87.77	8.69	3.55
SCS, units	92.61	5.48	1.91



**Figure 3.** Eigenfunctions (y-axis, unitless) associated with the largest ( $-\square-$ ), middle ( $-\blacklozenge-$ ), and smallest ( $-\triangle-$ ) eigenvalue for A) Ca content B) K content; C) Mg content; D) Na content; E) P content.

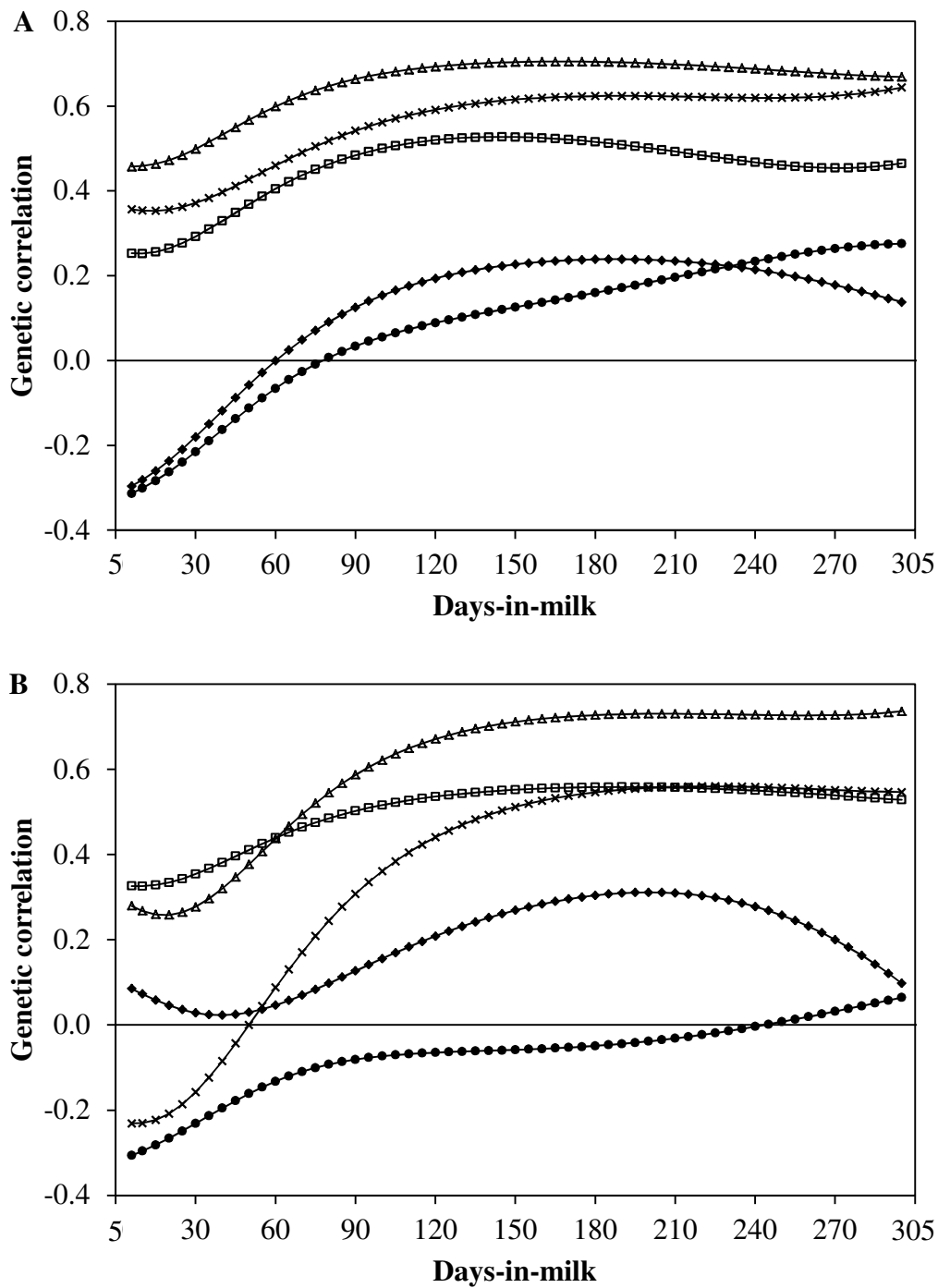


**Figure 4.** Within trait genetic correlations (SE in parentheses) between 5 DIM ( $-\square-$ ), 150 DIM ( $-\blacklozenge-$ ), and 305 DIM ( $-\triangle-$ ) and the rest of lactation for A) Ca content (0.00 to 0.07); B) K content (0.00 to 0.06); C) Mg content (0.00 to 0.25); D) Na content (0.00 to 0.10); E) P content (0.00 to 0.10).

***Between trait genetic correlations.*** Estimates of genetic correlations between milk minerals obtained from the repeatability animal model (i.e., averaged across all DIM) were weak (0.20 between Ca and K, and between Na and P) to strong (0.71 between K and P, and between Mg and P; Table 3). The only exception was the almost null genetic correlation between Ca and Na (Table 3). The genetic correlations between protein percentage and minerals across DIM were all positive and tended to become stronger as lactation lengthened (Figure 5). The genetic correlations of protein percentage with K and Na contents were turning from negative to positive at 61 DIM and 78 DIM, respectively. Milk fat percentage was positively genetically correlated across all DIM with Ca, K, and Mg, with the strongest correlations estimated at 190 DIM ( $0.56 \pm 0.03$  between fat and Ca), 200 DIM ( $0.31 \pm 0.07$  between fat and K), and 305 DIM ( $0.74 \pm 0.23$  between fat and Mg). The genetic correlations between fat and Na were all negative until 243 DIM, and those between fat and P changed sign at 50 DIM (Figure 5). The genetic correlations of lactose and urea contents with milk minerals were negative, with very few exceptions (Figure 6); the strongest genetic correlations were estimated at 5 DIM ( $-0.62 \pm 0.06$  between lactose percentage and K content, and  $-0.78 \pm 0.07$  between urea and Mg contents; Figure 6). Finally, the genetic correlations between minerals and SCS are depicted in Figure 7. The genetic correlations between SCS and Na ranged from  $0.38 \pm 0.21$  (5 DIM) to  $0.79 \pm 0.18$  (305 DIM), and they varied from  $-0.29 \pm 0.21$  at 5 DIM between SCS and K content, to  $0.18 \pm 0.07$  at 37 DIM between SCS and Mg content (Figure 8).

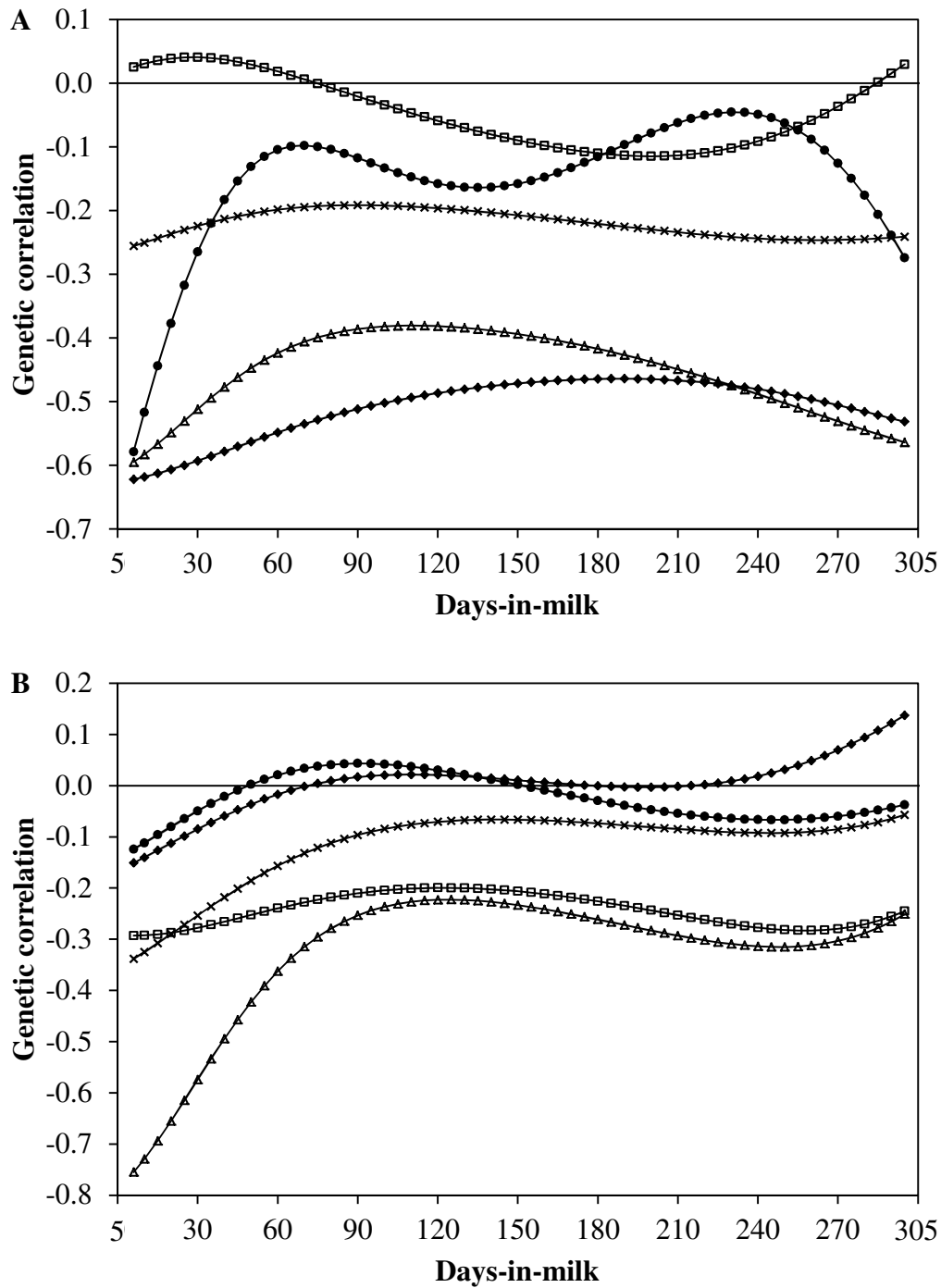
**Table 3.** Average genetic correlation coefficients calculated from the random regression models between contents of Ca, K, Mg, Na, and P, and milk chemical composition and somatic cell score (SCS).

	Ca	K	Mg	Na	P
K	0.20				
Mg	0.65	0.62			
Na	-0.01	0.45	0.27		
P	0.48	0.71	0.71	0.20	
Protein	0.46	0.12	0.65	0.09	0.56
Fat	0.51	0.19	0.62	-0.07	0.37
Lactose	-0.04	-0.51	-0.46	-0.15	-0.22
Urea	-0.24	0.01	-0.32	-0.02	-0.11
pH	0.52	-0.41	-0.29	-0.41	-0.05
SCS	-0.10	-0.01	0.03	0.48	0.01

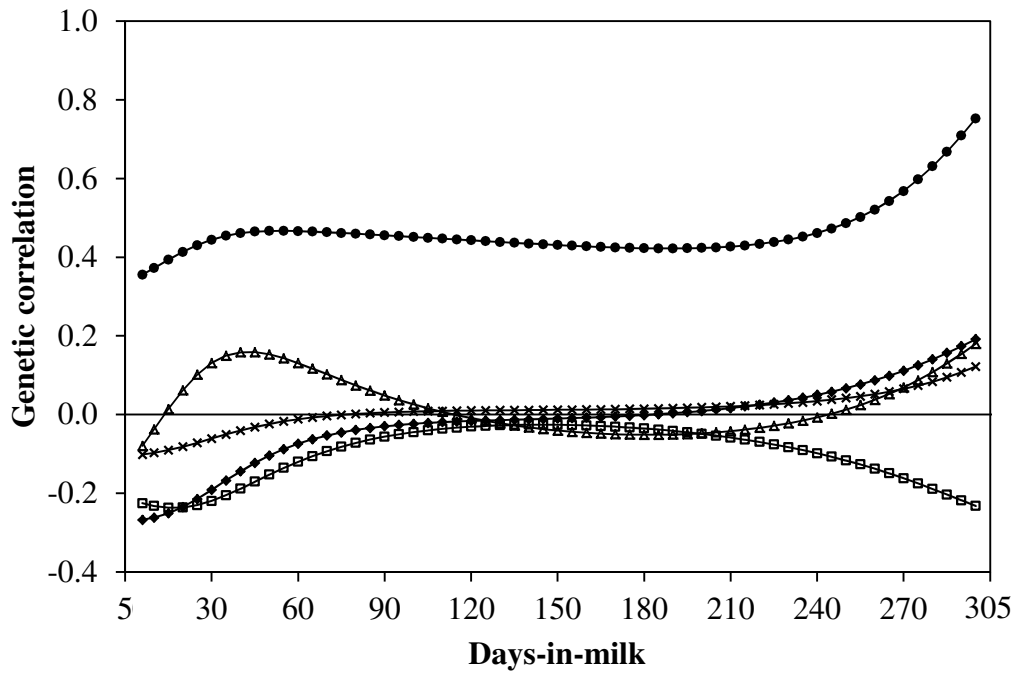


**Figure 5.** Genetic correlations (SE in parentheses) between A) protein percentage and contents of Ca (□; 0.03 to 0.12), K (◆; 0.06 to 0.14), Mg (Δ; 0.03 to 0.17), Na (●; 0.05 to 0.15), and P (×; 0.03 to 0.13), and B) fat percentage and contents of Ca (□; 0.03 to 0.13), K (◆; 0.07 to 0.17), Mg (Δ; 0.03 to 0.23), Na (●; 0.07 to 0.17), and P (×; 0.04 to 0.16).





**Figure 6.** Genetic correlations (SE in parentheses) between A) lactose percentage and contents of Ca ( $-\square-$ ; 0.05 to 0.11), K ( $-\blacklozenge-$ ; 0.06 to 0.08), Mg ( $-\triangle-$ ; 0.05 to 0.12), Na ( $-\bullet-$ ; 0.06 to 0.12), and P ( $-\times-$ ; 0.06 to 0.12), and B) urea content and contents of Ca ( $-\square-$ ; 0.06 to 0.11), K ( $-\blacklozenge-$ ; 0.09 to 0.13), Mg ( $-\triangle-$ ; 0.07 to 0.11), Na ( $-\bullet-$ ; 0.09 to 0.14), and P ( $-\times-$ ; 0.06 to 0.11).



**Figure 7.** Genetic correlations (SE in parentheses) between SCS and contents of Ca (□; 0.05 to 0.18), K (◆; 0.09 to 0.21), Mg (△; 0.07 to 0.31), Na (●; 0.05 to 0.21), and P (×; 0.06 to 0.21).

## DISCUSSION

The objective of the present study was to estimate (co)variance components of milk minerals predicted using mid-infrared spectroscopy in a large dataset of Holstein-Friesian dairy cattle. Because all traits were heritable and had substantial genetic variation, between-animals differences due to superior (or poor) genetic merit indeed exist. Heritable genetic variation of milk minerals was already demonstrated in the literature (van Hulzen et al., 2009; Buitenhuis et al., 2015; Toffanin et al., 2015) but the results from the present only partially agreed with previous findings. Indeed, heritability estimates for Ca and P (from the repeatability model) of the present study were larger than results of Toffanin et al. (2015), who reported estimates of 0.10 and 0.12 for Ca and P, respectively. Buitenhuis et al. (2015) reported null genetic variance for K and Na, whereas van Hulzen et al. (2009) reported larger heritability of K (0.46) and Mg (0.60) contents compared to the findings of the present study. However, such inconsistency may be probably due to the lower sample size used in the literature, which may have not fully captured the biological variability of milk minerals within Holstein cattle. Moreover, the lack of non-repeated records could have potentially inflated the genetic variance and therefore heritability estimates.

The use of random regressions as carried out in the current study did not assumed unity of the genetic variance throughout lactation, and therefore facilitated the estimation of the genetic variance at each DIM. Such statistical approach can facilitate more effective breeding schemes for genetically improving the studied traits (Kirkpatrick et al., 1990) and are nowadays commonly used in the analysis of longitudinal data in dairy cattle (van der Werf et al., 1998; Berry et al., 2007; Hurley et al., 2017).

### ***Practical implications***

Because mid-infrared spectra are routinely available on all milk samples taken during milk testing, one of the beneficial of using mid-infrared predicted phenotypes is the possibility of obtaining multiple measurements on a large number of animals. Such advantage facilitates the estimation of the additive genetic and permanent environmental variances as two separates entities, but also is expected to increase the response to selection as multiple records are available per individual. However, the magnitude of the increment of the genetic gain is also a function of the repeatability. Following a single trait selection on each milk mineral, using the evidences from the current research, for the minerals with the greatest repeatability estimates the response to selection increases by 21% (Ca) and 33% (P) when 15 measurements are taken individually compared to when only record per animal is available. In the same scenario, the response to selection increases by 83%, 77%, and 52% for K, Mg, and Na contents. Therefore, as for all production traits, measuring multiple times milk minerals on each individual can be extremely advantageous from a breeding perspective. However, because of the economical disadvantages of recording milk minerals on a large scale, such an hypothetical breeding program may prove little (or none) advantage to the dairy industry as the potential increment in profit is hindered by the increment of recording costs. To date, the only viable solution to overcome this obstacle is the use of mid-infrared spectroscopy as, once prediction models are developed and validated, the cost of recording new phenotypes becomes negligible.

Results from the present study clearly demonstrated that breeding strategies could also potentially be adopted in order to potentially alter the lactation profile of milk minerals and to identify animals with great deviations from a standard lactation profile (Kirkpatrick et al., 1990; van der Werf et al., 1998). Such strategies, which are

commonly used in the genetic evaluation of production traits in dairy cattle worldwide (Interbull, 2017), are extremely advantageous in production systems characterized by seasonal calving in which milk supply, and consequently milk composition, is subjected to substantial variability across calendar months of the year (Berry et al., 2013). In all instances, in the present study the first eigenfunction was positive all throughout lactation and associated with the largest proportion of genetic variance. This evidence suggests that most of the potential of breeding for milk minerals relies on the ability to change the height of the lactation profile at all DIM. The alteration of the lactation profile, although still possible, would require more selection pressure on the relevant eigenfunction. Such evidence was also reported by Visentin et al. (2017b) on the estimation of variance components using random regression models on milk processing characteristics, such as coagulation properties, heat stability, and acidity, predicted through mid-infrared spectroscopy. Similar findings on the analysis of the geometry of the additive genetic covariance matrix were reported also by Hurley et al. (2017) for feed efficiency traits in grazing dairy cows.

Given the positive genetic correlations between milk minerals reported in the present study, with the exception of the null genetic correlation between Ca and Na, the hypothetical selection for one milk minerals is expected to increase the content also of the other minerals considered in the present study. Such an implication may have negative consequence from a nutritional point of view as the current nutritional guidelines recommend to reduce the ingestion of Na (Whelton and He, 2014). Therefore, negative emphasis should be placed to Na content to hold at constant, or eventually reduce, Na content through breeding. The results from the present study demonstrated that positive genetic correlations existed between milk minerals and fat and protein percentages. These last two traits are part of the breeding objectives of

Holstein cattle in various countries, such as Switzerland, Germany, Spain, France, and Italy (Miglior et al., 2005), in which also there is indirect selection on milk yield through direct selection on fat and protein yield. Although such breeding objectives may be partially contributing to a positive (indirect) response to selection of milk minerals, still the full potential to genetically improve milk minerals can be only achieved with direct selection.

## **CONCLUSIONS**

The present study demonstrated that exploitable genetic variation exists for all milk minerals content. Breeding strategies can contribute to increase milk minerals in dairy cow milk, with positive implications for dairy processors, although an indirect positive response to selection of milk minerals is already achieved through the current selection objective in the Italian Holstein cattle. Breeding however can be a viable solution to increase such response to selection to permanently halter the mineral contents within the national dairy population. Breeding strategies can be potentially used to halter the lactation profile on milk minerals content in order to suit a particular breeding scheme. Yet, the economic value of milk minerals requires further quantification.

## **AKNOWLEDGMENTS**

The authors would like to thank Luis Kerschbaumer and Thomas Kerschbamer (South Tyrol Dairy Association – Sennereiverband Südtirol, Bolzano, Italy), Christian Plitzner (Breeders Association of Bolzano province – Vereinigung der Südtiroler Tierzuchtverbände, Bolzano, Italy) and Claudio Stecchi (CTS informatica srl, Bolzano, Italy) for providing data used in this study and for technical support. This

study was supported by the University of Padova (Ricerca Scientifica fondi quota EX 60% - 2014, project 60A08-7955/14, Italy).

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## *General conclusions*

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In order to be included in a breeding scheme, a trait must fulfill three criteria: i) be (economically or socially) important; ii) be measurable, possible quickly and at low-cost; iii) be heritable, or be genetically correlated with an heritable trait.

The main obstacle to genetically improve milk processability was the absence of an on-line method to measure milk processing traits important for both cheese and dried milk production. In the present thesis it was demonstrated that milk processability is predictable using mid-infrared spectroscopy with moderate to strong accuracy. The use of this method, for the quick generation of low-cost predicted phenotypes, could be used as an on-line method by the dairy industry to potentially partition milk to different dairy products and therefore to identify more efficiently the best use of milk. Within lactation, milk was more adapted to cheese production when characteristics describing the aptitude of milk to produce milk powder were poorest. Specialized dairy breeds produced milk less adapted to cheese production. These predicted milk processing phenotypes expressed moderate heritability estimates and, with the exception of milk pH, exploitable genetic variation. Such traits were also antagonistically correlated with milk production suggesting that emphasis should be placed on milk processability in order to genetically improve, or at least hold at constant, milk processing characteristics; oppositely, milk minerals were positively correlated with milk composition. Breeding strategies could be also adapted to alter the lactation profile of milk technological characteristics to make it more consistent with the national production system.



## *List of publications*

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*During my PhD experience I had the chance to meet and engage with many people: I would like to thank them all!*

*A big thanks goes to my supervisors, Prof. Mauro Penasa, Dr. Donagh Berry, and Prof. Massimo De Marchi as they have been the reference point from which to learn every single day of my PhD. I would like to thank also Dr. Sinead McParland, Dr. Mark Fenelon and Prof. Martino Cassandro for their support during my experiences in Teagasc and University of Padova.*

*Having an office in Italy and Ireland has been a great opportunity to have a lot of colleagues who revealed to be good friends! Thanks a lot to Giovanni, Giovanni Battista, Paolo, Alba, Giulia, Elisabetta, Claudio, Alan H., Steven, Alan T., Aine, Siobhan, Michelle, Nuria, Alessia, Bruno, Lorenzo, Andrea...each of them have contributed to a little piece of this thesis.*

*A very very big thank goes to my girlfriend Iudita, for her daily dose of kindness, patience, niceness, affection, but above all for everyday simply being the Iudita I love...a big piece of thesis is thank to her!*

*Thank you also to my parents, Elsa and Venanzio, and my sisters, Donatella and Stefania, and my bother-in-law Amedeo. They have teach me the meaning of hard-working, the difference between the good and bad, they made me grown up from a child to a man, and they kicked my...when it was the right time to do it!*

*Thanks also to the Italian Holstein-Friesian breeders association for having given me the opportunity to start a new job experience in their R&D office.*

*Every single little part of my work during my PhD, and all my future work on the genetics of dairy cattle, goes to honor the memory of my beloved grandfather Carlo. You left too early, but you are always and everywhere here with us.*