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CICLO XXXII

GENETIC AND NONGENETIC VARIATION OF BLOOD METABOLITES PREDICTED FROM MILK INFRARED SPECTRA IN DAIRY CATTLE

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CONTENTS

Abstract

The negative energy balance experienced by dairy cows in early lactation caused by the lack of trade-off between energy intake (input) and requests for lactogenesis (output) is responsible for the occurrence of metabolic disorders. Blood metabolites are important indicators to monitor nutritional and energy status of the cows, and to detect the presence of metabolic disorders. In particular, the hyperketonemia (HYK) is one of the most frequent and costly metabolic diseases in early-lactation dairy cows, and it is commonly diagnosed through the determination of β-hydroxybutyrate (BHB) concentration in blood.

With this background, the overall objectives of the present thesis were: i) to summarise literature results on phenotypic and genetic aspects of BHB concentration in blood and milk of dairy cows; ii) to develop mid-infrared (MIR) spectroscopy prediction models for routine determination of blood metabolites; iii) to describe phenotypic variation of MIR-predicted blood metabolites in Brown Swiss, Holstein-Friesian and Simmental cattle breeds; iv) to assess the genetic variation of blood BHB and nonesterified fatty acids (NEFA) predicted by MIR spectroscopy, and their correlations with milk production and composition traits in early-lactation Italian Holstein dairy cows.

Hyperketonemia is an abnormal concentration of circulating ketone bodies in the blood; in particular, concentration of blood BHB $\geq$ 1.2 mmol/L is commonly recognized as indicator of HYK. In general, HYK impairs health of dairy cows by increasing the risk of the onset of other early-lactation diseases, and it negatively affects reproductive performance. Although the relationship with milk yield is still controversial, HYK has a detrimental effect on milk composition. Costs of HYK are mainly imputable to impaired fertility and milk loss. From a genetic point of view,

results from the literature suggested the feasibility of selecting cows with low susceptibility to HYK. Milk is the most promising matrix to monitor HYK, taking advantage of using MIR spectroscopy during routine milk recording.

The effectiveness of using routine milk MIR spectra to predict main blood metabolites in early-lactation dairy cows was evaluated. Blood BHB, urea and NEFA were the most predictable traits. Predicted blood BHB showed an improved performance in detecting cows with HYK, compared with commercial calibration equation for milk BHB.

Factors associated with the phenotypic variation of MIR-predicted blood metabolites were investigated on a large spectral multi-breed database. Holstein-Friesian cows had the greatest concentration of blood BHB and NEFA, and the lowest blood urea content. Blood BHB and NEFA concentrations generally increased with parity. The greatest BHB concentration was observed in the first 10 days of lactation, except for Simmental cows. From 5 to 35 days in milk, NEFA concentration decreased, whereas urea content increased for all considered breeds. The maximum levels of blood BHB and NEFA concentrations were recorded in spring and early summer. Blood urea generally increased across the year, from spring to winter.

Genetic parameters for MIR-predicted blood BHB and NEFA concentrations were estimated. The greatest heritability for both metabolites was assessed in the first 10 days after calving (0.32 for BHB and 0.23 for NEFA), and their genetic correlation varied from 0.50 to 0.60. Moreover, an unfavourable trend of estimated breeding values for both blood BHB and NEFA concentrations across year of birth of the bulls was detected. Genetic correlations of BHB and NEFA with milk yield, somatic cell score, protein, lactose and urea content were similar or at least in the same direction, whereas opposite correlations were observed with fat content and fat-to-protein ratio.

Riassunto

Ad inizio lattazione, le vacche da latte vanno comunemente incontro ad un bilancio energetico negativo, causato da uno squilibrio tra energia fornita dalla dieta e richieste della lattogenesi. Tale condizione può portare all'insorgenza di diversi disordini metabolici. I metaboliti presenti nel sangue fungono da importanti indicatori per monitorare lo stato nutrizionale ed energetico degli animali e per rilevare la presenza di disordini metabolici. Ad esempio, l'iperchetonemia (HYK) è una tra le più frequenti e costose malattie metaboliche, e viene individuata misurando la concentrazione di β-idrossibutirrato (BHB) nel sangue.

Gli obiettivi del presente lavoro di tesi sono stati: i) riassumere i risultati presenti in letteratura riguardo gli aspetti fenotipici e genetici del contenuto di BHB nel sangue e nel latte bovino; ii) sviluppare modelli di predizione basati sulla spettroscopia nel medio infrarosso (MIR) per determinare parametri metabolici nel sangue; iii) descrivere la variazione fenotipica dei metaboliti del sangue predetti con spettroscopia MIR in bovine di razza Bruna, Frisona e Pezzata Rossa; iv) stimare l'ereditabilità della concentrazione di BHB e acidi grassi non esterificati (NEFA) nel sangue predetti con tecnologia MIR a partire dagli spettri del latte, e la loro correlazione con caratteri di produzione e composizione di bovine di razza Frisona.

Un'elevata concentrazione di corpi chetonici circolanti è definita HYK. L'HYK compromette la salute delle vacche da latte aumentando il rischio di insorgenza di altre malattie. Sebbene la relazione con la produzione di latte sia ancora controversa, l'HYK ha un effetto negativo sulla composizione. I costi per l'HYK sono principalmente imputabili alla ridotta fertilità e alla perdita di latte. La letteratura suggerisce la possibilità di selezionare animali con ridotta suscettibilità all'HYK. Il

latte è la matrice più promettente per il monitoraggio dell'HYK, avvantaggiandosi dell'utilizzo della spettroscopia MIR nei controlli funzionali.

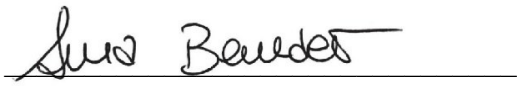
È stata valutata l'efficacia dell'utilizzo degli spettri MIR raccolti in condizioni di routine per la predizione dei principali metaboliti nel sangue di vacche ad inizio lattazione. I caratteri predicibili con maggiore accuratezza sono stati BHB, urea e NEFA. Il BHB predetto nel sangue ha mostrato performance migliori nell'individuare i soggetti con HYK rispetto al BHB predetto nel latte dall'equazione di calibrazione commerciale.

I fattori associati alla variazione fenotipica dei metaboliti del sangue predetti con spettroscopia MIR sono stati valutati utilizzando un dataset di spettri derivanti dalle analisi del latte di bovine di razza Frisona, Bruna e Pezzata Rossa. La razza Frisona si è caratterizzata per la più elevata concentrazione di BHB e NEFA e per il valore più basso di urea. Mediamente le concentrazioni di BHB e NEFA sono aumentate con l'ordine di parto. I livelli più alti di BHB sono stati raggiunti nei primi 10 giorni di lattazione, ad eccezione della Pezzata Rossa. Tra i 5 e i 35 giorni di lattazione, le concentrazioni di NEFA si sono ridotte, mentre quelle di urea sono aumentate. I livelli massimi di BHB e NEFA si sono registrati in primavera e inizio estate. Le concentrazioni di urea sono aumentate nel corso dell'anno.

Sono stati stimati i parametri genetici delle predizioni MIR di BHB e NEFA nel sangue. Per entrambi i metaboliti l'ereditabilità più elevata si è registrata nei primi 10 giorni dopo il parto (0.32 per BHB e 0.23 per NEFA). La correlazione genetica tra loro è risultata compresa tra 0.50 e 0.60. Le correlazioni genetiche di BHB e NEFA con la maggior parte dei caratteri del latte considerati sono risultate simili tra loro o almeno nella stessa direzione, mentre correlazioni in senso opposto si sono riscontrate con il contenuto di grasso e il rapporto grasso-proteina nel latte.

Declaration

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.



General introduction

Metabolic disorders

Metabolic disorders are the manifestation of a dysfunction or physiological imbalance of one or more metabolic processes, that involve the release and conversion of metabolites either used in production processes or excreted as waste (Pryce et al., 2016). Breeding strategies for increased milk production have led to a dependence on body reserves to support early lactation. As a consequence, dairy cows experience a negative energy balance at the beginning of lactation, which leads to an imbalance in hormones, immune functions and metabolites, involving the potential onset of metabolic diseases (LeBlanc et al., 2010; White, 2015).

Approximately 75% of health and metabolic disorders in dairy cattle occur in the first month postpartum (LeBlanc, 2010), and the most important ones are retained placenta, hypocalcemia, metritis, mastitis, displaced abomasum and ketosis (Suthar et al., 2013). Several studies have demonstrated that these diseases cause substantial farm financial losses by increasing diagnosis and treatment costs and decreasing milk production and fertility in dairy herds (Dohoo and Martin, 1984; Liang et al., 2017). For this reason, accurate and easy diagnostic strategies and opportunities to select for general disease resistance have been investigated in the last years.

Metabolic parameters

Prediction or early detection of cows with health, nutritional or metabolic problems is an important goal. Several metabolic parameters can be tested and used to support or anticipate veterinary diagnoses during early lactation. For instance, the severity and duration of the negative energy balance is reflected by the rise in circulating nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) and by the

level of decreased glucose concentrations (Esposito et al., 2014). On the other hand, urea mirrors protein intakes and nitrogen utilization, providing information on the diet (Kohn et al., 2005; Macrae et al., 2006; Kume et al., 2008). Therefore, circulating concentrations of these metabolites can be used as indicators of nutritional and metabolic status of the animals. The monitoring of the herd, early detection of potential diseases, large-scale data collection and feasible access to phenotypes for genetic evaluations, are only some of the several advantages derive from using the determination of specific indicators instead of veterinary diagnosis.

Determination of metabolic indicators

Blood and milk are the most important matrixes for metabolites determination. Related reference tests commonly rely on blood analyses performed in laboratory. However, laboratory analyses require a considerable amount of time and economic resources, and thus, they are not applicable on a large-scale for routine monitoring. In addition, several cow-side tests have been implemented for an easier but semi-quantitative measurement in field conditions (Bach et al., 2016; Ruoff et al., 2017). Milk mid-infrared (MIR) spectroscopy has been considered as alternative method for assessing and monitoring health traits (De Marchi et al., 2014; Grelet et al., 2016), as it is a quick and cost-effective technique allowing the prediction of numerous traits on a large-scale. Several efforts have been made to predict either milk metabolites such as acetone, BHB, fat-to-protein ratio and urea, or blood metabolites such as NEFA, BHB, and urea, and to investigate these traits as potential indicators of metabolic status at herd level (van Knegsel et al., 2010; Santschi et al., 2016; Luke et al., 2019). Using routine MIR spectroscopy data to predict and record information about cow

metabolic status at individual cow level is a great challenge for researches in the dairy sector.

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Aims of the thesis

The overall aim of the present thesis was to develop prediction models for blood BHB and other relevant metabolites, and to investigate phenotypic and genetic aspects of the predicted traits. The specific aims were:

1. to summarise results on the associations of BHB concentration in blood and milk with cow health, milk production and composition, and reproductive performance, and to describe its genetic aspects and economic implications in dairy cattle;
2. to assess the feasibility of using routine milk MIR spectra for the prediction of blood metabolites in dairy cows using partial least squares regression analysis coupled with spectral variable selection;
3. to investigate the associations between measured blood metabolites and predicted milk traits in early lactation;
4. to identify factors associated with blood BHB, NEFA and urea predicted by MIR spectroscopy in a large database of Brown Swiss, Holstein-Friesian, and Simmental cattle breeds;
5. to assess the genetic variation of MIR-predicted blood BHB and NEFA, and their correlations with milk production and composition traits in early-lactation Holstein cows.

Chapter 1

Invited review: β-hydroxybutyrate concentration in blood and milk and its associations with cow performance

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ABSTRACT

Hyperketonemia (HYK) is one of the most frequent and costly metabolic disorders in high-producing dairy cows and its diagnosis is based on β-hydroxybutyrate (BHB) concentration in blood. In the last 10 years, the number of papers that have dealt with the impact of elevated BHB levels in dairy cattle has increased. Therefore, this paper reviewed the recent literature on BHB concentration in blood and milk, and its relationships with dairy cow health and performance, and farm profitability. Most studies applied the threshold of 1.2 mmol/L of BHB concentration in blood to indicate HYK; several authors considered BHB concentrations between 1.2 and 2.9 mmol/L as subclinical ketosis, and values ≥ 3.0 mmol/L as clinical ketosis. Results on HYK frequency (prevalence and incidence) and cow performance varied according to parity and days in milk, being greater in multiparous than in primiparous cows, and in the first 2 weeks of lactation than in later stages. Hyperketonemia has been associated with greater milk fat content, fat-to-protein ratio and energy-corrected milk, and lower protein and urea nitrogen in milk. The relationships with milk yield and somatic cell count are still controversial. In general, HYK impairs health of dairy cows by increasing the risk of the onset of other early lactation diseases, and it negatively affects reproductive performance. The economic cost of HYK is mainly due to impaired reproductive performance and milk loss. From a genetic point of view, results from the literature suggested the feasibility of selecting cows with low susceptibility to HYK. The present review highlights that milk is the most promising matrix to identify HYK, because it is easy to sample and allows a complete screening of the herd through BHB concentration predicted using mid-IR spectroscopy during routine milk recording. Further research is needed to validate accurate and convenient methods to discriminate between cows in risk of

HYK and healthy animals in field conditions and to support farmers to achieve an early detection and minimise the economic losses.

Keywords: cattle, health, hyperketonemia, ketone body, milk production

INTRODUCTION

Ketosis is one of the most detrimental metabolic diseases in dairy cows. It occurs in early lactation when animals experience a negative energy balance (NEB), defined as the lack of trade-off between energy intake (input) and demands for increased milk production (output) (Herdt, 2000). During this period, cows use alternative energy sources to supply the decrease of available glucose that comes from gluconeogenesis; glucose is a fundamental nutrient strictly linked to the maintenance of normal functions in most of body tissues and lactogenesis. The inability of cows to cope with NEB and glucose drop due to an inadequate metabolic adaptation leads to an excessive mobilisation of adipose reserves, releasing abnormal concentrations of non-esterified fatty acids (NEFA) and ketone bodies (acetone, acetoacetate and BHB) in the blood. Thus, biochemical analyses are fundamental to help with the diagnosis of this complex disease. Elevated concentrations of ketone bodies in blood, defined as HYK, negatively affect immune function, health and milk production (McArt et al., 2013). The BHB is the most common ketone body used to diagnose HYK (Oetzel, 2004) because it is the predominant and more stable circulating ketone body in cow fluids (Duffield et al., 2009). However, BHB measurements vary for several reasons such as diurnal variation in body fluids (Nielsen et al., 2003), and methods of sampling and analysis (Krogh et al., 2011; Bach et al., 2016).

Hyperketonemia can result in subclinical ketosis (SCK) or clinical ketosis (CK). Some signs described in literature in animals suffering from CK are ketone smell in breath, reduced activity and appetite, excessive loss of body condition, weakness and apparent blindness (Berge and Vertenten, 2014), but those signs are unspecific and/or difficult to be detected; this increases the error of diagnosis and makes arduous to precisely discriminate between CK and SCK. Both CK and SCK affect milk production, reproduction performance and health of dairy cattle (McArt et al., 2013; Raboisson et al., 2014), and thus, they are responsible of increased culling rate (Seifi et al., 2011) and costs at herd level (Liang et al., 2017; Mostert et al., 2018). Given this background, the reinforcement of farmers' awareness of HYK effects, the search for new opportunities of genetic improvement (Pryce et al., 2016) and the implementation of herd management strategies against HYK have recently gained more and more importance in the scientific community and dairy sector. The aim of this review is to summarise results on the associations of BHB concentration in blood and milk with cow health, milk production and composition, and reproductive performance, and to describe its genetic aspects and economic implications in dairy cattle.

CRITERIA AND METHODOLOGY OF THE REVIEW

Papers included in the present review were retrieved from Scopus (www.scopus.com) and ISI Web of Science (www. webofknowledge.com) databases for the period January 2007 to January 2018. The keywords used in the literature search were: ketosis, dairy cows, cattle, bovine, beta-hydroxybutyrate, β-hydroxybutyrate, BHB, milk betahydroxybutyrate, milk β-hydroxybutyrate, milk BHB, milk composition, milk yield, milk production, genetic, genomic, economic,

cost, hyperketonemia, subclinical ketosis, performance, health, reproduction, fertility, ketone body and metabolic disease. Even if ketosis is a complex disease, only papers that focussed on the effects of blood and milk BHB concentrations on milk production, milk composition, reproduction and health performance, and on economic and genetic aspects of this ketone body and HYK or ketosis were considered. The determination of the most accurate blood BHB cut-off to diagnose HYK was not an aim of this review because it has been previously discussed in McArt et al. (2013).

In the reviewed papers ketosis and HYK have been often used as synonyms (McArt et al., 2012; Vanholder et al., 2015; Rutherford et al., 2016), and thus, we decided to use the term HYK because we consider it more adequate when investigating BHB concentration in blood or milk. The terms SCK and CK have been used when referring to studies that made clear reference to SCK and CK in the text. The unification of terminology such as HYK, CK and SCK, or frequency defined as prevalence or incidence, was sometimes very problematic and made difficult the comparison among studies. Data quality when analysing health events differed among the reviewed studies because some of them were based on voluntary declaration from farmers (Ospina et al., 2010a; Koeck et al., 2014; Parker Gaddis et al., 2018) and others were from veterinarians (Suthar et al., 2013). Moreover, although efforts have been made to standardise the clinical diagnosis, the participation of several observers leads to an unquantifiable error of diagnosis, involving a difficult interpretation of the results.

Studies on BHB effects on cow production have increased since 2012 (Figure 1), with an increasing incidence of papers dealing with genetic parameters and economic aspects. The increment of BHB studies underlines that metabolic disorders have been assuming more and more relevance in dairy industry and scientific

community. Moreover, the increase of papers on genetic and economic aspects of HYK is likely related to the more recent availability of large data including BHB concentration as a routinely recorded trait in some production systems, which is necessary, for example, to estimate its genetic parameters.

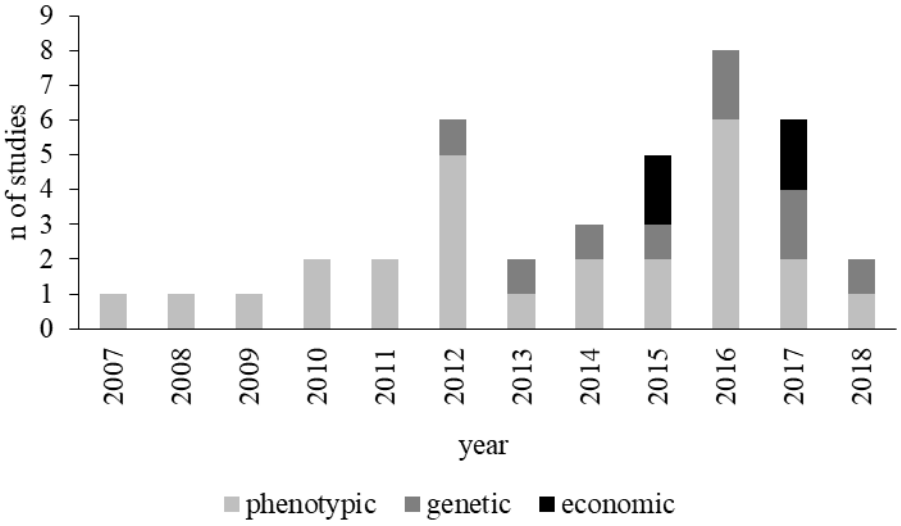

Figure 1. Number of reviewed studies in dairy cattle per area of interest and year of publication. Phenotypic area includes milk production and composition, reproduction and health performance.

Table 1. Description of reviewed studies on hyperketonemia in Holstein cows detected from blood or milk analysis

Reference	Country[1]	Herds (n)	Cows (n)[2]	Parity	Observation period
Blood					
Walsh et al. (2007)	CDN	25	796	2, 3	-3 to 9 weeks from calving
van Haelst et al. (2008)	NL	1	16	2, 3, 4	9 weeks from calving
Duffield et al. (2009)	CDN	25	1,010	1, 2, 3	2 weeks from calving
Ospina et al. (2010a)	USA	100	2,758	1, ≥2	-2 to 2 weeks from calving
Ospina et al. (2010b)	USA	91	2,290	1, ≥2	-2 to 2 weeks from calving
McArt et al. (2011)	USA	4	1,717	1, 2, ≥3	3 to 16 days in milk
Seifi et al. (2011)	CDN	16	8,49	1, 2, ≥3	3 weeks from calving
Chapinal et al. (2012a)	CDN/USA	45	1,919	1, ≥2	-1 to 3 weeks from calving
Chapinal et al. (2012b)	CDN/USA	55	2,365	1, ≥2	-1 to 1 weeks from calving
McArt et al. (2012)	USA	4	1,717	1, 2, ≥3	3 to 16 days in milk
Roberts et al. (2012)	CDN/USA	69	5,979	1, 2, ≥3	-1 to 2 weeks from calving
van der Drift et al. (2012a)	NL	118	1,678	1, 2, 3, ≥4	5 to 60 days in milk
van der Drift et al. (2012b)	NL	122	1,615	1, 2, 3, ≥4	5 to 60 days in milk
Suthar et al. (2013)	several[4]	528	5,884	1, 2, 3, ≥4	2 to 15 days in milk
Vanholder et al. (2015)	NL	23	1,715	1, 2, ≥3	7 to 14 days in milk
Kaufman et al. (2016)	CDN	4	339	1, 2, ≥3	-2 to 4 weeks from calving
Mann et al. (2016)	USA	1	84	2, ≥3	3 to 14 days in milk
Rutherford et al. (2016)	GB	3	203	1, 2, ≥3	7 to 21 days in milk
Song et al. (2016)	CHN	1	45	>1	-
Stangaferro et al. (2016)	USA	1	1,080	1, ≥2	-2 to 4 weeks from calving

Belay et al. (2017b)	N	2,828	179,691	1, 2, 3, 4	11 to 120 days in milk
Rathbun et al. (2017)	USA	1	570	1 to ≥6	5 to 18 days in milk
Ruoff et al. (2017)	D	6	621	1, ≥2	1 to 42 days in milk
Weigel et al. (2017)	USA	3	1,453	1 to ≥5	5 to 18 days in milk
Chandler et al. (2018)	USA	16	1,005	1, ≥2	5 to 20 days in milk
Milk					
van der Drift et al. (2012b)	NL	122	1,615	1, 2, 3, ≥4	5 to 60 days in milk
Buitenhuis et al. (2013)	DK	20	371	1, 2, 3	129 to 228 days in milk
Berge and Vertenten (2014)	D, F, GB, I, NL	131	4,709	1 to 12	7 to 21 days in milk
Koeck et al. (2014)	CDN	-	61,331	1	5 to 100 days in milk
Moyes et al. (2014)	DK	1	30	1	4 to 6 weeks from calving
Kayano and Kataoka (2015)	J	50	693	1 to 12	7 to 30 days in milk
Penasa et al. (2015)	I	299	19,980	1, 2, 3	5 to 305 days in milk
Jamrozik et al. (2016)	CDN	-	35,575	1 to 5	5 to 40 days in milk
Lee et al. (2016)	ROK	-	7895	1, 2, 3	4 to 305 days in milk
Santschi et al. (2016)	CDN	4242	498,310	1, 2, ≥3	5 to 35 days in milk
Rathbun et al. (2017)	USA	1	570	1 to ≥6	5 to 18 days in milk
Parker Gaddis et al. (2018)[3]	USA	-	23,865	1 to 5	1 to 60 days in milk

[1]CDN = Canada; CHN = China; D = Germany; DK = Denmark; F = France; GB = United Kingdom; I = Italy; J = Japan; NL = the Netherlands; N = Norway; ROK = South Korea; USA = United States.

[2]Berge and Vertenten (2014) (German Black Pied and other breeds); Suthar et al. (2013) (Holstein-Friesian crossbreds, Jersey and Brown Swiss); Belay et al. (2017b) (Norwegian Red); Chandler et al. (2018) (Jersey); Parker Gaddis et al. (2018) (Jersey).

[3]Producer-recorded cases.

[4]Denmark, Spain, Croatia, Hungary, Italy, Poland, Portugal, Slovenia, Serbia, Turkey.

Table 1 reports some information retrieved from the reviewed studies on HYK in dairy cattle, namely the country where the study was conducted, number of herds and cows, cow breed and parity, and observation period. Papers (n=4) that dealt with economic aspects were not included in Table 1 because they were based on literature values and data simulations. The papers mainly dealt with Holstein, as it is the most popular and productive cosmopolitan dairy cattle breed, and only few studies focussed on other breeds such as Jersey, Brown Swiss, Norwegian Red and local populations. The studies were conducted in 20 countries, with United States and Canada being the most represented with 15 and 10 papers, respectively (Table 1). As only few studies dealt with an observation period that started 1 to 3 weeks before calving, this review focused more on the *postpartum* period. Table 1 also shows that in recent years several papers have dealt with BHB concentration in milk, which is a more practical matrix than blood.

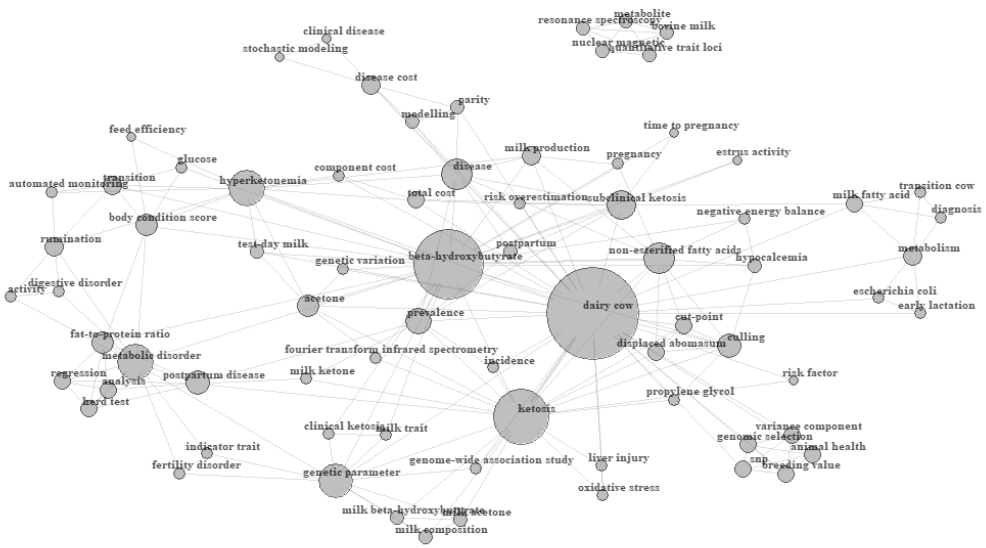

Figure 2. Author's keywords occurrence map for reviewed studies in dairy cattle. The closer two terms are located in the map, the stronger the relation between the terms and the bigger the nucleus, the more frequent the word has been used.

The keywords reported in each paper by their authors were extracted by adapting the Bibliometrix package (Aria and Cuccurullo, 2017) for R v. 3.4 (R Core Team, 2017), which produced the network of all recurrent keywords weighted by their frequency (Figure 2). The closer two terms are located in the map, the stronger the relationship between the terms, and the bigger the nucleus, more frequently the word has been used. The total number of keywords retrieved after editing of synonyms was 75 and 'dairy cows', 'β-hydroxybutyrate', 'ketosis', 'non-esterified fatty acids' and 'hyperketonemia' were the most recurrent keywords.

METHODS AND THRESHOLDS USED TO DEFINE HYPERKETONEMIA

The tested matrix, method of determination and diagnostic threshold affect the results about relationships between BHB concentration and HYK and, consequently, the impact on cow performance. However, universal method and threshold for defining HYK and linking it to ketosis have not been established yet, probably because of the difficulty of accurately diagnose CK, and therefore discriminate between CK and SCK. Moreover, the cut-offs used in the reviewed papers could increase the error of HYK detection because BHB concentration in blood and milk fluctuates during the day (Nielsen et al., 2003). Most reviewed studies did not specify sampling times, which could lead to unpredictable differences between outcomes. However, a clear pattern of the diurnal variation of BHB concentration in blood and milk has not been described yet, even if a relationship with the energy content of the diet has been reported by Nielsen et al. (2003). In particular, cows fed a total mixed ration with low-energy content showed a decrease of blood and milk BHB concentration in the evening and lower variation among cows, whereas cows fed a total mixed ration with high-energy content showed an increase of blood and milk

BHB concentration in the evening and greater variation among cows (Nielsen et al., 2003). In the same study (Nielsen et al., 2003), BHB concentrations in blood of a cow fed a total mixed ration with high-energy content exceeded the most common threshold used to define HYK in blood (1.2 mmol/L) for 37.5% of the day, which could lead to a misclassification of positive HYK. Therefore, the tested matrix, method of determination and threshold need to be taken into account to correctly interpret the results. Table 2 summarises the methods of determination of BHB concentration and the cutoffs of BHB used to establish HYK, or SCK and CK, in the reviewed papers.

The BHB can be detected in blood and milk. In particular, blood BHB concentration is the most common indicator to diagnose HYK and this explains why the majority of reviewed papers dealt with blood sampling (Table 2). The laboratory determination of BHB concentration is based on a colorimetric enzymatic reaction followed by a spectrophotometric analysis. Moreover, handheld blood ketone meters have been developed and validated in order to provide a more practical tool for on-field data collection. These on-farm testing systems include handheld devices and test strips based on an electrochemical reaction with a small amount of blood to determine the ketone concentration with acceptable specificity and sensitivity for HYK diagnosis (Bach et al., 2016; Sailer et al., 2018). However, it should be taken in consideration that blood BHB concentration is usually based on a single blood sample and it represents the status of the animal at that sampling point of the day.

Blood sampling is a labourious and time-consuming procedure, and it is stressful for the animals. The possibility of using milk BHB concentration to diagnose HYK has been investigated (Table 2) because milk recording is already a routine and non-invasive procedure, and it facilitates monitoring at herd level. Moreover, it differs

from blood measurement of BHB status of the animal as a milk sample represents a period of time. Although laboratory analyses such as photometrical method or enzymatic assays (Chandler et al., 2018) and cowside strip tests (Keto-Test; Berge and Vertenten, 2014) exist in this field, mid-IR spectroscopy (MIRS) is the most promising tool for the determination of milk BHB (Grelet et al., 2016). Indeed, the use of chemical analysis or handheld meter to measure BHB in routine milk recording is not feasible because it is too expensive and time consuming, whereas MIRS allows to collect data at population level cheaply and provide routine reports to farmers for monitoring their herd. Currently, the accuracy of the developed prediction equations for BHB concentration in milk is not high enough to quantify the exact content but it has proved to be useful for screening purposes to detect cows with elevated BHB concentrations in milk (Grelet et al., 2016; Lee et al., 2016; Santschi et al., 2016). Moreover, predicted milk acetone and BHB have been used to develop models for the prediction of blood BHB from test-day milk and performance traits (Chandler et al., 2018), and the use of MIRS milk spectra has been proposed to directly predict blood BHB concentration (Belay et al., 2017a and 2017b). Nevertheless, for this approach, we have to consider that usually blood BHB concentration is determined using a single blood sample that represents the status of the animal at the time of blood sampling, whereas a milk sample represents the BHB concentration of a period of time, which could interfere with the accuracy of the developed calibration model. To increase the accuracy of the prediction models using milk samples, several blood samples during the same period of time that the milk sample would represent could be collected.

For both blood and milk, different thresholds have been used (Table 2). In general, the optimum thresholds of BHB concentration for *postpartum* diseases

occurrence (Duffield et al., 2009; Ospina et al., 2010a; Suthar et al., 2013), fertility indicators (Chapinal et al., 2012a), and change in milk production and composition traits (Duffield et al., 2009) have been identified based on the highest combination of sensitivity and specificity of the analysis performed. However, most of reviewed studies have defined the cut-offs before starting the experiment (Koeck et al., 2014; Vanholder et al., 2015; Rutherford et al., 2016). The main difficulty was to unify terminology as some authors clearly specified a distinction between SCK and CK, whereas others considered only HYK. In general, regarding BHB cut-offs reported in the papers, HYK and SCK were defined from the same threshold. In blood, BHB ≥ 1.2 mmol/L is generally used as cut-off to identify cows with HYK, or affected by SCK. A deeper analysis of the adequacy of this cut-off in blood has been discussed in McArt et al. (2013). A less uniform BHB cut-off for CK has been proposed in literature. Although most papers have reported a direct relationship between blood BHB concentrations and CK incidence and established the threshold at blood BHB ≥ 3.0 mmol/L, some authors observed that CK occurrence can be associated to lower BHB concentration (e.g., BHB ≥ 1.1 mmol/L) (Seifi et al., 2011; Suthar et al., 2013; Song et al., 2016).

Only few papers have used milk BHB concentration to detect HYK (Table 2) and, considering the limitations of strip tests and MIRS prediction models, there is not a clear cut-off point. Ranges of milk BHB concentrations to classify cows with suspect HYK (0.15 to 0.19 mmol/L) or positive HYK (≥ 0.20 mmol/L) have been recently proposed by Koeck et al. (2014) and Santschi et al. (2016). On the other hand, Lee et al. (2016) considered cows as affected by SCK with milk BHB concentration between 0.01 and 0.20 mmol/L, and affected by CK with milk BHB concentration ≥ 0.20 mmol/L. However, even with elevated concentrations of milk

BHB, a blood test and/or a veterinary visit is necessary to help on the diagnosis of HYK.

Table 2. Thresholds of β-hydroxybutyrate (BHB) concentration in blood and milk (mmol/L) used in the literature to determine hyperketonemia (HYK), also defined as subclinical ketosis (SCK) and clinical ketosis (CK) in some cases, in dairy cattle

Reference	Method of analysis	HYK/SCK	CK
Blood BHB			
Walsh et al. (2007)	Automated analyser: Dacos 2 Analyzer (Coulter Electronics)	≥ 1.0 - 1.4[1]	-
van Haelst et al. (2008)	Automated analyser: Unicel DxC 600 (Beckman Instruments B.V.)	≥ 1.2	-
Duffield et al. (2009)	Automated analyser: Dacos 2 Analyzer (Coulter Electronics)	≥ 1.4	-
Ospina et al. (2010a and 2010b)	Automated analyser: Hitachi 917 (Roche Diagnostics)	≥ 1.0	-
Seifi et al. (2011)	Automated analyser: Hitachi 911 (Roche Diagnostics)	-	≥ 1.2
Chapinal et al. (2012a and 2012b)	Automated analyser: Hitachi 911 (Randox Laboratories)	≥ 1.4	-
McArt et al. (2011 and 2012); Weigel et al. (2017)	Handheld meter: Precision Xtra (Abbott Laboratories)	1.2 to 2.9	≥ 3.0
van der Drift et al. (2012a and 2012b)	Kit test: Ranbut kit; (Randox Laboratories)	≥ 1.2	-
Suthar et al. (2013)	Handheld meter: Precision Xtra (Abbott Laboratories)	≥ 1.2	≥ 1.1
Vanholder et al. (2015)	Handheld meter: Precision Xceed (Abbott Laboratories)	1.2 to 2.9	≥ 3.0
Kaufman et al. (2016); Mann et al. (2016); Rathbun et al. (2017)	Handheld meter: Precision Xtra (Abbott Laboratories)	≥ 1.2	-
Ruoff et al. (2017)	Handheld meter: NovaVet (Nova Biomedical)	≥ 1.2	-
Rutherford et al. (2016)	Handheld meter: Optium Xceed (Abbott Laboratories)	1.2 to 2.9	≥ 3.0
Song et al. (2016)	Not specified	1.2 to 1.5	≥ 1.5
Belay et al. (2017b)	FT-MIR spectrometer: Milkoscan Combifoss 6500 (Foss Electric)	≥ 1.2	-
Chandler et al. (2018)	Colorimetric assay	≥ 1.2	-
Milk BHB			
van der Drift et al. (2012a)	FT-MIR spectrometer: MilkoScan FT6000 (Foss Electric)	≥ 0.08	-
Berge and Vertenten (2014)	Keto-Test: Ketolac test strip (Sanwa Kagaku Kenkyusho Co. Ltd.)	≥ 0.10	-
Koeck et al. (2014); Santschi et al. (2016)	FT-MIR spectrometer: MilkoScan FT6000 (Foss Electric)	≥ 0.15 - 0.20	-
Lee et al. (2016)	FT-MIR spectrometer: CombiFoss FT+ (Foss Electric)	0.01 to 0.19	≥ 0.20

[1]Thresholds for first and second week of lactation, respectively.

FREQUENCY AND RISK FACTORS OF HYPERKETONEMIA

A disease frequency can be described through two different measures, prevalence and incidence, usually expressed as a percentage. Prevalence is defined as the number of existing cases of a specific disorder divided by the number of sampled animals at a given time, that is, a single test is required. Incidence is calculated as the number of new cases of a specific disease divided by the number of animals at risk during a defined period of time. Thus, to estimate incidence is necessary to test animals frequently enough to ensure that all cows that develop the disease during the observation period will be correctly identified. Moreover, incidence can be expressed as cumulative incidence or as incidence rate. Cumulative incidence is the most common one and provides a measure of risk in a given period of time. Incidence rate is the proportion of new cases calculated per unit of time and the result should be expressed per unit of time. Therefore, the ease of prevalence calculation explains why there is a lower number of papers that computed incidence (Table 3). Even if not clearly expressed in most of the cases, incidence values reported in reviewed studies corresponded to cumulative incidence, meaning that some misleading use of terminology to express frequency exists in literature. For instance, cumulative incidence was wrongly referred as rate in Weigel et al. (2017), whereas prevalence and incidence rate were used as synonyms in Lee et al. (2016), leading to a difficult interpretation of the results.

Prevalence and incidence of HYK reported in reviewed papers are displayed in Table 3. Prevalence ranged from 11.2% (van der Drift et al., 2012b) to 47.2% (Vanholder et al., 2015) when papers considered HYK or SCK. On the other hand, the percentage dropped off considerably when CK was considered, ranging from 3.7% (Seifi et al., 2011) to 11.6% (Vanholder et al., 2015). A similar situation was observed

in the studies reporting incidence, showing a range between 19.7% (McArt et al., 2012) and 44% (Kaufman et al., 2016) for HYK or SCK and a value of 2.4% for CK (Weigel et al., 2017).

As reported above, to interpret and compare correctly the measures of frequency, several factors such as method of detection, threshold used and biological fluid analysed should be taken into account. Moreover, these frequency expressions greatly depend on stage of lactation (observation period) and parity (Table 1). As HYK is mainly the consequence of NEB experienced by the cow after calving, the highest HYK prevalence has been detected in the first 2 weeks of lactation, declining greatly thereafter (van der Drift et al., 2012a; Koeck et al., 2014; Santschi et al., 2016). For example, a decrease of prevalence by 60% (from 18% to 7%) between the first and the second month of lactation has been reported by van der Drift et al. (2012a). Moreover, both HYK peak incidence (22.3% of cows with their first positive test) and prevalence (28.9% of cows with a positive test) have been detected at 5 days in milk in McArt et al. (2012). Considering these high frequencies in first days of lactation, the calculation of the risk of disease occurrence through multiple tests during early lactation would be an appropriate approach to measure HYK frequency.

Several authors observed a higher frequency of HYK in multiparous than primiparous cows (Santschi et al., 2016; Rathbun et al., 2017; Chandler et al., 2018), and suggested a direct relationship between increasing parity and HYK occurrence. Cumulative incidence from 8.6% to 26.2% (Rathbun et al., 2017) and prevalence from 18.8% to 27.6% (Santschi et al., 2016) have been detected moving from first to third or greater lactation. The increase of HYK occurrence with parity may be due to the concurrent needs of gestation and lactation, as indicated by Berge and Vertenten (2014). For this reason, it could be appropriate to record separately HYK risk-

estimates for different parity orders and then compute a herd level incidence risk by standardising parity-specific percentages. Conversely, the same pattern has not been observed in Jersey breed, as HYK was more prevalent in primiparous than multiparous cows (Chandler et al., 2018).

Other factors that should be considered for interpreting HYK occurrence are season of calving, breed and herd management. Authors generally agreed to identify spring as the season with greater prevalence of HYK, whereas contrasting results have been reported for late autumn and winter (Vanholder et al., 2015; Santschi et al., 2016) or summer (van der Drift et al., 2012a; Suthar et al., 2013). However, in most cases, no biological reason or evidence has been reported to justify the greater HYK prevalence in spring (Santschi et al., 2016). It has been suggested for Dutch farmers that the lower quality of the silage used during the first half of the year could explain the greater HYK prevalence in spring (Vanholder et al., 2015). Concerning breed, higher overall HYK prevalence in Jersey (19%) than Holstein cows (14%), with values that ranged from 11.4% to 25% in Jersey herds and 0% to 28% in Holstein herds, has been observed by Chandler et al. (2018). Management and feeding of gestating heifers, dry cows and cows in early lactation, as well as on-farm prevention approaches and incidence of other diseases contribute to HYK prevalence (Santschi et al., 2016). A negative association between the increase of herd size and HYK prevalence has been reported (Berge and Vertenten, 2014) as bigger herds usually implemented strategies such as grouping cows based on milk production to better meet nutritional requirements. Berge and Vertenten (2014) also observed a lower prevalence of HYK in systems with cubicles, cubicles and yards, or tie-up bars than in systems with straw yards, and a slightly greater frequency in systems in which cows were on pastures rather than housed indoor. Moreover, a lower prevalence of HYK

was reported in herds feeding forage and concentrate separately or total mixed ration compared with herds using partial mixed ration. Mixed ration refers to cows fed a total mixed ration between grazing periods. These differences in prevalence might be due to the fact that farmers cannot easily control animals in terms of nutritional level and health status when they are on pasture or housed in straw yards.

Table 3. Prevalence and incidence (%) of hyperketonemia (HYK), also defined as subclinical ketosis (SCK) and clinical ketosis (CK) in some cases, in dairy cattle

Reference	HYK/SCK	CK
Prevalence		
Walsh et al. (2007)	18.8 to 36.2	-
Duffield et al. (2009)	16.6 to 18.6	
Seifi et al. (2011)	-	3.7
Chapinal et al. (2012a)	12 to 20	-
van der Drift et al. (2012a)	11.2	-
Suthar et al. (2013)	21.8	-
Berge and Vertenten (2014)	39	-
Koeck et al. (2014)	14	-
Vanholder et al. (2015)	47.2	11.6
Mann et al. (2016)	30.5	-
Rutherford et al. (2016)	17	-
Santschi et al. (2016)	22.9	-
Chandler et al. (2018)	14 to 19	-
Incidence		
McArt et al. (2012)	43.2	-
Kaufman et al. (2016)	44	-
Rathbun et al. (2017)	19.7	-
Weigel et al. (2017)	24	2.4

ASSOCIATIONS OF B-HYDROXYBUTYRATE WITH DAIRY COW PERFORMANCE

Health

Cows with BHB concentration ≥ 1.2 or 1.1 mmol/L in blood (Seifi et al., 2011; Suthar et al., 2013) or ≥ 0.10 mmol/L in milk (Berge and Vertenten, 2014) are from 4.7 to 14.7 times more likely to manifest clinical signs of HYK. In these studies, CK was diagnosed by veterinarians (Seifi et al., 2011; Suthar et al., 2013; Berge and Vertenten, 2014) or herd managers (Suthar et al., 2013) according to the following definitions decreased feed intake or appetite, decreased milk production, a positive urine or milk ketone test, low rumen fill, reduced activity or demeanour, excessive loss of body condition, constipation or hard/dry faeces, ketone odour in breath/milk and nervous signs. However, in the reviewed papers, it is not clear how authors used all those variables to establish CK. Moreover, it is commonly agreed that HYK increases the risk of the onset of other early lactation diseases, such as displaced abomasum (odds ratio (OR) = 1.6 to 19.3; Seifi et al., 2011; McArt et al., 2011 and 2012; Suthar et al., 2013; Berge and Vertenten, 2014), metritis (OR=1.5 to 1.7; Suthar et al., 2013; Berge and Vertenten, 2014) and lameness (OR = 1.7 to 1.8; Suthar et al., 2013; Berge and Vertenten, 2014), and the risk increased with blood BHB concentration (Ospina et al., 2010a; McArt et al., 2012; Suthar et al., 2013). Overall, reviewed studies supported the hypothesis of Roberts et al. (2012) that cows with high blood BHB concentrations, especially multiparous animals, had greater probability of being removed from the herd in early lactation. In particular, in McArt et al. (2012) cows diagnosed with HYK were three times more likely to die or be culled than non-hyperketonemic cows, observing also that each 0.1 mmol/L increment of blood BHB concentration during the first month of lactation increased the risk of culling by 1.4

times. Although most studies have focussed on the very early lactation ($\leq$ 21 days in milk), consequences of elevated BHB levels on health can be observed until 60 days in milk. Some authors noted that a greater disease risk occurred for cows diagnosed with HYK in the first week after calving (Seifi et al., 2011; McArt et al., 2012). For instance, in McArt et al. (2012) cows diagnosed with HYK from 3 to 5 days in milk were 6.1 times more likely to develop displaced abomasum than cows diagnosed with HYK after the first week. Furthermore, several authors highlighted that the risk of HYK occurrence (Berge and Vertenten, 2014; Kaufman et al., 2016) or being culled after its detection (Roberts et al., 2012) was more likely for multiparous cows, probably due to the greater milk yield and to possible problems experienced during the previous lactation and dry period.

The relationships between early lactation disorders are complex. From the outcomes of reviewed papers, displaced abomasum appears as a result of HYK. Despite this, in the studies of McArt et al. (2011 and 2012) some cows developed displaced abomasum before being diagnosed positive for HYK. Displaced abomasum and HYK are both generated by a poor adaptive response to early lactation requirements. Indeed, after calving cows (especially high-producing animals) do not assume the appropriate amount of energy to face the requirements of high production, mainly because the maximum intake capacity is reached 7 to 8 weeks postpartum. In addition, since HYK leads to hypoglycaemia in multiparous cows (Ruoff et al., 2017), and to reduced rumination time and activity (Duffield et al., 2009; Kaufman et al., 2016; Stangaferro et al., 2016), HYK can be considered as a cause of displaced abomasum.

In a recent study, high levels of blood BHB have been described to be significantly correlated to oxidative stress and liver apoptosis damage (Song et al.,

2016). Thus, it is reasonable to conclude that the NEB status in early lactation and the physiological stress occurring during HYK have a role in the depression of the immune system. As a consequence, cows with HYK are more likely to be affected by metritis and lameness during the early lactation (Duffield et al., 2009; Ospina et al., 2010a; Suthar et al., 2013; Berge and Vertenten, 2014). Regarding mastitis, controversial results have been reported. Although Berge and Vertenten (2014) found that cows diagnosed with HYK were almost twice as likely to have a mastitis event in the first month of lactation compared with healthy cows and Moyes et al. (2014) observed that udder inflammation caused an increase of milk BHB concentration, Duffield et al. (2009) and Suthar et al. (2013) did not detect any association between mastitis and HYK.

Milk production

The effects of elevated blood or milk BHB levels on milk yield are controversial. While a decrease of daily milk production between 1% and 18% has been observed in several studies, an increase of daily milk yield from 5% to 11% in hyperketonemic cows has been reported by other authors (Figure 3). Besides, van der Drift et al. (2012a) and Chandler et al. (2018) did not report significant differences between cows with or without HYK. Generally, HYK affects more negatively milk production of multiparous than primiparous cows (Ospina et al., 2010b; Chapinal et al., 2012b; Kayano and Kataoka, 2015; Santschi et al., 2016), which is reasonable because first lactation cows do not have NEB status of the previous lactation as potential risk factor, and on average they have better body condition and yield less milk than multiparous animals. However, Rathbun et al. (2017) reported that the onset of HYK is not related to milk yield in previous lactation or to genetic potential for

milk production. They suggested that HYK in high-producing cows is indeed due to energy requirements of current lactation. Further research is needed to confirm this hypothesis which seems contradictory to the results observed for primiparous and multiparous cows. The negative impact of HYK on milk yield is more pronounced when detected in the first week rather than in the second week of lactation, even if cows show the same blood BHB concentration (Duffield et al., 2009; Chapinal et al., 2012a; McArt et al., 2012). The difference of milk yield between cows with HYK and without HYK increases during lactation (Kayano and Kataoka, 2015; Santschi et al., 2016), probably due to the cumulative NEB in hyperketonemic cows.

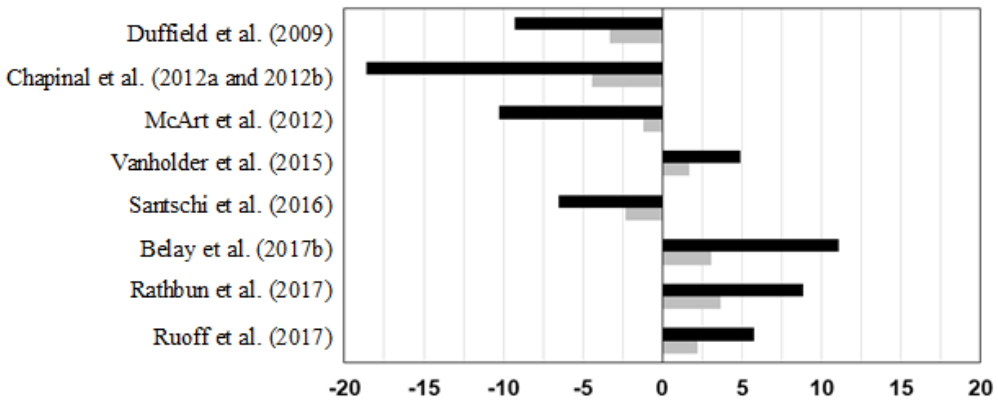

Figure 3. Greatest significant differences between normal and hyperketonemic cows for daily milk yield. Black bars express milk in %/day per cow and grey bars express milk in kg/day per cow. Negative and positive values indicate lower and higher values in hyperketonemic cows, respectively.

Milk composition

Hyperketonemia has been associated with greater milk fat content, fat-to-protein ratio (F:P) and energy-corrected milk, and lower protein, lactose and urea nitrogen in milk (Table 4; Kayano and Katatoka, 2015). An increment in fat content

between 2.4% (Vanholder et al., 2015) and 23.9% (Santschi et al., 2016) has been reported in hyperketonemic compared with healthy cows. Generally, greater differences of fat content between hyperketonemic and healthy cows have been observed in very early lactation (Koeck et al., 2014; Rathbun et al., 2017). However, a greater increment of fat percentage for cows with HYK in the second rather than in the first week of lactation was reported in the study of Duffield et al. (2009). This discrepancy could be related to the fact that Duffield et al. (2009) defined a greater BHB concentration threshold for the second week (2 mmol/L) than for the first week of lactation (1.2 mmol/L). Moreover, while Santschi et al. (2016) reported that differences in milk fat content between hyperketonemic and healthy cows increased with parity, no significant differences were observed by Chandler et al. (2018). Hyperketonemia negatively affects milk protein content; hyperketonemic animals produced milk with 0.3% (Santschi et al., 2016) to 11.6% (Chandler et al., 2018) less protein compared with healthy animals. Moreover, the greatest differences between hyperketonemic and healthy cows were detected in primiparous animals (Santschi et al., 2016; Chandler et al., 2018) and, when the week effect was considered, in the second week of lactation (Rathbun et al., 2017).

The F:P has been reported to be 10% to 32.8% higher in hyperketonemic than in healthy animals (Chandler et al., 2018). As it has been indicated for protein, those differences were greater for primiparous than multiparous cows. In addition, F:P and fatty acids (FA) have been proposed as indicators of HYK in early lactation (van Haelst et al., 2008; Mann et al., 2016). A significant decrease of several *de novo* (C6:0, C8:0, C10:0, C12:0, C14:0) and a medium-chain (C15:0) FA has been observed in milk of cows with HYK (Mann et al., 2016). Moreover, Chandler et al. (2018) reported increased concentrations of long-chain as well as total unsaturated

and trans-FA in hyperketonemic Jersey cows. In both studies, monounsaturated FA increased with HYK. The decrease of the synthesis of *de novo* FA in milk might suggest a less metabolically active mammary gland, while the increment of long-chain FA and total unsaturated FA could be related to a greater acidogenic ruminal fermentation due to lower dry matter intake and higher passage rate. Overall, the association between milk FA and elevated concentrations of BHB needs further investigation.

High-producing cows suffer a pronounced lipomobilisation in early lactation, concurrently with low serum concentrations of glucose, total proteins and urea which could explain the results of Table 4. Hyperketonemic cows had from 4.6% to 16.6% less milk urea nitrogen compared with healthy animals. The greatest decrease between hyperketonemic and healthy cows has been observed in multiparous cows (Santschi et al., 2016). The decrease of milk urea nitrogen could be related to the reduced feed intake, the oxidative stress and the liver apoptosis damage in cows affected by HYK, which leads to a low dietary protein availability and a lower protein biosynthesis in the liver, respectively (Duffield et al., 2009; Song et al., 2016). Energy-corrected milk has been reported to increase from 2% (Santschi et al., 2016) to 12.6% (Rathbun et al., 2017) in hyperketonemic compared with healthy cows. Moreover, the greatest differences in energy-corrected milk between hyperketonemic and healthy cows were observed in the first week of lactation (Rathbun et al., 2017) and in pluriparous cows (Santschi et al., 2016). The increase of energy-corrected milk in cows with HYK within the first month of lactation is mostly influenced by elevated fat percentage (Santschi et al., 2016; Rathbun et al., 2017) and in some cases by greater milk yield (Rathbun et al., 2017). Regarding lactose content, the inverse relationship between circulating BHB and glucose at metabolic level reduces the availability of this

fundamental precursor for lactose synthesis in epithelial cells of the mammary gland. Thus, for lactose content a reduction between 0.6% (Belay et al., 2017b) and 3.7% (Santschi et al., 2016) has been reported in hyperketonemic compared with healthy cows. As indicated for the other traits, the difference in lactose concentration between hyperketonemic and healthy cows was greater in primiparous than pluriparous cows (Santschi et al., 2016). As hyperketonemic cows have higher incidence of clinical mastitis compared with healthy cows (Berge and Vertenten, 2014), greater somatic cell count is expected in hyperketonemic animals. Although results of Santschi et al. (2016), who reported a 61.3% increment of mastitis incidence in hyperketonemic multiparous cows compared with healthy animals, supported this hypothesis, HYK and somatic cell count were uncorrelated in Vanholder et al. (2015) and Chandler et al. (2018), and were negatively associated in Rathbun et al. (2017), who observed that the incidence of mastitis decreased by 3.2% in hyperketonemic compared with healthy cows. In addition, milk acetone concentration has shown a coefficient of correlation with blood BHB between 0.50 and 0.79, and thus it has been proposed as an additional milk indicator of HYK, similarly to milk BHB (van der Drift et al., 2012a and 2012b; Chandler et al., 2018).

Table 4. Significant differences between normal and hyperketonemic cows for milk composition[1]

Reference	Fat (%)	Protein (%)	F:P	Lactose (%)	MUN (mg/dL)	SCC	ECM (kg/day)
Duffield et al. (2009)[2]	+0.22 to +0.48	-0.09	-	-	-	-	-
van der Drift et al. (2012a)	+0.66	-0.11	+0.26	-	-	-	-
Koeck et al. (2014)[2,3]	-	-	0 to +0.33	-	-	-	-
Vanholder et al. (2015)[4]	+0.10 to +0.31	-0.10 to -0.22	-	-	-	-	-
Santschi et al. (2016)[3,5]	+0.46 to +0.98	-0.01 to -0.10	+0.17 to +0.33	-0.05 to -0.17	-0.50 to -1.70	+55 to +184[6]	+0.60 to +2.10
Belay et al. (2017b)[2]	+0.40 to +0.50	-0.13 to -0.15	-	-0.03 to -0.06	-	-	-
Rathbun et al. (2017)[2]	+0.25 to +0.36	-0.16 to -0.24	-	-	-	-0.06[7]	+3.29 to +5.51
Chandler et al. (2018)[5]	+0.59	-0.12 to -0.39	+0.12 to +0.43	-	-	-	-

Negative and positive values indicate lower and higher values in hyperketonemic cows, respectively. Values are mean, or minimum and maximum.

[1]F:P = fat to protein ratio; MUN = milk urea nitrogen; SCC = somatic cell count; ECM = energy-corrected milk calculated as in NRC (2001).

[2]Values represent differences between days or weeks of lactation.

[3]Values represent differences between suspect or positive cows for hyperketonemia.

[4]Values represent differences between subclinical and clinical ketosis.

[5]Values represent differences between parities.

[6]SCC expressed as SCC $\times 10^3$/mL.

[7]SCC expressed as $\log_{10}$ of SCC.

Reproductive performance

The cow has to be in positive energy balance to fully express oestrus behaviour and become pregnant (Rutherford et al., 2016). All reviewed studies on the effect of HYK on reproductive performance dealt with HYK diagnosed with the analysis of blood BHB (Table 5). Animals with elevated blood BHB in the first 2 weeks after calving had lower pregnancy success at first artificial insemination than healthy cows (OR = 0.47, P = 0.003; Walsh et al., 2007), whereas no effects were observed by Chapinal et al. (2012a) and McArt et al. (2012). However, a decrease of pregnancy success within 70 days post-voluntary waiting period with a hazard ratio of 0.87 (P = 0.10) was reported by Ospina et al. (2010b). Moreover, greater number of inseminations per pregnancy (2.8 vs. 2.0, respectively; $P < 0.05$), lower peak activity (35% less activity), shorter activity at oestrus (14% less hours) and longer interval from calving to first observed oestrus in HYK than healthy cows were observed by Rutherford et al. (2016), who reported also prolonged days open for multiparous cows.

Table 5 Associations between hyperketonemia and reproductive performance in dairy cattle

Reference	Days open	Successful to first insemination	Oestrus duration	Oestrus activity
Walsh et al. (2007)	prolonged	reduced	-	-
Ospina et al. (2010b)	-	reduced	-	-
Chapinal et al. (2012a)	-	no difference	-	-
McArt et al. (2012)	no difference	no difference	-	-
Rutherford et al. (2016)	prolonged	reduced	reduced	reduced

Genetic aspects

In recent years, genetic investigations on BHB have become more relevant, leading to an increased number of papers on this topic (Figure 1). Several authors described blood and milk BHB as heritable traits, with estimates that ranged from 0.09 to 0.37 and 0.04 to 0.29, respectively (Table 6). On average, estimates of heritability of both traits increased during lactation (Koeck et al., 2014; Lee et al., 2016; Belay et al., 2017b), probably because the environmental and residual factors play a stronger role in early rather than in mid or late lactation. Some authors (Penasa et al., 2015; Jamrozik et al., 2016) observed that heritability of milk BHB decreased with increasing parity. On the other hand, an increase of heritability from first to second parity, and a slight decrease in third parity have been reported by Lee et al. (2016).

Table 6. Heritability of blood and milk β-hydroxybutyrate (BHB) in dairy cattle

Reference	Breed	blood BHB	milk BHB
van der Drift et al. (2012b)	Holstein	0.17	0.16
Koeck et al. (2014)	Holstein		0.14 to 0.29[1]
Penasa et al. (2015)	Holstein		0.08 to 0.14[2]
Jamrozik et al. (2016)	Holstein		0.07 to 0.13[2]
Lee et al. (2016)	Holstein		0.04 to 0.17[1,2]
Belay et al. (2017b)	Norwegian Red	0.25 to 0.37[1]	
Weigel et al. (2017)	Holstein	0.09	

[1]Values in different stages of lactation.

[2]Values in different parities.

Heritability estimates of blood and milk BHB were greater than estimates of CK assessed by Koeck et al. (2014), Jamrozik et al. (2016) and Belay et al. (2017b) using linear animal models (Table 7); this could depend not only on the less variability of CK, which is a dichotomous variable (presence/absence of disease), but also on the possible discrepancy of health data, which were recorded by more than one observer in the studies of Koeck et al. (2014), Jamrozik et al. (2016) and Belay et al. (2017b). The greater heritability of blood and milk BHB compared with CK, coupled with positive moderate to strong genetic correlations between these traits suggest that BHB is a useful indicator to select against ketosis (Koeck et al., 2014; Jamrozik et al., 2016; Belay et al., 2017b). Although BHB has been reported as a relatively good genetic indicator for metabolic disorders, it did not exhibit any potential as a predictor of fertility problems (Table 7; Jamrozik et al., 2016).

Considering genetic correlations of BHB with milk yield and composition traits (Table 7), some controversial results have been reported in studies that considered the early (van der Drift et al., 2012b; Koeck et al., 2014; Jamrozik et al., 2016) or entire lactation (Penasa et al., 2015) rather than mid lactation (Buitenhuis et al., 2013). Weak to moderate positive (Buitenhuis et al., 2013; Belay et al., 2017b) and negative (Penasa et al., 2015) relationships were observed between milk or blood BHB and milk yield. Negative genetic associations with milk protein, lactose and urea content were generally consistent in literature (Buitenhuis et al., 2013; Belay et al., 2017b), and a positive genetic correlation with milk fat percentage has been reported by Belay et al. (2017b), probably due to the larger fat mobilisation required in early lactation by selecting for high milk production. A general consensus in the literature described milk BHB and F:P as positively genetically correlated (Koeck et al., 2014;

Penasa et al., 2015; Jamrozik et al., 2016) and blood or milk BHB to be strongly positively correlated with acetone (van der Drift et al., 2012b).

On average, genetic correlations of BHB with diseases and milk production or composition traits were stronger in early lactation (Penasa et al., 2015; Belay et al., 2017b) and for primiparous cows (Penasa et al., 2015; Jamrozik et al., 2016) compared with later stages of lactation and parity orders. Nevertheless, a clear explanation for the stronger correlations in early lactation and primiparous cows has not been provided yet.

Genetic perspectives for analysis of HYK and ketosis have been emerging in recent years. For instance, Weigel et al. (2017) reported that the incorporation of genomic data to pedigree-based analyses enhanced estimates of HYK heritability, breeding values and predicted phenotypes. Parker Gaddis et al. (2018) observed that susceptibility to ketosis in Jerseys was affected by numerous regions along the genome, involving genes related with several pathways as immune system, insulin regulation and lipid metabolism. However, in this study ketosis events were retrieved from a voluntary producer-recorded database which increases the error of diagnosis because of the several producers involved and the declarations collected on a voluntary basis.

Table 7. Heritability and genetic correlations (r_g) of early-lactation diseases, milk yield, fat, protein and lactose percentages, fat to protein ratio (F:P), and acetone with blood and milk β-hydroxybutyrate (BHB) in dairy cattle

Trait	Heritability	r_g with blood BHB	r_g with milk BHB	Reference
Disease				
Clinical ketosis	0.02	-	0.48	Koeck et al. (2014)
	0.02 to 0.04	-	0.25 to 0.63	Jamrozik et al. (2016)[2]
	0.08	0.18 to 0.47	-	Belay et al. (2017b)[3]
Displaced abomasum	0.04	-	0.07	Koeck et al. (2014)
	0.02 to 0.06	-	0.05 to 0.36	Jamrozik et al. (2016)[2]
Metritis	0.02	-	0.09 to 0.37	Jamrozik et al. (2016)[2]
Retained placenta	0.02 to 0.03	-	0.12 to 0.16	Jamrozik et al. (2016)[2]
Milk trait				
Milk yield, kg/day	0.31	-	0.45	Buitenhuis et al. (2013)
	-	-	-0.21 to -0.09	Penasa et al. (2015)[2]
	0.16 to 0.23	0.05 to 0.19/-0.03 to 0.28[1]	-	Belay et al. (2017b)[3]
Fat, %	0.39	-	-0.94	Buitenhuis et al. (2013)
	0.10 to 0.17	-0.01 to 0.17/0.03 to 0.08[1]	-	Belay et al. (2017b)[3]
Protein, %	0.27 to 0.44	-0.28 to -0.23/-0.36 to -0.22[1]	-	Belay et al. (2017b)[3]
F:P	0.12	-	0.49	Koeck et al. (2014)
	-	-	0.28 to 0.33	Penasa et al. (2015)[2]
	0.10 to 0.16	-	0.15 to 0.49	Jamrozik et al. (2016)[2]
Lactose, %	0.41 to 0.46	-0.23 to -0.15/-0.19 to -0.16[1]	-	Belay et al. (2017b)[3]
Acetone, mmol/L	0.10	0.52	0.90	van der Drift et al. (2012b)

[1]Correlations within and across stages of lactation.

[2]Values represent differences between parities.

[3]Values represent differences between stages of lactation.

Economic aspects

Recent studies have been conducted to evaluate the economic impact of HYK in the dairy herd. The HYK cost in European countries has been evaluated using a stochastic model with distribution laws as input parameters (Raboisson et al., 2015) and a dynamic stochastic simulation model (Mostert et al., 2018). The economic impact of HYK in United States has been assessed using a deterministic model (McArt et al., 2015; Liang et al., 2017), even if in the deterministic model of Liang et al. (2017) several variables were modelled stochastically. While McArt et al. (2015) calculated the cost for HYK with blood BHB concentration ≥ 1.2 mmol/L, Liang et al. (2017) did not clearly define the diagnostic method.

All the studies observed that the cost of HYK, which can be increased twice considering diseases related to HYK (mastitis, metritis, displaced abomasum, lameness and CK), is mainly due to impaired reproductive performance and milk loss. Despite the variation of prices (e.g. milk, feed, replacement and slaughter prices) on the market of each region and year, observed results followed the same trend. The total average cost of HYK has been estimated between $77 (Liang et al., 2017) and $289 (McArt et al., 2015) per case and year in United States, and between €130 (Mostert et al., 2018) and €257 (Raboisson et al., 2015) per case and year in Europe. In general, the cost is at least twice greater in multiparous than primiparous cows (Liang et al., 2017; Mostert et al., 2018). However, a higher cost for primiparous ($374) than multiparous animals ($256) has been reported by McArt et al. (2015). Although cost distribution was difficult to compare among studies due to the different variables considered in each formula, McArt et al. (2015) and Mostert et al. (2018) clearly reported that the most important cost was related to impaired reproductive performance (34% to 36%) and milk production loss (24% to 26%). Interestingly, the

cost of the milk that was discarded following the treatment of HYK-related diseases (14%) has been additionally considered by Mostert et al. (2018). Most of HYK total costs (80%) were attributable to several HYK-related diseases (e.g. displaced abomasum, lameness, clinical mastitis and metritis) and consequently early culling, allocating less relative importance to milk production loss (11%) and prolonged days open (9%) in Raboisson et al. (2015). On the other hand, Liang et al. (2017) assigned most of the total costs to veterinary interventions and treatments (68%) or to extended days open (47%) for primiparous or multiparous cows, respectively.

CONCLUSIONS AND PERSPECTIVES

The present review summarised the major impacts of elevated blood or milk BHB concentrations on productive, fertility and economic aspects in early lactation dairy cows. A general consensus defined HYK as cause of increased risk of health problems during early lactation, with consequent negative effects on herd profitability. Nevertheless, controversial results have been observed for milk production and somatic cells count. The associations between BHB concentrations and milk yield are still not well defined both from a phenotypic and genetic point of view, and further studies are necessary to better understand the mechanisms underlying these relationships. Although the reviewed literature is consistent in reporting that elevated blood BHB concentration is detrimental to reproductive performance, Ospina et al. (2010a and 2010b) highlighted that NEFA concentration is a stronger predictor of fertility depletion than BHB. A debate about the most convenient indicator concerns also milk composition traits, for which routinely predicted traits as acetone, F:P and FA profile have been assuming increasing importance. Moreover, the interest of using multiple measurable indicators to

determine HYK has been emerging because until now most papers established HYK on the basis of a single predictor.

Therefore, the following relevant points deserve further investigations:

(i) Identification and validation of trustworthy methods to discriminate between cows affected by HYK and healthy animals in field conditions. Although the determination of blood BHB concentration is the most common method to identify HYK, it is not a useful tool in field conditions both from the economic and animal welfare point of view. The possibility to predict BHB in milk using MIRS is currently the most concrete and feasible way to collect phenotypes at population level and some studies have demonstrated that this approach is useful for monitoring HYK in dairy cows. Nevertheless, MIRS does not allow to collect detailed individual milk BHB concentrations with enough accuracy; this issue has been widely discussed in several papers and it is one of the main topics that lead researches on MIRS and metabolic-related indicators. The combination of several milk test-day predicted traits (e.g. BHB, acetone, F:P, FA) and performance variables has been proposed as an interesting strategy to predict HYK (Chandler et al., 2018). Another approach that has been recently suggested is the use of BHB concentration in blood rather than in milk as reference method to calibrate MIRS devices (Belay et al., 2017b). The possibility to predict the metabolic profile of cow by MIRS is a great challenge for the dairy sector.

(ii) Some of the controversial results highlighted in this review between the effect of elevated BHB concentration and health, milk production and composition, and reproductive performance, as well as HYK economic cost and genetic aspects could be related to data quality. Considering that ketosis is a complex metabolic disease, the diagnosis by using a single cut-off could be not enough to correctly discriminate

between healthy and hyperketonemic animals, or to properly separate between SCK and CK. Further research should focus on BHB variability within and between cows, in order to provide essential information for the development of a more accurate diagnosis method. Moreover, when dealing with health events, results could vary if they come from a voluntary declaration, if the declaration comes from the farmers or the veterinarians, or if the health event is registered by only one person or different persons. In addition, further investigation using ordinal or multinomial logistic regression to assess the relation between HYK (or CK) and various predictors is needed. A more detailed review focussed on data quality could help to better understand how the quality of recorded data may affect the impact of HYK on cow's health and performance.

(iii) Quantification of phenotypic and genetic variation of HYK in different breeds and environmental conditions. This issue is very relevant for scientists and technicians, and the possibility of recording BHB concentration or other predicted traits at population level, as mentioned above, would help investigate this topic. Several impacts of HYK on cow performance are controversial or not quantified yet, and the difficulties to have large and accurate data in the first days after calving is one of the main concerns for future research. Moreover, the possibility of combining metabolic information of dairy cows in early lactation with milk production and composition will support farmers to achieve an early detection of metabolic problems minimizing the economic losses.

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Chapter 2

Prediction of blood metabolites from milk mid-infrared spectra in early-lactation cows

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ABSTRACT

Dairy cows commonly experience an unbalanced energy status in early lactation, and this condition can lead to the onset of several metabolic disorders. Blood metabolic profile testing is a valid tool to monitor and detect the most common early lactation disorders, but blood sampling and analysis are time-consuming and expensive, and the procedure is invasive and stressful for the cows. Mid-infrared (MIR) spectroscopy is routinely used to analyze milk composition, being a cost-effective and nondestructive method. The present study aimed to assess the feasibility of using routine milk MIR spectra for the prediction of main blood metabolites in dairy cows, and to investigate associations between measured blood metabolites and milk traits. Twenty herds of Holstein Friesian, Brown Swiss or Simmental cows located in Northeast Italy were visited 1 to 4 times between December 2017 and June 2018, and blood and milk samples were collected from all lactating cows within 35 d in milk. Concentrations of main blood metabolites and milk MIR spectra were recorded from 295 blood and milk samples and used to develop prediction models for blood metabolic traits through backward interval partial least squares analysis. Blood β-hydroxybutyrate (BHB), urea, and nonesterified fatty acids were the most predictable traits with coefficients of determination of 0.63, 0.58, and 0.52, respectively. On the contrary, predictive performance for blood glucose, triglycerides, cholesterol, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase were not accurate. Associations of blood BHB and urea with their respective contents in milk were moderate to strong, whereas all other correlations were weak. Predicted blood BHB showed an improved performance in detecting cows with hyperketonemia (blood BHB ≥ 1.2 mmol/L), compared with commercial calibration equation for milk BHB. Results highlighted the opportunity of using milk MIR spectra to predict blood

metabolites and thus to collect routine information on the metabolic status of early-lactation cows at a population level.

Keywords: dairy cow, metabolic disorder, β-hydroxybutyrate, milk infrared spectroscopy

INTRODUCTION

Dairy cows experience an unbalanced energy status in early lactation as a consequence of the transition from gestation and dry-off to lactogenesis. Metabolic adaptations and high energy demands required for milk production cause a negative energy balance (McArt et al., 2013), which in turn is responsible for the occurrence of several metabolic and reproductive disorders (LeBlanc, 2010; Suthar et al., 2013; Raboisson et al., 2014).

Serum metabolic profile testing is a common method to monitor the metabolic health and nutritional status of dairy cows. Among blood metabolites, glucose, nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHB), and blood urea nitrogen (BUN) are commonly used as key indicators of metabolic status. Glucose is the main metabolic energy source and the precursor of lactose synthesis pathway, and thus its demand increases around the time of calving, especially for high-producing cows (Drackley et al., 2001; LeBlanc, 2010). If glucose demand exceeds the gluconeogenesis, circulating glucose concentration decreases, impairing the use of this monosaccharide as an energy supply (Ingvartsen, 2006). Insufficient blood glucose levels lead to the mobilization of body energy reserves to cope with negative energy balance; this induces an increase of blood NEFA, which are oxidized in liver to produce energy. When oxidizing capacity of liver is overloaded, circulating ketone

bodies are released (Esposito et al., 2014). The abnormal concentration of ketone bodies is known as hyperketonemia (HYK) and it is commonly identified through blood BHB quantification, BHB being the predominant and most stable circulating ketone body (McArt et al., 2012). On the other hand, BUN provides indication about effective RDP intakes, nitrogen utilization efficiency, and nitrogen excretion (Kohn et al., 2005; Macrae et al., 2006; Kume et al., 2008). Moreover, relevant metabolites such as triglycerides, cholesterol, and liver enzymes [glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT)] are often included in serum metabolic profile testing to have information on hepatic functionality and identify potential physiological imbalance (González et al., 2011; Bjerre-Harpøth et al., 2012).

Although blood metabolic profile testing is a valid tool to detect common disorders in early lactation, blood sampling and analysis are time-consuming and expensive, and the procedure is invasive and stressful for cows. For this reason, large-scale blood metabolic profiling is not feasible. Thanks to routine data availability, milk has been widely investigated as alternative biological matrix to blood for assessing and monitoring health traits through mid-infrared (MIR) spectroscopy (Grelet et al., 2016). Predicted milk metabolic indicators, such as acetone, BHB, and fat-to-protein ratio (F/P), have been used to monitor metabolic status at herd level (van Knegsel et al., 2010; Santschi et al., 2016; Tatone et al., 2017), but given their moderate diagnostic accuracy, they have been considered improper diagnostic tools at an individual cow level (van Knegsel et al., 2010; Chandler et al., 2018). To increase the diagnostic capability of data derived from milk MIR spectra, van der Drift et al. (2012b) and Chandler et al. (2018) have developed linear and logistic regression models combining available test-day information such as milk production and

composition, milk ketone bodies, days in milk, and parity to assess metabolic disorders. Moreover, several efforts have been made to directly predict blood metabolites at individual cow level using milk MIR spectra (Belay et al., 2017; Grelet et al., 2019; Luke et al., 2019) or merging them with milk composition and producer-reported variables (Pralle et al., 2018; Grelet et al., 2019). Using routine milk MIR data to predict cow metabolic status would not only prevent the need for expensive analyses but also allow recording of information at a population level. Therefore, the aims of the present study were to assess the feasibility of using routine milk MIR spectra for the prediction of blood metabolites in dairy cows and to investigate the associations between measured blood metabolites and predicted milk traits.

MATERIALS AND METHODS

Data Collection

Animal sampling and handling protocols were approved by the Ethical Committee for the Care and Use of Experimental Animals of the University of Padova, and carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

Twenty herds of Holstein Friesian, Brown Swiss, or Simmental cows (herd size from 15 to 500 cows) located in Northeast Italy were visited 1 to 4 times from December 2017 to June 2018. On average, 10 lactating cows, from 5 to 35 days in milk (DIM) and from parity 1 to 10, were sampled at each visit. Four herds were visited more than once, with a time interval between visits ranging from 5 to 28 d. In total, 194 lactating cows were sampled, from a minimum of 3 to a maximum of 41 cows per herd. Moreover, 64% of the animals were sampled once, and 20%, 15%, and 1% were sampled two, three, and four times, respectively. Milk and blood samples

were collected from the same cow at each sampling. Individual milks were collected following the same procedure used during routine cow milk testing, in which each sample is representative of the composition of the entire milking. Samples were immediately added with preservative (Bronopol, 2-bromo-2-nitropropan-1,3-diol, Knoll Pharmaceuticals, Nottingham, UK) and stored in portable refrigerators at 4°C. One blood sample was collected within 1 h from the end of morning (259 samples) or afternoon milking (36 samples) by jugular venipuncture into vacuum-sealed tubes (9-mL Vacutainer; Becton Dickinson and Company, Franklin Lakes, NJ) containing lithium heparin and gently inverted several times to prevent blood clotting.

Milk and Blood Analysis

Milk samples were transferred to the laboratory of the South Tyrolean Dairy Association (Bolzano, Italy) and analyzed for fat, protein, casein (CN), and lactose percentages, milk urea nitrogen (MUN) and BHB concentration (mBHB) using MilkoScan FT7 (Foss, Hillerød, Denmark). The device was calibrated with the equations developed and commercialized by Foss, and milk MIR spectra were standardized according to manufacturer guidelines. Fat-to-protein ratio was calculated from predicted milk composition traits. Somatic cell count was analyzed by Fossomatic (Foss) and values were transformed to SCS through the formula $SCS = 3 + \log_2(SCC/100{,}000)$.

Blood samples were centrifuged at 1,800 x *g* for 15 min at 4°C to separate plasma, which was stored in 2-mL Eppendorf tubes at -20°C. Frozen plasma samples were sent to the Clinical Biochemistry Laboratory of the Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (IZSLER, Brescia, Italy) and analyzed for metabolic parameters through an ILab 650 chemistry analyser

(Instrumentation Laboratory SpA, Milano, Italy) using colorimetric assay for NEFA, enzymatic kinetic colorimetric assay for BHB, kinetic assay (IFCC, International Federation of Clinical Chemistry and Laboratory Medicine) for GOT and GPT, dichromatic colorimetric end point assay (Allain et al., 1974) for total cholesterol, colorimetric trinder end point assay for glucose, colorimetric end point assay for triglycerides, and urease test for BUN.

Associations Between Blood Metabolic Parameters and Milk Traits

The first observation available for each cow was used to investigate the phenotypic associations between blood metabolites, as well as the phenotypic associations of blood metabolites with milk metabolic parameters and quality traits. In particular, Pearson correlations between the residuals were assessed after adjusting metabolite concentrations for the effects of parity, week in milk, breed, and herd-month of sampling. The effect of sampling time (morning and afternoon milking) was also tested, but it was not statistically significant in explaining the variation of the studied traits and thus it was excluded from the final model. Statistical analysis was performed using the MIXED procedure of SAS software version 9.4 (SAS Institute Inc., Cary, NC), according to the following linear model:

$$y_{ijklm} = \mu + P_i + W_j + B_k + HM_l + \varepsilon_{ijklm},$$

where y is the dependent variable (blood metabolites, milk production, or quality traits); μ is the overall intercept of the model; P_i is the fixed effect of the *i*th parity of the cow (*i* = primiparous and multiparous); W_j is the fixed effect of the *j*th class of week of lactation of the cow (*j* = 1 to 5); B_k is the fixed effect of the *k*th breed of the cow (*k* = Brown Swiss, Holstein-Friesian, and Simmental); HM_l is the random effect of the *l*th herd-month of sampling (*l* = 1 to 22); and ε is the random residual.

Prediction Models

Milk MIR spectra collected by the South Tyrolean Dairy Association were used to develop MIR prediction models for blood metabolites. Milk MIR spectra were paired with the reference values for blood and transformed from transmittance (**T**) to absorbance (**A**) by applying the formula: $A = \log_{10}(1/T)$. The dataset was checked for spectral outliers using Mahalanobis distance (threshold = 3.0), and no outliers were detected. Following infrared instrument manufacturer specifications (Foss), spectral wavelengths in regions commonly associated with high variability and low repeatability induced by water content of milk were removed. Thus, the final dataset comprised 450 spectral variables in the intervals 964.5 to 1,562.5 cm^{-1}, 1,720.7 to 2,291.7 cm^{-1}, and 2,415.1 to 2,970.7 cm^{-1}, from 295 samples. Blood BHB was normalized via $\log_{10}$ transformation.

Prediction models and fitting statistics were computed using a macro developed in SAS software version 9.4 (SAS Institute Inc.). Backward interval partial least squares (BiPLS) analysis was performed according to Zou et al. (2007), to improve predictive ability of the developed models. Milk MIR spectra were divided into 45 intervals across the MIR range from 964.5 to 2,970.7 cm^{-1}, each including 10 spectral variables, and the partial least squares (PLS) procedure of SAS was iteratively performed excluding one interval at a time. Predicted residual error sum of squares (PRESS) was calculated for each iteration. The interval to be excluded from the subsequent BiPLS round was the one resulting in the lowest PRESS statistic when left out. The procedure was iterated until only one interval remained (Xiaobo et al., 2010). For each iteration, the number of latent variables to perform the PLS procedure was defined as the minimum number of latent variables from 1 to 10 to achieve the

lowest PRESS, with $P > 0.10$. Root mean square error in leave-one-out cross validation was calculated for each BiPLS round, and the model with the best performance was selected as the final prediction model. For comparison, PLS was performed using the same parameters considered for BiPLS, including the whole spectrum to develop the prediction model. Calibration outliers were defined as observations having a residual between predicted and observed values in calibration that deviated more than 3 standard deviations (SD) from the residual average. The BiPLS procedure was repeated after calibration outliers exclusion.

The external validation was conducted by randomly assigning 1 third of the sampled cows to the validation set and 2 thirds of the samples to the calibration set. The external validation was iterated 3 times, each time over a different third of cows, and reported fitting statistics were the average of the fitting statistics of the 3 iterations. Fitting statistics of PLS and BiPLS models were the standard error in cross validation and in external validation, the coefficient of determination in cross validation (R^2_{cv}) and in external validation (R^2_v), and the ratio performance to deviation in cross validation and external validation, calculated as the ratio between SD and root mean square error in leave-one-out cross validation and between SD and root mean square error in external validation, respectively.

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics of blood metabolites and milk traits are summarized in Table 1. Among blood components, NEFA and BHB were the most variable traits, with coefficient of variation of 73% and 63%, respectively. Considering blood NEFA concentration ≥ 0.70 mmol/L as a critical threshold to identify cows with high body

reserves mobilization (McArt et al., 2013), 22.4% of blood samples were above this threshold. On average, descriptive statistics of NEFA and prevalence of high NEFA concentrations were in agreement with findings of Luke et al. (2019) in early-lactation Holstein cows. The percentage of hyperketonemic samples (BHB ≥ 1.2 mmol/L; McArt et al., 2012) was 9.8%. Although mean blood BHB and the percentage of samples with elevated BHB concentrations agreed with Pralle et al. (2018), lower and more variable statistics were reported by Grelet et al. (2019) and Luke et al. (2019) in Holstein cows, probably because of the longer observation period considered in Grelet et al. (2019; 1 to 50 DIM) and Luke et al. (2019; 5 to 49 DIM) compared with the present study. The BUN averaged 3.82 mmol/L and had coefficient of variation of 36%, which is lower and less variable compared with results of Luke et al. (2019). Only 3 samples showed abnormal concentrations of BUN (1 sample below 1.7 mmol/L and 2 samples above 6.8 mmol/L; Butler et al., 1996; Macrae et al., 2006), indicating a low prevalence of RDP-related disorders. Glucose was the least variable metabolic trait, with coefficient of variation of 23%. Average glucose concentration was 3.03 mmol/L, which is slightly lower than that reported by Grelet et al. (2019). In our data set, 9.5% of samples had glucose concentrations ≤ 2.2 mmol/L, which is a critical cutoff for hypoglycaemia (Gordon et al., 2013).

Means of milk production and composition traits were comparable with those reported in a recent study conducted among multibreed herds of Northeast Italy (Visentin et al., 2018). The mBHB averaged 0.02 mmol/L, which is lower than concentrations from previous reports (around 0.07 to 0.08 mmol/L; van der Drift et al., 2012a; Chandler et al., 2018; Pralle et al., 2018).

Table 1. Descriptive statistics of blood metabolites, milk yield, and composition traits

Trait[1]	n[2]	Mean	SD	CV, %	Minimum	Maximum
Blood						
BHB, mmol/L	295	0.73	0.46	63	0.28	3.55
NEFA, mmol/L	295	0.48	0.35	73	0.01	2.23
BUN, mmol/L	295	3.82	1.38	36	1.20	20.80
Glucose, mmol/L	295	3.03	0.71	23	1.70	11.30
Triglycerides, mmol/L	295	0.09	0.05	56	0.03	0.93
Cholesterol, mmol/L	295	3.19	1.08	34	0.49	7.00
GOT, IU/L	295	97.93	35.61	36	54.00	352.00
GPT, IU/L	295	18.64	4.60	25	3.00	31.00
Milk						
Milk yield, kg/d	286	18.69	5.38	29	7.00	36.10
Fat, %	295	4.13	0.93	22	1.56	7.93
Protein, %	295	3.32	0.41	12	2.38	5.03
F/P	295	1.25	0.27	22	0.48	2.37
Casein, %	295	2.55	0.34	13	1.67	4.14
Lactose, %	295	4.76	0.22	13	4.08	5.27
MUN, mg/dL	293	22.61	8.55	5	7.40	126.60
SCS	294	2.24	1.88	84	-1.32	8.85
mBHB, mmol/L	295	0.02	0.05	250	-0.10[3]	0.34

[1]BHB = β-hydroxybutyrate; NEFA = nonesterified fatty acids; BUN = blood urea nitrogen; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; F/P = fat-to-protein ratio; mBHB = milk β-hydroxybutyrate.

[2]n = number of samples.

[3]Fourier-transform infrared spectroscopy analysis may lead to negative values.

Associations Between Blood Metabolic Parameters and Milk Traits

Pearson correlations between blood metabolic parameters corrected for environmental and genetic (breed) effects are summarized in Table 2. The strongest positive correlation was observed between BHB and NEFA (0.45; $P < 0.001$); this estimate was close to that reported by Luke et al. (2019) and much higher than the one assessed by González et al. (2011), who observed a weak and not different from zero association (0.11) between measured blood BHB and NEFA in early-lactation Holstein cows. The lower correlation estimated by González et al. (2011) could be due to the much higher mean BHB concentration calculated in their study (1.45

mmol/L), which notably exceeded both the mean BHB concentration of the present study (0.73 mmol/L) and the cutoff commonly used to identify HYK (1.2 mmol/L). On the other hand, average NEFA reported in González et al. (2011; 0.54 mmol/L) was similar to that reported in the current study (0.48 mmol/L). The second strongest relationship was observed between NEFA and triglycerides (0.42; $P < 0.001$); moreover, triglycerides showed weak but significant correlation with BHB (0.22; $P < 0.001$). Conversely, González et al. (2011) concluded that triglycerides cannot be considered adequate indicators of lipomobilization, reporting nonsignificant correlations of triglycerides with NEFA and BHB. Moderate to weak correlations of GOT with BHB (0.33; $P < 0.001$) and NEFA (0.28; $P < 0.01$) were observed in the present study, whereas González et al. (2011) estimated correlations of 0.16 between GOT and BHB, and -0.46 between GOT and NEFA.

As expected, glucose concentration was negatively associated with blood BHB (-0.54; $P < 0.001$), NEFA (-0.23; $P < 0.01$), and BUN (-0.23; $P < 0.01$), highlighting a significant association between hypoglycaemia, fat mobilization, and circulating nitrogen. The relationship between BHB and glucose was also reported by González et al. (2011), who estimated a significant negative correlation (-0.63) between these 2 traits. Similar to results reported in previous studies (González et al., 2011; Luke et al., 2019), a weak negative association was estimated between BUN and NEFA (-0.17; $P < 0.05$), and a very low and nonsignificant correlation was assessed between BUN and BHB (0.08).

Table 2. Pearson correlations between blood metabolites at first observation of each cow (n = 194), corrected for parity, week in milk, breed, and herd-month of sampling

Trait[1]	NEFA	Trigl	Chol	BUN	Gluc	GOT	GPT
BHB	0.45***	0.22**	0.01	0.08	-0.54***	0.33***	-0.09
NEFA		0.42***	-0.19**	-0.17*	-0.23**	0.28***	-0.23**
Triglycerides			0.05	-0.06	0.05	0.11	-0.07
Cholesterol				0.30***	-0.03	-0.17*	0.31***
BUN					-0.23**	-0.09	0.14*
Glucose						-0.15*	0.02
GOT							0.23**

[1]BHB = β-hydroxybutyrate, mmol/L; NEFA = nonesterified fatty acids, mmol/L; Trigl = Triglycerides, mmol/L; Chol = Cholesterol, mmol/L; BUN = blood urea nitrogen, mmol/L; Gluc = Glucose, mmol/L; GOT = glutamic oxaloacetic transaminase, IU/L; GPT = glutamic pyruvic transaminase, IU/L.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Pearson correlations between the linear mixed model residuals of blood metabolites and those of milk BHB, F/P, and quality traits are reported in Table 3. The correlation between mBHB and blood BHB was 0.58 ($P < 0.001$). Similarly, van der Drift et al. (2012a) estimated a correlation of 0.52 between blood and milk BHB, and Chandler et al. (2018) reported correlations between 0.40 and 0.62 for Holstein cows. Like blood BHB, mBHB showed significant moderate correlations with glucose (−0.41; $P < 0.001$) and NEFA (0.38; $P < 0.001$), and weak associations with GOT (0.20; $P < 0.01$) and triglycerides (0.16; $P < 0.05$). Associations between F/P and other blood metabolites were in the same direction but weaker than those between mBHB and blood parameters (Table 3), suggesting that mBHB could be used as a more accurate indicator of metabolic disorders than F/P, supporting previous findings (van Knegsel et al., 2010). The strongest association was observed between MUN and BUN (0.70; P < 0.001), similar to results reported by Wittwer et al. (1999). On the other hand, a weak correlation between MUN and glucose was observed (−0.25; $P <$

0.001), which resembled the association between BUN and glucose (Table 2). The moderate associations of protein and CN percentage with blood BHB (−0.29 and −0.32, respectively; $P < 0.001$) can be explained by the detrimental effect that HYK exerts on milk protein content (Benedet et al., 2019). Moreover, CN percentage was the trait most strongly correlated with GOT (−0.25; $P < 0.001$), suggesting that impaired hepatic functionality leads to reduced milk protein synthesis. Lactose was moderately ($P < 0.001$) associated with NEFA (−0.31) and cholesterol (0.26). The negative association between milk lactose content and NEFA could be explained by the detrimental effect that metabolic imbalance has on milk quality (Benedet et al., 2019), whereas the correlation between lactose and cholesterol could be related to increased milk production, as cholesterol showed weak but significant ($P < 0.05$) negative correlations with fat and protein percentage (Table 3).

Table 3. Pearson correlations between blood metabolites, milk β-hydroxybutyrate and milk quality traits at first observation of each cow (n = 194), corrected for parity, week in milk, breed, and herd-month of sampling

Trait[1]	mBHB, mmol/L	F/P	Fat, %	Protein, %	Casein, %	Lactose, %	MUN, mg/dL	SCS
BHB, mmol/L	0.58***	0.34***	0.23**	-0.29***	-0.32***	-0.25***	0.18*	-0.06
NEFA, mmol/L	0.38***	0.23**	0.17*	-0.21**	-0.23**	-0.31***	-0.07	0.03
Triglycerides, mmol/L	0.16*	0.14	0.08	-0.17*	-0.20**	-0.10	0.04	-0.05
Cholesterol, mmol/L	-0.10	-0.10	-0.16*	-0.16*	-0.12	0.26***	0.14*	-0.16*
BUN, mmol/L	-0.06	0.08	0.02	-0.14	-0.15*	0.08	0.70***	-0.02
Glucose, mmol/L	-0.41***	-0.19**	-0.13	0.18*	0.16*	0.10	-0.25***	0.19**
GOT, IU/L	0.20**	0.16*	0.08	-0.21**	-0.25***	-0.08	-0.03	-0.09
GPT, IU/L	-0.13	-0.14	-0.16*	-0.05	-0.04	0.22**	0.01	-0.14

[1]BHB = β-hydroxybutyrate; NEFA = nonesterified fatty acids; BUN = blood urea nitrogen; mBHB = milk β-hydroxybutyrate; F/P = fat-to-protein ratio; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Prediction of Blood Metabolites

For each blood metabolite, specific spectral regions were selected through BiPLS procedure to develop the prediction models. Useful wavenumbers for the most important metabolic traits are depicted in Figure 1. Wavenumbers selected by BiPLS for BUN, NEFA, and BHB calibration models included the spectral region between 1,450 and 1,200 cm^{-1}, known to be associated with acetone content in milk, which is an indicator of subclinical ketosis (Heuer et al., 2001). Moreover, the calibrations of BUN, NEFA, and BHB include spectral regions typical of milk lactose (1,250 to 1,000 cm^{-1}) and fat content (1,450 to 1,390 cm^{-1}; Grelet et al., 2015). Considering the negative association between milk lactose percentage and BHB in blood and milk (Benedet et al., 2019), the inclusion of spectral regions typical for lactose content in the BHB prediction model was somewhat expected (Table 3). Finally, BiPLS for BHB prediction also included wavelengths between 1,500 and 1,400 cm^{-1}, typical of milk protein content (De Marchi et al., 2014). Comprehensive descriptions of selected wavelengths with associated compositions are listed in Supplemental Table S1.

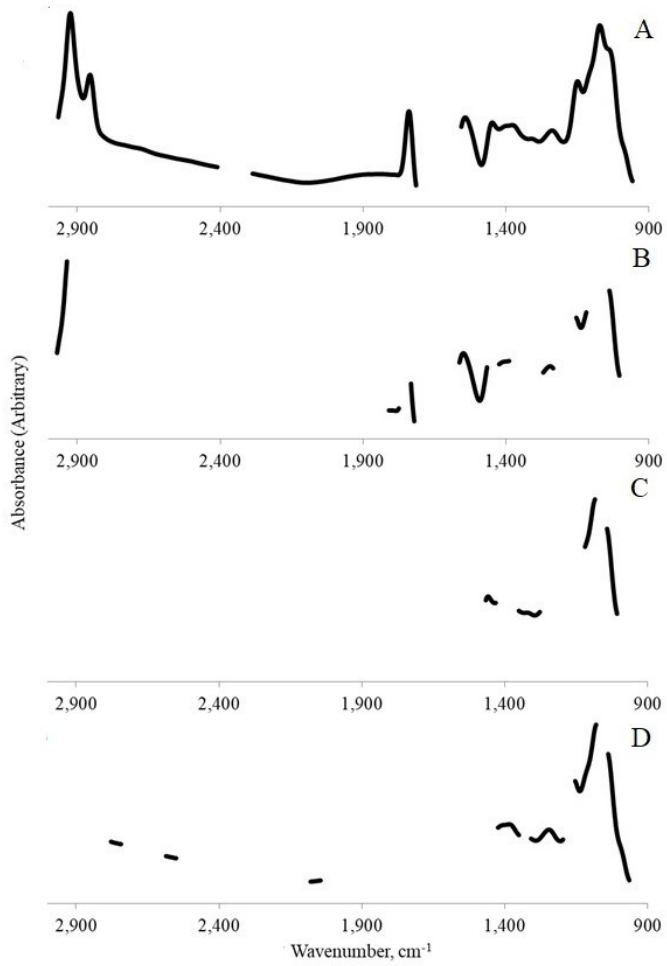

Figure 1. Whole (A) and selected wavenumber variables/spectral regions for predicting blood urea nitrogen (B), nonesterified fatty acids (C), and β-hydroxybutyrate (D).

Table S1. Selected wavenumber variables/spectral regions for predicting blood β-hydroxybutyrate (BHB), nonesterified fatty acids (NEFA), and urea nitrogen (BUN)

Trait	Spectral region	NV[1]	Assignment[2]
BHB, mmol/L	964.5 – 1,037.802	20	Lactose
	1,080.24 – 1,153.542	20	Lactose
	1,195.98 – 1,307.862	30	Lactose/Acetone
	1,350.3 – 1,423.602	20	Acetone/Fat
	2,044.74 – 2,079.462	10	Protein Backbone
	2,550.138 – 2,584.86	10	Thiols/Fat
	2,743.038 – 2,777.76	10	Fat
NEFA, mmol/L	1,003.08 – 1,037.802	10	Lactose
	1,080.24 – 1,114.962	10	Lactose
	1,273.14 – 1,346.442	20	Acetone
	1,427.46 – 1,462.182	10	Acetone/Fat
BUN, mmol/L	1,003.08 – 1,037.802	10	Lactose
	1,118.82 – 1,153.542	10	Lactose
	1,234.56 – 1,269.282	10	Lactose/Acetone
	1,388.88 – 1,423.602	10	Acetone/Fat
	1,466.04 – 1,732.242	30	Protein
	1,774.68 – 1,809.402	10	Fatty acids
	2,935.938 – 2,970.66	10	Fatty acids

[1]NV = number of variables.

[2]Dufour et al. (2009); Grelet et al. (2015).

Fitting statistics of developed models greatly varied among blood metabolites (Table 4). Prediction models for BHB, BUN, and NEFA had R^2_{cv} of 0.64, 0.54, and 0.53, and R^2_v of 0.63, 0.58, and 0.52, respectively. The ratio of performance to deviation in cross validation ranged from 1.45 (NEFA) to 1.61 (BHB), and the ratio of performance to deviation in external validation from 1.41 (NEFA) to 1.58 (BHB). Glucose, triglycerides, cholesterol, GOT, and GPT were predicted with less accuracy compared with BHB, BUN, and NEFA; indeed, R^2_{cv} and R^2_v were smaller than 0.50. The accuracy of prediction of BHB was slightly higher compared with other studies (Belay et al., 2017; Pralle et al., 2018; Luke et al., 2019), and similar to the results of Grelet et al. (2019), who obtained R^2_{cv} of 0.70. Grelet et al. (2019) concluded that the model was not appropriate for providing exact BHB values, but it was accurate enough for distinguishing between high and low blood BHB concentrations. Similarly, our results suggest that prediction models could be used to discriminate between low, medium, and high BHB concentrations and, thus, between cows with or without HYK. The moderate accuracy of blood NEFA prediction was reported also by Luke et al. (2019), who observed R^2 from 0.45 to 0.61 in external and random validation, respectively, whereas Grelet et al. (2019) reported lower R^2_{cv} (0.39) for NEFA. Despite the moderate accuracy, predicted blood NEFA may reasonably be used to facilitate the detection of negative energy balance in sampled cows. The prediction model for BUN showed lower-than expected performance, especially compared with the findings of Luke et al. (2019), who reported R^2_{cv} and R^2_v of 0.90. This could be due to the limited range of variation observed in our study for BUN concentration compared with that of Luke et al. (2019). This hypothesis is supported by the fact that external validation conducted by Luke et al. (2019) on a data set with means and variation similar to the present study showed a low R^2_v (0.35).

Nevertheless, MUN analysis is routinely provided in milk recording systems, and a moderate to strong positive Pearson correlation exists between MIR-predicted MUN and measured BUN in the present study (r = 0.70 for corrected data and r = 0.85 for raw data; $P < 0.001$). For this reason, MUN can be considered a reliable tool to monitor nitrogen utilization (Jonker et al., 1998; Wittwer et al., 1999). To our knowledge, this is the first study that attempted to predict bovine blood triglycerides, cholesterol, GOT, and GPT using milk MIR spectra; the prediction models for these traits were not accurate. Regarding glucose, low R^2_{cv} for this trait was already observed by Grelet et al. (2019), who attributed the difficulties of developing a reliable model to the limited variability of glucose. As a matter of fact, the low variability of glucose observed by Grelet et al. (2019), expressed by a coefficient of variation of 15%, was close to the coefficient of variation (17%) observed in the present study after removal of outliers (Table 4). Limited variability could also be a possible reason for low predictive accuracy for the other traits (triglycerides, cholesterol, GOT, and GPT), along with the difficulties of detecting low blood concentrations (Table 1 and Table 4) and the absence of a direct correspondence or relationship with milk traits (Table 3).

Overall, the comparison of MIR models developed in different studies is difficult; prediction performances of blood traits are influenced by blood sampling techniques (e.g., time between milk and blood collection, and standardization) and the validation procedures used. In our study, similar to that of Pralle et al. (2018), a stringent protocol for milk and blood samples collection was applied to obtain blood and milk samples within 1 h. Conversely, in other recent studies (Grelet et al., 2019; Luke et al., 2019), sampling procedures were characterized by longer intervals between milk and blood collection. Moreover, only a few studies included an external

validation step, using different approaches (Belay et al., 2017; Pralle et al., 2018; Luke et al., 2019). For instance, Luke et al. (2019) used data from an independent farm as external validation dataset. A similar or a herd-by-herd approach is useful to avoid possible overly optimistic results from cross-validation (Wang and Bovenhuis, 2019). However, due to the limited number of early-lactation cows available in each herd, a herd-by-herd validation approach was not possible in the present study. This is a potential limitation that should be considered in future investigations.

Table 4. Fitting statistics[1] of blood metabolites predictions in calibration, cross-validation, and external validation

Trait[2]	n	Mean	SD	NV	LV	SEC	R^2	SECV	R^2_{cv}	RPD_{cv}	SEPV	R^2_v	RPD_v
BHB, mmol/L	295	0.73	0.46	120	9	0.26	0.70	0.28	0.64	1.61	0.29	0.63	1.58
NEFA, mmol/L	294	0.48	0.34	50	7	0.22	0.57	0.23	0.53	1.45	0.24	0.52	1.41
BUN, mmol/L	294	3.76	0.96	90	10	0.60	0.61	0.65	0.54	1.47	0.62	0.58	1.55
Glucose, mmol/L	294	3.00	0.52	60	8	0.44	0.29	0.47	0.20	1.11	0.47	0.20	1.11
Triglycerides, mmol/L	293	0.09	0.02	50	7	0.02	0.25	0.02	0.16	1.12	0.02	0.18	1.10
Cholesterol, mmol/L	295	3.19	1.08	50	5	0.82	0.42	0.85	0.39	1.28	0.80	0.44	1.32
GOT, IU/L	294	97.06	32.43	50	4	27.49	0.28	28.34	0.24	1.14	28.29	0.24	1.15
GPT, IU/L	295	18.64	4.60	40	10	4.15	0.18	4.72	0.05	0.97	4.69	0.07	0.98

[1]n = number of samples; NV = number of variables; LV = latent variables; SEC = standard error of calibration; R^2 = coefficient of determination in calibration; SECV = standard error of prediction in cross-validation; R^2_{cv} = coefficient of determination in cross-validation; RPD_{cv} = ratio of performance to deviation in cross-validation; N_v = number of samples in external validation; SEPV = standard error of prediction in external validation; R^2_v = coefficient of determination in external validation; RPD_v = ratio of performance to deviation in external validation.

[2]BHB = β-hydroxybutyrate; NEFA = nonesterified fatty acids; BUN = blood urea nitrogen; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase.

Discriminant Ability of Predicted Blood and Milk Indicators

Predicted blood and milk BHB and F/P were tested for their performance in discriminating between cows affected or not affected by HYK. To achieve this goal, samples with predicted BHB in cross-validation (**BHB_{cv}**) of 1.2 mmol/L or higher were classified as indicating HYK, according to McArt et al., (2013). On the other hand, a concentration of 0.14 mmol/L for mBHB was selected as the cutoff to identify cows with potential HYK, as reported in Renaud et al. (2019). Although recognized as a milk HYK indicator with lower accuracy than that of ketone bodies (van Knegsel et al., 2010), F/P has the advantage of being an easy-access indirect indicator of HYK, computed from robustly predicted milk composition traits (fat and protein content). A cutoff of 1.5 was proposed by van Knegsel et al. (2010), and we applied this on our recorded data to discriminate between cows with or without HYK. Classification obtained through this test was compared with results from reference blood analyses to determine the discriminant capabilities of BHB_{cv}, mBHB, and F/P.

Accuracy, sensitivity, specificity, positive predictive value, and negative predictive value of HYK determination were computed for each metabolic indicator (Table 5). Accuracy is the proportion of correctly assigned observations (HYK or not) among all observations tested, and it ranged from 0.85 (F/P) to 0.92 (BHB_{cv}) for the predictors tested in this analysis. Although different but satisfactory accuracies were estimated, a poor sensitivity (0.14 to 0.28), defined as the ability to detect cows with HYK, was observed for all metabolic indicators. The BHB_{cv} showed the best positive predictive value (0.67), which is the probability that an animal predicted positive for HYK has a measured blood BHB $\geq$ 1.2 mmol/L, whereas F/P showed the worst positive predictive value (0.26) and, consequently, a high proportion of false positives. This is in accordance with the findings of van Knegsel et al. (2010), who

reported less capability of F/P than ketone bodies to detect cows affected by HYK. Overall, BHB_{cv} showed the most promising combination of specificity (0.98) and negative predictive value (0.93), these being the probability that the test result is negative for HYK when the disorder is not present (specificity) and that HYK is not present when the cow is tested negative for HYK (negative predictive value).

Several attempts to classify cows with or without HYK through prediction models based on milk MIR spectra, traits, and performance variables have been described in literature. Considering mBHB, although using thresholds lower than 0.14 mmol/L to identify HYK, van der Drift et al. (2012b; 0.07 mmol/L) and Chandler et al. (2018; 0.10 mmol/L) did not report results more promising than ours, observing high false positive rates and limited accuracy and efficiency. Renaud et al. (2019) obtained greater sensitivity and specificity than we observed in the present study. Nevertheless, the same authors concluded that the low prevalence of HYK in the population limited the statistical robustness of the test and that a larger number of cows should have been enrolled to achieve adequate performance. Similar limitations should be taken into account in considering the low sensitivity of BHB_{cv}. True positive cases of HYK detected through this test had measured blood BHB concentrations $\geq$ 1.37 mmol/L, averaging 2.55 mmol/L. Conversely, the average measured BHB of misclassified HYK cases was 1.53 mmol/L, suggesting that the higher the concentration, the greater the ability to discriminate samples with BHB below or above the HYK threshold. Thus, a much higher number of cows with HYK would have contributed to achieve more power and accuracy in predicting elevated BHB concentrations. To increase MIR predicting power, Pralle et al. (2018) combined producer-reported variables and milk composition traits with milk MIR spectra. However, due to the marginal improvement in model performance, Pralle et al. (2018)

concluded that there are more advantages in considering these variables for predicting HYK status separately.

Table 5. Accuracy, sensitivity, specificity, and predictive values (95% confidence interval) of predicted metabolic indicators for hyperketonemia[1] diagnosis

	Metabolic indicator[2]		
Diagnostic statistic	BHB_{cv}	mBHB	F/P
Recommended threshold[3]	1.2 mmol/L	0.14 mmol/L	1.5
Accuracy	0.92 (0.88-0.95)	0.89 (0.86-0.93)	0.85 (0.81-0.89)
Sensitivity	0.28 (0.11-0.44)	0.14 (0.05-0.33)	0.28 (0.11-0.47)
Specificity	0.98 (0.97-1.00)	0.98 (0.96-1.00)	0.91 (0.88-0.95)
Positive predictive value	0.67 (0.40-0.93)	0.40 (0.10-0.70)	0.26 (0.10-0.41)
Negative predictive value	0.93 (0.90-0.96)	0.91 (0.88-0.95)	0.92 (0.89-0.95)

[1]Defined as blood β-hydroxybutyrate ≥ 1.2 mmol/L (McArt et al., 2013).

[2]BHB_{cv} = blood β-hydroxybutyrate concentration predicted in cross-validation; mBHB = milk β-hydroxybutyrate; F/P = fat-to-protein ratio.

[3]Thresholds proposed by Renaud et al. (2019) for milk BHB and van Knegsel et al. (2010) for F/P.

CONCLUSIONS

In the present study, milk and blood samples of early-lactation dairy cows were collected and analyzed to assess metabolic status information. Moderate to strong correlations between blood metabolites, and weak to moderate associations between blood and milk metabolic indicators in early lactation were observed. Although the first attempt at predicting bovine blood triglycerides, cholesterol, GOT, and GPT using milk MIR spectra did not show accurate results, milk MIR spectra demonstrated potential for predicting important blood metabolites, in particular blood BHB. Predicted blood BHB was more strongly correlated with measured blood BHB than with mBHB, showing the most powerful ability to discriminate hyperketonemic cows. Therefore, blood metabolites predicted through milk MIR spectra are an important source of routine information on the metabolic status of early-lactation

cows. This is a prelude to large-scale phenotyping of predicted blood BHB for use in breeding programs to reduce cows' susceptibility to HYK postpartum. We aim to increase the sample size in the near future, to increase the variability of the calibration data set and improve the accuracy of prediction models.

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Chapter 3

Variation of blood metabolites of Brown Swiss, Holstein-Friesian, and Simmental cows

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ABSTRACT

Early lactation is a critical period in which dairy cows usually experience severe metabolic changes that can lead to the occurrence of metabolic diseases. Serum metabolic profile is the most common method to monitor metabolic health and nutritional status of early-lactation dairy cows, but blood analysis is time-consuming and expensive, and requires blood sampling, which is an invasive and stressful procedure for the animals. Mid-infrared (MIR) prediction models for blood metabolites have been recently developed, allowing the prediction of metabolic traits on a large scale. The current study aimed to investigate factors associated with blood β-hydroxybutyrate (BHB), nonesterified fatty acids (NEFA), and blood urea nitrogen (BUN) predicted from milk MIR spectra in a large database of Brown Swiss, Holstein-Friesian, and Simmental cows. The database consisted of the first test-day record (n = 43,201) of early-lactation cows from 5 to 35 days in milk (DIM) farmed in multi-breed herds. Sources of variation of predicted blood metabolites were investigated using linear mixed models, including the fixed effects of herd, year and month of sampling, breed, parity, stage of lactation, and interactions between the effects. Random factors were cow nested within breed and the residual. Holstein-Friesian cows exhibited the greatest concentration of blood BHB and NEFA, followed by Simmental and Brown Swiss cows. An opposite situation was detected for BUN, with the greatest and the lowest concentrations in Brown Swiss and Holstein-Friesian, respectively. Blood BHB and NEFA concentrations generally increased with parity. The greatest BHB concentration was observed between 5 and 15 DIM, except for Simmental cows, which exhibited a slightly increasing trend across early lactation. From 5 to 35 DIM, NEFA concentration declined, whereas BUN increased for all considered breeds. The maximum levels of blood BHB and NEFA were recorded

during spring and early summer. Trends of BUN generally increased across the year, from spring to winter. Environmental effects identified in the present study can be included as adjusting factors in within-breed estimation of genetic parameters for major blood metabolites.

Key words: dairy cattle, milk mid-infrared spectroscopy, blood metabolite, metabolic profile

INTRODUCTION

During early lactation, dairy cows experience severe metabolic changes due to the transition from gestation to milk production and may suffer a negative energy balance (NEB) which makes cows prone to develop metabolic disorders and health issues (LeBlanc et al., 2010; McArt et al., 2013). The NEB induces the mobilisation of body reserves and the oxidation of nonesterified fatty acids (NEFA) in liver to produce energy, which results in their increased concentration in blood. If the maximum oxidizing capacity of liver is reached, ketone bodies are produced and released in blood (Esposito et al., 2014). Thus, elevated serum concentrations of NEFA and BHB are key indicators of the mobilisation of body energy reserves and the presence of hyperketonemia (HYK), i.e., an abnormal concentration of circulating ketone bodies in body fluids (McArt et al., 2013). On the other hand, BUN is an indicator of protein status and provides information on the effective RDP intakes, and nitrogen utilization efficiency and excretion (Kohn et al., 2005; Macrae et al., 2006; Kume et al., 2008). The concentration of BUN normally increases during the first weeks of lactation (Luke et al., 2019), being associated with an increased feed intake (Seifi et al., 2007).

To monitor the metabolic status of farmed animals and promptly intervene in the possible occurring of metabolic disorders, the availability of information on metabolic indicators is crucial. A common method to monitor the metabolic health and nutritional status of dairy cows is the metabolic profiling, which is based on the determination of blood metabolites such as NEFA, BHB, and BUN. Nevertheless, blood sampling is invasive, stressful, and time-consuming, and the laboratory analyses are expensive to be performed routinely. The semi-quantitative cow-side tests available to measure metabolites (Iwersen et al., 2009; McArt et al., 2013) are also laborious and costly if used as a whole-herd screening tool.

Recently, calibration models to predict blood metabolites using milk mid-infrared (MIR) spectra have been developed (Benedet et al., 2019a; Grelet et al., 2019; Luke et al., 2019). The MIR spectroscopy is of particular interest to collect information about HYK, considering that test-day milk recording procedures are widely used to analyse milk gross composition. Thus, the new calibration models for blood metabolites from milk MIR spectra have the advantage of providing routine data useful to monitor cow metabolic status, and thus to prevent invasive samplings and expensive analyses. Moreover, the availability of large data allows to investigate phenotypic variation of blood metabolic traits at population level. Currently, there is a paucity of large-scale studies that have investigated sources of variation of blood BHB, NEFA, and BUN in early-lactation cows of different breeds (Urdl et al., 2015; Chandler et al., 2018). Therefore, the aim of the present research was to investigate sources of variation of blood BHB, NEFA, and BUN in multi-breed herds of Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows using data predicted from routine test-day milk MIR spectra.

MATERIALS AND METHODS

Data Collection

The initial data comprised spectra of individual milk samples of BS, HF, and SI cows from multi-breed herds collected during the monthly test-day recording in Bolzano province (Italy) between January 2011 and December 2018. The study area is mostly characterized by small farms with traditional feeding (forage or hay and concentrates), and approximately 20% of the herds move cows to mountain pastures in summer (Visentin et al., 2018; Franzoi et al., 2019).

During the test-day, milk samples were collected, immediately added with preservative (Bronysolv; ANA.LI.TIK Austria, Vienna, Austria), and processed according to International Committee for Animal Recording (ICAR, 2012) recommendations at the milk laboratory of the South Tyrolean Dairy Association (Sennereiverband Südtirol, Bolzano, Italy). For each sample, fat (%), protein (%), casein (%), lactose (%), and MUN (mg/dL) were determined, and spectral information containing 1,060 infrared transmittance wavelengths in the region between 900 and 5,000 cm^{-1}, were stored using MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Values of SCC were assessed by Fossomatic (Foss Electric A/S, Hillerød, Denmark) and transformed to SCS through the formula $SCS = 3 + \log_2(SCC/100{,}000)$.

Prediction of Blood Metabolites

Mid-infrared prediction models previously developed by Benedet et al. (2019a) were applied to the stored spectra of the present study to predict concentrations of blood BHB, NEFA, and BUN. Briefly, between December 2017 and June 2018, 295 individual bovine blood and milk samples were collected from early-

lactation BS, HF, and SI cows in Northeast Italy, also including herds belonging to the dataset of the current study. Reference analyses on blood samples for the determination of BHB, NEFA, and BUN concentrations were conducted in the Clinical Biochemistry Laboratory of the Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (IZSLER, Brescia, Italy) through an ILab 650 chemistry analyser (Instrumentation Laboratory SpA, Milano, Italy) using colorimetric assay for NEFA, enzymatic kinetic colorimetric assay for BHB, and urease test for BUN. Prediction models were developed using partial least squares regression approach after a backward interval partial least squares procedure as described in Benedet et al. (2019a). Coefficients of determination in cross-validation were 0.64 for BHB, 0.54 for BUN, and 0.53 for NEFA.

Data Editing

The initial dataset was edited to ensure that considered multi-breed herds were under test-day recording scheme for at least 4 yr during the study period (2011 to 2018). Moreover, only herds with at least two breeds and two cows per breed were considered. The first test-day record between 5 and 35 DIM of each lactation was retrieved from cows of parity 1 to 13. For each milk trait and blood metabolite, values that deviated more than 3 SD from the respective mean were considered inconsistent information and treated as missing values. After data editing, 43,201 test-day records of 24,566 cows in 765 herds were available for statistical analysis. Frequencies for each breed-herd combination were: BS+HF, 278 herds, 9,633 cows, 16,849 records; BS+SI, 216 herds, 4,823 cows, 8,683 records; HF+SI, 168 herds, 5,943 cows, 10,585 records; BS+HF+SI, 103 herds, 4,167 cows, 7,084 records. Herd size ranged from 4 to 159 cows. Parity and DIM averaged 2.78 ± 1.76 and 20.70 ± 8.55 d, respectively.

Statistical Analysis

Sources of variation of the studied traits were investigated using the MIXED procedure of SAS software ver. 9.4 (SAS Institute Inc., Cary, NC), according to the following linear mixed model:

$$y_{ijklmnop} = \mu + B_i + P_j + D_k + M_l + Y_m + H_n + (B \times P)_{ij} + (B \times D)_{ik} + (B \times M)_{il} + (P \times D)_{jk} + cow_o(B_i) + \varepsilon_{ijklmnop},$$

where $y_{ijklmnop}$ is the dependent variable (milk yield, fat percentage, protein percentage, casein percentage, lactose percentage, MUN, SCS, BHB, NEFA, or BUN); μ is the overall intercept of the model; B_i is the fixed effect of the *i*th breed of the cow (*i* = Brown Swiss, Holstein-Friesian, and Simmental); P_j is the fixed effect of the *j*th parity of the cow (*j* = first, second, third, and fourth and later parities); D_k is the fixed effect of the *k*th class of stage of lactation of the cow (*k* = 1 to 6, the first being a class from 5 to 10 d, followed by 5 classes of 5 d each); M_l is the fixed effect of the *l*th month of sampling (*l* = January to December); Y_m is the fixed effect of the *m*th year of sampling (*m* = 2011 to 2018); H_n is the fixed effect of the *n*th herd (n = 1 to 765); $(B \times P)_{ij}$ is the fixed interaction effect between breed and parity; $(B \times D)_{ik}$ is the fixed interaction effect between breed and stage of lactation; $(B \times M)_{il}$ is the fixed interaction effect between breed and month of sampling; $(P \times D)_{jk}$ is the fixed interaction effect between parity and stage of lactation; cow_o is the random effect of the *o*th cow (n = 1 to 24,566) nested within the *i*th breed; and $\varepsilon_{ijklmnop}$ is the random residual. A multiple comparison of means for the fixed effects was performed using Bonferroni's test ($P < 0.05$).

Single-Breed Analysis

A single-breed analysis was performed in addition to the multi-breed investigation to compare results of cattle breeds farmed in multi- or single-breed herd types. Thus, after the same editing procedure used for multi-breed data, 78,762 first milk test-day records between 5 and 35 DIM and related predicted blood BHB, NEFA, and BUN of BS, HF, or SI cows in 1,859 single-breed herds were available for statistical analysis. For the considered 43,236 cows, parity and DIM averaged 2.87 ± 1.84 and 20.76 ± 8.51, respectively. Three subsets were created according to the breed and the frequencies for each breed were: BS, 912 herds, 17,845 cows, 31,488 records; HF, 234 herds, 7,138 cows, 12,285 records; SI, 713 herds, 18,253 cows, 34,989 records.

For each breed, data on blood metabolites were analyzed using the MIXED procedure of SAS software ver. 9.4 (SAS Institute Inc., Cary, NC), according to the following linear mixed model:

$$y_{ijklmno} = \mu + P_i + D_j + M_k + Y_l + H_m + (P \times D)_{ij} + cow_n + \varepsilon_{ijklmno},$$

where $y_{ijklmno}$ is the dependent variable (BHB, NEFA, or BUN); μ is the overall intercept of the model; P_i is the fixed effect of the ith parity of the cow (i = first, second, third, and fourth and later parities); D_j is the fixed effect of the jth class of stage of lactation of the cow (j = 1 to 6, the first being a class from 5 to 10 d, followed by 5 classes of 5 d each); M_k is the fixed effect of the kth month of sampling (k = January to December); Y_l is the fixed effect of the lth year of sampling (l = 2011 to 2018); H_m is the fixed effect of the mth herd (m = 1 to 912 for BS, 1 to 234 for HF, 1 to 713 for SI); $(P \times D)_{ij}$ is the fixed interaction effect between parity and stage of lactation; cow_n is the random effect of the nth cow (n = 1 to 17,845 for BS, 1 to 7,138 for HF, 1 to 18,253 for SI); and $\varepsilon_{ijklmno}$ is the random residual. A multiple comparison of means for the fixed effects was performed using Bonferroni's test ($P < 0.05$).

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics of predicted blood metabolites and milk traits are summarised in Table 1. Average BHB (0.65 mmol/L) was intermediate between mean BHB reported by Luke et al. (2019; 0.54 mmol/L) and Pralle et al. (2018; 0.80 mmol/L) for HF cows, whereas NEFA (0.36 mmol/L) was lower than that observed by Luke et al. (2019; 0.49 mmol/L) in HF but similar to the value obtained by Djoković et al. (2017; 0.38 mmol/L) in early-lactation SI cows. On average, BUN (2.87 mmol/L) was lower than previous findings in early-lactation cows (Djoković et al., 2015; Luke et al., 2019). The lower blood metabolites concentration compared with results reported in literature may be due to the management of the considered multi-breed farms, which were located in a mountain area characterized by traditional feeding and lower productivity than herds in the plain. As a matter of fact, in the current analysis, the percentages of records with abnormal blood metabolites concentrations were very low, meaning that herds had low prevalence of metabolic disorders. The percentage of records suggesting HYK (BHB $\geq$ 1.2 mmol/L; McArt et al., 2013) was 2.4%. Also, 5.3% of the data exhibited NEFA concentration $\geq$ 0.70 mmol/L, which is considered a critical threshold to identify cows with high body reserves mobilisation (McArt et al., 2013). About 10% of samples showed abnormal concentrations of BUN (< 1.7 mmol/L or > 6.8 mmol/L; Butler et al., 1996; Macrae et al., 2006), suggesting possible wrong RDP feeding strategies.

Results for milk yield and composition traits were in agreement with those reported in recent studies conducted in multi-breed herds of Northeast Italy (Penasa et

al., 2014; Manuelian et al., 2019), bearing in mind that only the first month of lactation was investigated in the present study.

Table 1. Descriptive statistics of blood metabolites, milk yield, and composition traits of early-lactation cows in multi-breed herds (43,201 observations)

Trait[1]	Mean	SD	CV, %	Minimum	Maximum
Blood metabolite, mmol/L					
BHB	0.65	0.23	35.05	0.19	2.78
NEFA	0.36	0.19	53.27	0.01	1.32
BUN	2.87	0.84	29.37	1.20	11.53
Milk trait					
Milk yield, kg/d	30.95	6.98	22.54	9.10	56.50
Fat, %	4.10	0.74	17.93	1.68	6.87
Protein, %	3.28	0.35	10.77	2.18	4.52
F/P	1.26	0.24	18.68	0.54	2.32
Casein, %	2.57	0.27	10.54	1.65	3.51
Lactose, %	4.81	0.17	3.51	4.06	5.36
MUN, mg/dL	18.87	7.17	38.00	0.10	43.40
SCS	2.08	1.94	93.12	-3.64	9.61

[1]NEFA = nonesterified fatty acids; F/P = fat-to-protein ratio.

Breed Effect

Least squares means of predicted blood metabolites, milk yield, and composition traits for BS, HF, and SI breeds are in Table 2. Small but significant ($P < 0.05$) differences were observed between BHB concentrations in blood of the three breeds; the greatest (0.65 mmol/L; $P < 0.05$) and the lowest (0.62 mmol/L; $P < 0.05$) BHB concentrations were detected for HF and BS, respectively, whereas SI was intermediate (0.63 mmol/L; $P < 0.05$). Moreover, HF exhibited the greatest NEFA (0.42 mmol/L; $P < 0.05$) and the lowest BUN concentrations (2.67 mmol/L; $P < 0.05$), which could suggest that HF cows are more prone to incur abnormal metabolite concentrations and metabolic disorders than other breeds. In fact, elevated BHB and NEFA, and low BUN commonly indicate an insufficient energy and protein intake

due to the diet or inability of the animal to cope with the NEB that characterizes the first month of lactation (Macrae et al., 2006; LeBlanc, 2010). Brown Swiss and SI showed the same concentration of NEFA (0.35 mmol/L), but different BUN concentration (3.18 vs. 2.80 mmol/L, respectively; $P < 0.05$). These results partially agreed with Urdl et al. (2015), who reported similar concentrations for BUN but opposite for BHB in early-lactation BS, HF, and SI cows. Moreover, the same authors did not observe a significant breed effect on NEFA, concluding that breed is a less important effect than energy intake or milk production on blood metabolites. On the contrary, our findings suggested that breed is an important source of variation for early-lactation blood metabolites, even if the reason of some differences among breeds are still unclear. For instance, the dual-purpose SI breed was expected to be the best to cope with the energy stress for milk production and thus we expected the lowest NEFA and BHB concentrations for this breed. However, SI is probably characterized by a different but not well-known metabolic pathway following the slower milk production increase than specialized dairy breeds (HF and BS).

Holstein-Friesian yielded greater milk (32.83 kg/d; $P < 0.05$) than the other breeds, and BS exhibited the greatest percentages of fat (4.13%), protein (3.33%), and casein (2.61%) ($P < 0.05$). In general, SI cows had an intermediate level of these milk traits, with the exception of fat percentage which was very similar to fat percentage of HF cows. The greatest (20.80 mg/dL; $P < 0.05$) and the lowest (16.42 mg/dL; $P < 0.05$) MUN contents were estimated for BS and HF, respectively, reflecting the same situation depicted by BUN concentration in each breed ($P < 0.05$; Table 2). The lowest SCS was observed for SI breed (1.74; $P < 0.05$) and the greatest for HF (2.24; $P < 0.05$). Overall, although considering the whole lactation, Penasa et al. (2014) and

Manuelian et al. (2019) obtained similar trends for milk production and composition traits of BS, HF, and SI cattle breeds.

Table 2. Least squares means[1] (standard error) of blood metabolites, milk yield, and composition traits of Brown Swiss, Holstein-Friesian, and Simmental cows in multi-breed herds

Trait[2]	Brown Swiss	Holstein-Friesian	Simmental
Blood metabolite, mmol/L			
BHB	0.62 (0.001)[a]	0.65 (0.002)[c]	0.63 (0.002)[b]
NEFA	0.35 (0.002)[a]	0.42 (0.002)[b]	0.35 (0.002)[a]
BUN	3.18 (0.01)[c]	2.67 (0.01)[a]	2.80 (0.01)[b]
Milk trait			
Milk yield, kg/d	29.28 (0.06)[a]	32.83 (0.07)[b]	29.40 (0.07)[a]
Fat, %	4.13 (0.007)[b]	4.06 (0.008)[a]	4.05 (0.009)[a]
Protein, %	3.33 (0.003)[c]	3.16 (0.003)[a]	3.30 (0.004)[b]
F/P	1.25 (0.002)[a]	1.30 (0.003)[b]	1.24 (0.003)[a]
Casein, %	2.61 (0.002)[c]	2.47 (0.003)[a]	2.58 (0.003)[b]
Lactose, %	4.82 (0.002)[b]	4.77 (0.002)[a]	4.81 (0.002)[b]
MUN, mg/dL	20.80 (0.07)[c]	16.42 (0.08)[a]	18.47 (0.09)[b]
SCS	2.08 (0.02)[b]	2.24 (0.02)[c]	1.74 (0.03)[a]
Herds, n	597	549	487
Cows, n	9,992	8,203	6,371
Records, n	17,600	13,854	11,747

[1]Least squares means with different superscript letters within a row are significantly different according to Bonferroni's test ($P < 0.05$).

[2]NEFA = nonesterified fatty acids; F/P = fat-to-protein ratio.

Interactions between Effects of Breed, Parity, Stage of Lactation, and Month of Sampling

In addition to multi-breed analysis, a dataset of single-breed herds was considered and analyzed for blood metabolites to compare results. In fact, although considering multi-breed herds is necessary to properly disentangle breed and herd effects, management and feeding strategies adopted in these herds could have smoothed the typical characteristics of different cattle breeds, which could be more detectable in single-breed farms.

Figure 1 depicts the least squares means of predicted blood metabolites across parities in multi-breed herds, and results for single-breed analysis are given in Supplemental Figure S1. In both analyses, BHB and NEFA concentrations were generally greater in third and later than first and second parities for all considered breeds ($P < 0.05$). Focusing on HF, primiparous cows had similar or greater BHB and NEFA than cows in second lactation. For BS and SI, the lowest BHB concentrations were observed for primiparous cows ($P < 0.05$). On the other hand, the trend of NEFA across parities of BS and SI was similar to that of HF, in which NEFA was greater in first than second lactation ($P < 0.05$), similarly to Mäntysaari et al. (2019). In contrast with these findings, an increasing blood BHB and NEFA concentration with increasing parity was generally expected (Benedet et al., 2019b; Gärtner et al., 2019). However, elevated concentrations of BHB and NEFA in blood of first-lactation cows could be due to their increased energy demands for growth occurring concurrently with the requirements of lacto-genesis or a worse energy status than multiparous cows before calving (Meikle et al., 2004; Wathes et al., 2007). Overall, BUN decreased slightly across parities in all considered breeds (Figure 1 and Supplemental Figure S1). Even in this case, no significant association or an opposite weak tendency between parity and BUN (Wathes et al., 2007) or milk (Yoon et al., 2004) was expected.

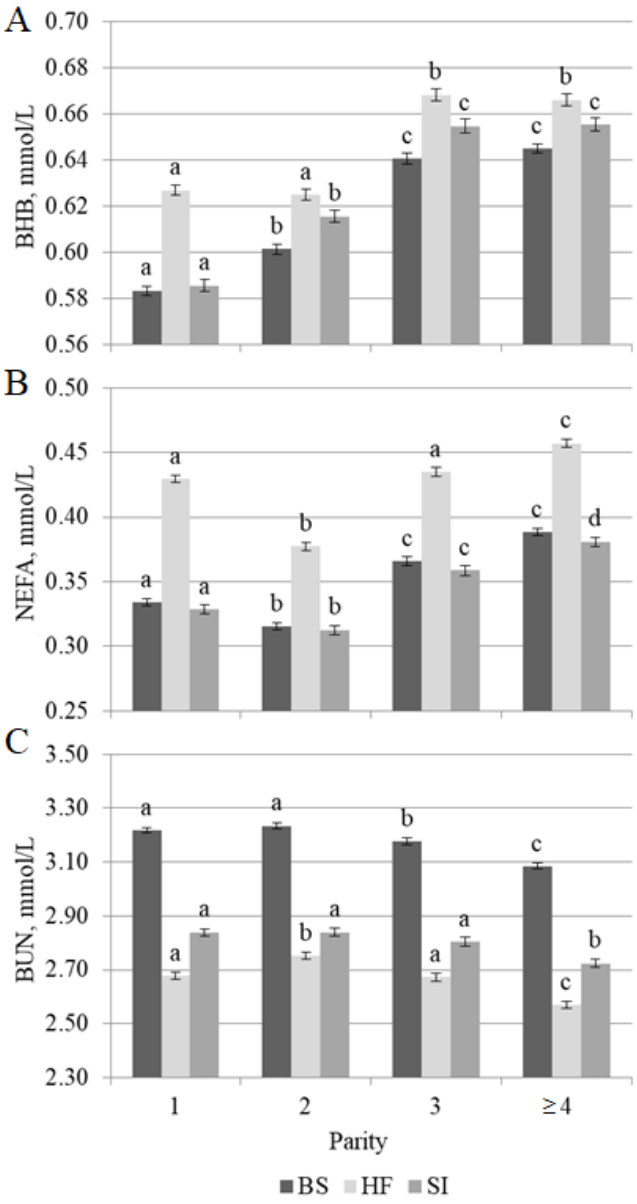

Figure 1. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across parities in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in multi-breed herds. Different superscript letters indicate significantly different least squares means within breed according to Bonferroni's test ($P < 0.05$).

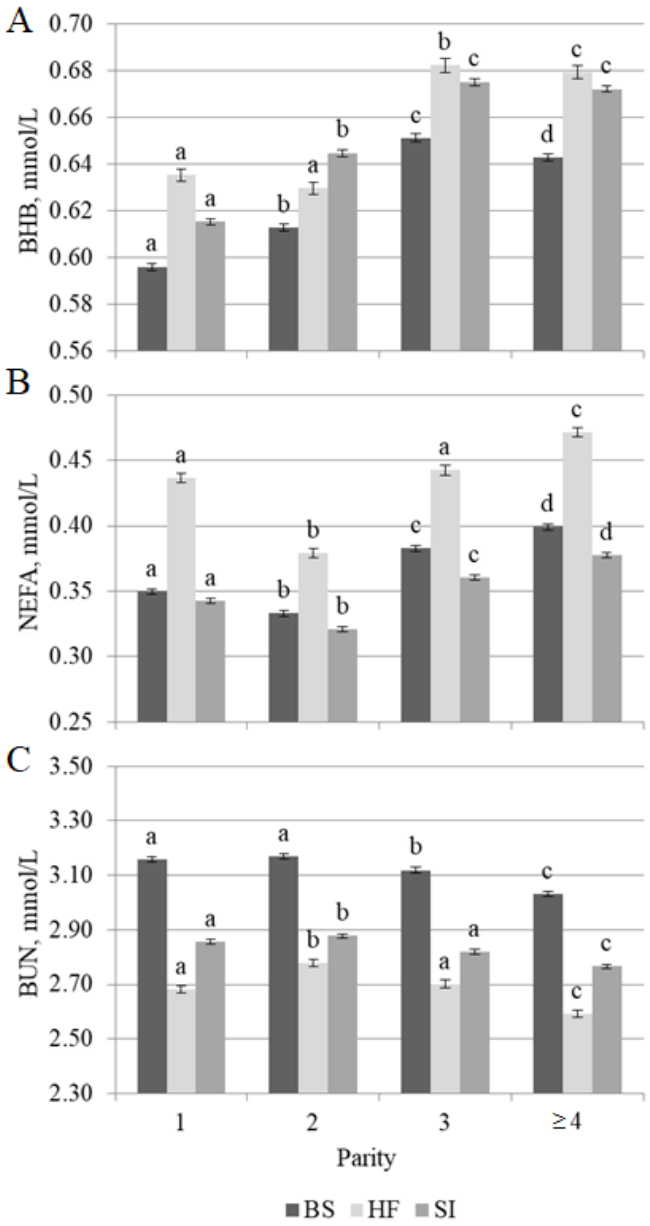

Figure S1. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across parities in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in single-breed herds. Different superscript letters indicate significantly different least squares means within breed according to Bonferroni's test ($P < 0.05$).

Trends of predicted blood metabolites across the first month of lactation in multi-breed analysis are shown in Figure 2. Although with greater concentrations, HF exhibited a pattern for BHB that was comparable to that of BS, showing a peak between 11 and 15 DIM and a fluctuating decline thereafter. On the other hand, SI exhibited a nonlinear but generally consistent increase across early lactation. Considering NEFA and BUN, a decreasing and an increasing linear trend was observed, respectively (Figure 2). Holstein-Friesian had the greatest NEFA concentrations, whereas BS and SI had similar NEFA concentrations from 5 to 35 DIM. The greatest and the lowest BUN concentrations were observed for BS and HF, respectively, with intermediate values for SI. Overall, trends of predicted blood metabolites detected in the current study agreed with previous findings for HF cows (Seifi et al., 2007; Weber et al., 2013; Barletta et al., 2017). The opposite direction of NEFA and BHB patterns between 5 and 15 DIM may be explained by the different utilization of these metabolites by liver and other body tissues (Wathes et al., 2007), whereas the increase of BUN concentration after calving is probably associated with the increase of feed intake across the lactation (Seifi et al., 2007).

Focusing on the breed effect, while NEFA and BUN exhibited similar trends in the first classes of DIM in multi- and single-breed analyses, different patterns of BHB were observed for BS, HF, and SI farmed in single-breed herds (Supplemental Figure S2). In the single-breed analysis, trends of BHB in blood were more accentuated across DIM classes for HF and SI breeds, in opposite directions, whereas BS exhibited a flatter BHB pattern than in multi-breed analysis. Although a stationary (Valergakis et al., 2011) or increasing (Seifi et al., 2007) BHB trend was previously observed in the first month of lactation of HF cows, there is a lack of information about BS and SI breeds in literature.

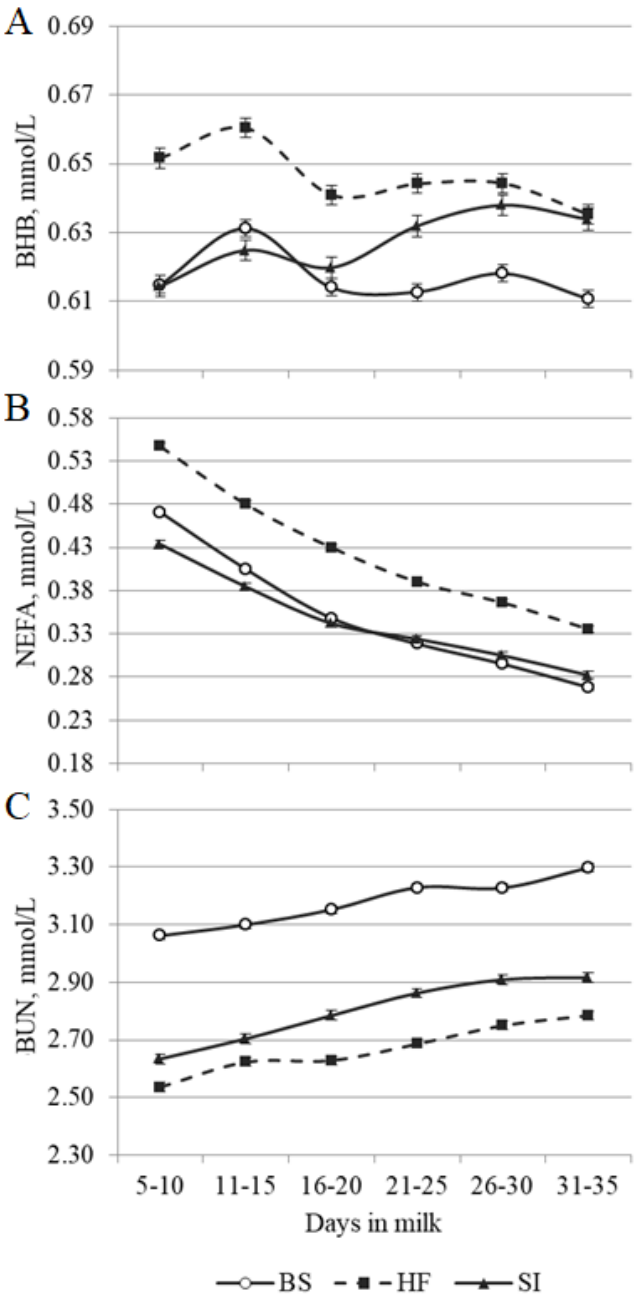

Figure 2. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across early-lactation in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in multi-breed herds.

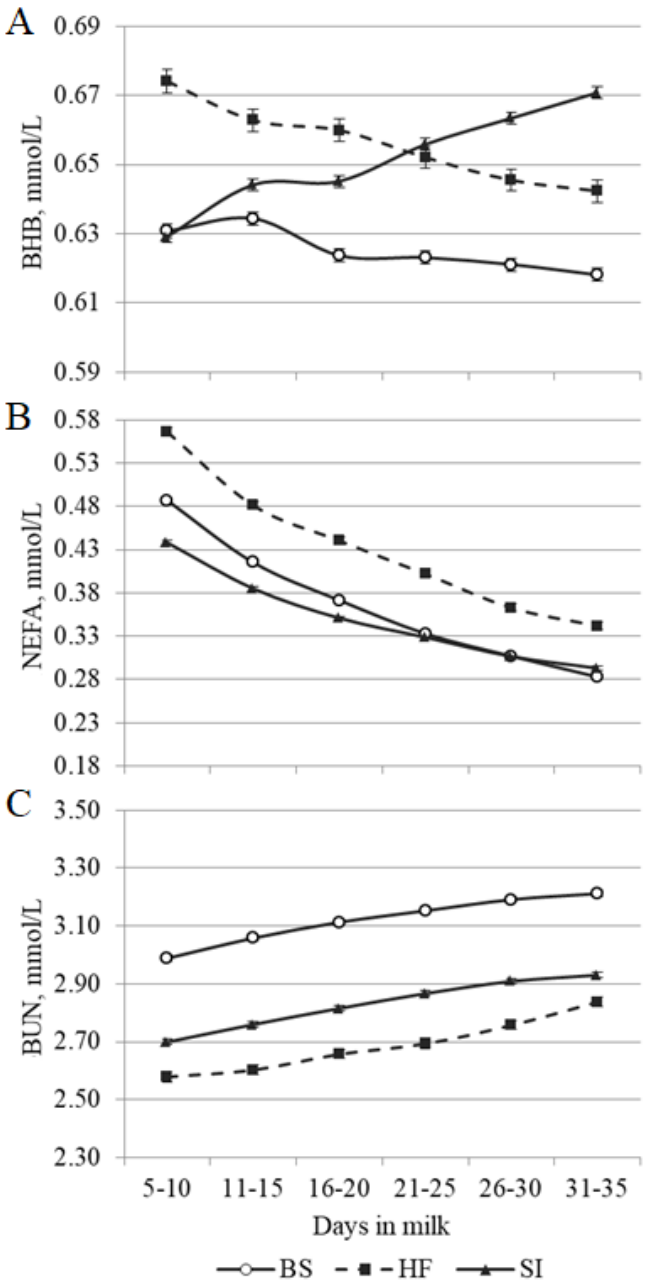

Figure S2. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across early lactation in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in single-breed herds.

Least squares means of blood metabolites across months of sampling for HF, BS, and SI breeds are depicted in Figure 3 and Supplemental Figure S3. Concentration of blood BHB fluctuated across the year, peaking in early summer and then decreasing towards autumn (Figure 3 and Supplemental Figure S3). Similarly, but with a more linear trend, NEFA concentrations increased from January to June and then decreased until winter (Figure 3 and Supplemental Figure S3). The increased fat mobilisation denoted by increased BHB and NEFA concentrations may have been caused by the beginning of grazing season, with the correlated metabolic changes. Moreover, the same reason could have affected trends of BUN across months for all studied breeds (Figure 3 and Supplemental Figure S3). In fact, following a slightly increasing trend from February to May, especially for SI and HF cows, BUN dropped in June and then increased again during summer. The influence of pasture and calving on DMI could have caused a negative protein balance in early summer (Yoon et al., 2004). On the other hand, diet used to feed cows in winter could be the reason of increased BUN concentrations during cold months.

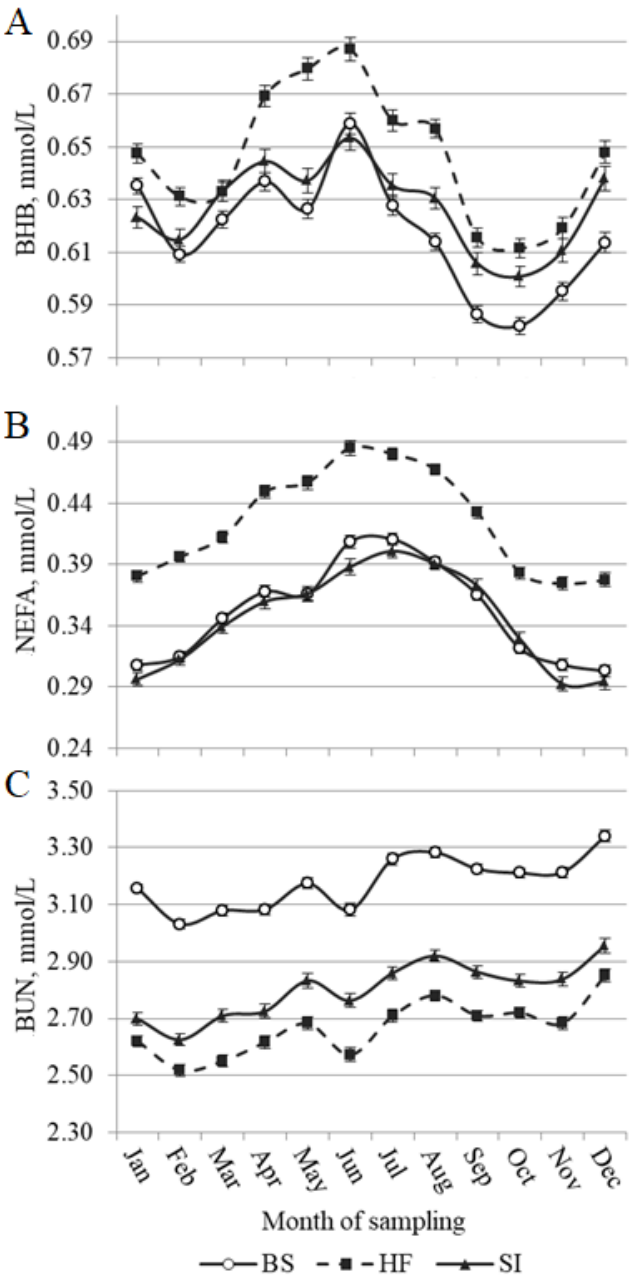

Figure 3. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across months of sampling in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in multi-breed herds.

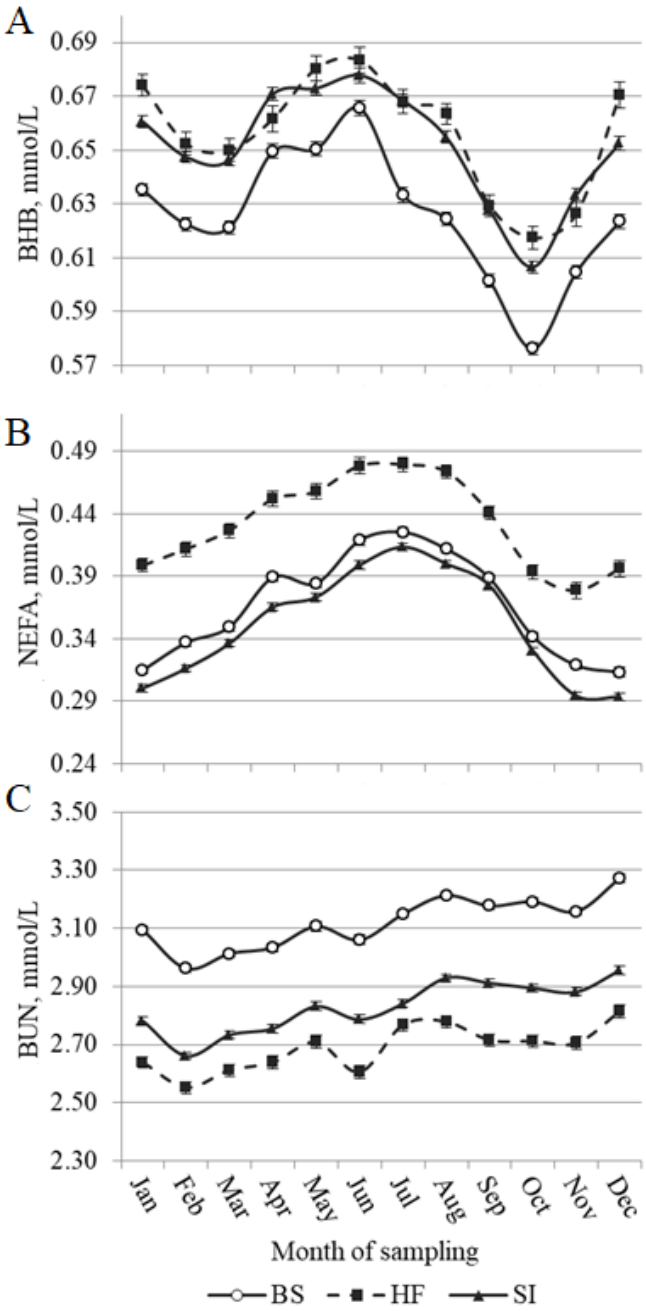

Figure S3. Least squares means of blood (A) β-hydroxybutyrate (BHB), (B) nonesterified fatty acids (NEFA), and (C) urea nitrogen (BUN) across months of sampling in Brown Swiss (BS), Holstein-Friesian (HF), and Simmental (SI) cows in single-breed herds.

CONCLUSIONS

In the present study, significant differences were observed for blood metabolites in the most important Italian cattle breeds. The greatest concentration of BHB and NEFA in blood were detected for HF cows, followed by SI and BS. Conversely, the greatest and the lowest BUN were observed in blood of BS and HF, respectively. Blood BHB and NEFA concentrations increased with parity. The greatest BHB was observed in the first days of lactation, except for SI, which exhibited a small increase across early lactation. In all breeds, NEFA declined and BUN increased in the first month of lactation. The maximum concentrations of blood BHB and NEFA were recorded during spring and early summer, whereas BUN generally increased from spring to winter. Environmental effects identified in the present study can be considered as adjusting factors in within-breed estimation of genetic parameters of major blood metabolites. In perspective, it would be interesting to conduct the same investigation using data from highly intensive mixed herds, characterized by greater prevalence of metabolic disorders and thus greater variability of blood metabolites.

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Chapter 4

Heritability estimates of predicted blood β-hydroxybutyrate and nonesterified fatty acids and relationships with milk traits in early-lactation Holstein cows

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ABSTRACT

At the beginning of lactation, high-producing cows commonly experience an unbalanced energy status, generally responsible for the onset of metabolic disorders and impaired health and performances. Blood β-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) are indicators of excessive fat mobilisation and circulating ketone bodies. Recently, prediction models based on mid-infrared (MIR) spectroscopy have been developed to assess blood BHB and NEFA from routinely collected individual milk samples. This study aimed to estimate genetic parameters of blood BHB and NEFA predicted from milk MIR spectra and to assess their phenotypic and genetic correlations with milk production and composition traits in early-lactation Holstein cows.

The data set comprised the first test-day record of each cow and lactation, and spectra of individual milk samples (n = 24,894) of 14,379 Holstein cows collected from 5 to 35 days in milk (DIM). Blood BHB and NEFA were predicted from milk MIR spectra using previously developed prediction models. Blood metabolites and milk traits were analysed across the whole observational period (5 to 35 DIM) and within 6 classes of DIM using univariate and bivariate animal models to assess heritabilities and genetic correlations, respectively.

Blood BHB and NEFA showed similar trends of genetic variation across DIM, with the greatest heritability in the first 10 days after calving (0.32 and 0.23 for BHB and NEFA, respectively). These two metabolites were moderately to strongly genetically correlated each other (0.50 to 0.60). Moreover, bulls' estimated breeding values for NEFA and BHB showed an unfavourable trend across year of birth. Genetic correlations of BHB and NEFA with milk yield, somatic cell score, protein percentage, lactose percentage and urea content were similar or at least in the same

direction, whereas opposite correlations were observed with fat percentage and fat-to-protein ratio.

Results of the current study suggest that blood BHB and NEFA predicted from milk MIR spectra have genetic variation that is potentially exploitable for breeding purposes. Therefore, both predicted BHB and NEFA would be useful indicator traits of hyperketonemia in a selection index aimed to reduce the susceptibility of dairy cows to metabolic disorders in early lactation.

Key words: blood metabolite, mid-infrared spectroscopy, milk, genetic correlation, dairy cow

INTRODUCTION

Early lactation is a critical period for the dairy cow because it commonly coincides with an unbalanced energy status due to a disequilibrium between energy intake (input) and increased requirements for milk production (output). In particular, the energy demand necessary to support lactogenesis in early lactation affects body reserves (Pryce et al., 2016) and leads to a negative energy balance often responsible for increased incidence of metabolic disorders and reproductive issues (LeBlanc, 2010; Suthar et al., 2013; Esposito et al., 2014). The excessive mobilisation of body reserves in cows is reflected by the increase in circulating β-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) (McArt et al., 2013). The determination of the concentration of these metabolites in blood is generally considered the reference test to monitor cow metabolic and nutritional status. For instance, NEFA concentration ≥ 0.70 mmol/L is potential alert for post-partum health problems (McArt et al., 2013),

and BHB concentration ≥ 1.2 mmol/L is used to define hyperketonemia and has been associated with ketosis (Benedet et al., 2019a).

Although blood metabolic profile testing relies on laboratory analyses, it requires blood sampling and is thus expensive, time-consuming and invasive. To limit costs and labour, milk mid-infrared (MIR) spectroscopy has been exploited to develop prediction models for blood metabolites (Grelet et al., 2019; Luke et al., 2019; Benedet et al., 2019b). Mid-infrared spectroscopy allows large-scale data collection and has been successfully implemented in the routine milk recording system to determine milk composition (De Marchi et al., 2014). Moreover, the phenotypes assessed from routinely collected data could be exploited at both phenotypic and genetic level. In fact, blood metabolites may be used to monitor and diagnose metabolic issues in dairy farms, and could be evaluated as indicator traits in breeding programs to reduce the prevalence of ketosis in dairy herds (Pryce et al., 2016). For instance, blood BHB is more heritable than ketosis (0.09 to 0.37 vs 0.02 to 0.08; Benedet et al., 2019a), and shows moderate genetic correlation with the observed disease (Belay et al., 2017a). Considering that veterinary diagnoses of ketosis are scarce in Italy, an indirect selection based on predicted blood BHB could be feasible and effective.

Few genetic studies have been conducted on blood BHB measured by reference methods (Oikonomou et al., 2008; van der Drift et al., 2012; Cecchinato et al., 2018) or predicted using milk MIR spectra (Belay et al., 2017a). Therefore, the present study aimed to estimate heritability of blood BHB and NEFA concentrations predicted from milk MIR spectra, and to assess their genetic correlations with milk production and composition traits in early-lactation Holstein cows.

MATERIALS AND METHODS

Data

The initial data comprised 536,685 spectra of individual milk samples of Holstein cows collected during monthly test-day recording procedures in Bolzano province (Italy) between January 2011 and December 2018. The study area is mostly characterised by small farms, with an average herd size of 22 lactating cows present throughout the year (Zuliani et al., 2018) and traditional feeding (forage or hay and concentrates), some with access to highland pastures in summer season. After milk collection, preservative (Bronysolv; ANA.LI.TIK Austria, Vienna, Austria) was immediately added and samples were processed according to International Committee for Animal Recording recommendations (ICAR, 2019) in the milk laboratory of the South Tyrolean Dairy Association (Sennereiverband Südtirol, Bolzano, Italy). For each milk sample, fat, protein, casein, and lactose percentage, and urea nitrogen content (MUN, mg/dL) were determined. Fat-to-protein ratio (F/P) was calculated. Spectral information containing 1,060 infrared transmittance data in the region between 5,000 and 900 cm^{-1} were stored using a MilkoScan FT6000 (Foss Electric A/S, Hillerød, Denmark). Values of somatic cell count (SCC) were determined using Fossomatic (Foss Electric A/S, Hillerød, Denmark) and transformed to somatic cell score (SCS) through the conventional formula: $SCS = 3 + \log_2(SCC/100{,}000)$.

Mid-infrared prediction models were applied on the stored spectral data to predict concentrations of blood BHB and NEFA (for full details see Benedet et al., 2019b). Briefly, between December 2017 and June 2018, 295 blood and milk samples were collected from early-lactation dairy cows in 20 herds of Northeast Italy. Reference analyses were performed on blood samples for the determination of BHB and NEFA concentrations and milk spectra were used to develop the prediction

models through partial least squares regression after a backward interval partial least squares. Coefficients of determination in cross-validation for BHB and NEFA were 0.64 and 0.53, respectively.

Days in milk (DIM) were restricted to be between 5 and 35, and only the first test-day of each lactation of a cow was kept in the dataset. Parity ranged from 1 to 10 and 47% of the cows had repeated observations, i.e., they had one test-day in more than 1 lactation. Moreover, for each trait, observations that exceeded 3 standard deviations from the mean were not considered in subsequent analyses. Herds with less than 5 cows sampled and present for less than 4 years (between 2011 and 2018) were removed from the dataset. After editing, 24,894 test-day records of 14,379 cows in 634 herds were available for genetic analyses.

Estimation of genetic parameters

The pedigree of cows with phenotypic information was traced back to 6 generations of ancestors, ending up with 43,943 animals. Variance and covariance components of blood metabolites and milk traits were estimated in ASReml 4.1 (Gilmour et al., 2015) using univariate and bivariate repeatability animal models, respectively. The general form of the model for the entire dataset (5 to 35 DIM), in matrix notation, was:

$$y = \mathbf{X}b + \mathbf{Z}a + \mathbf{W}w + e,$$

where y was the vector of observations for blood BHB, NEFA, and milk traits; b was the vector of fixed effects of parity (4 classes: 1, 2, 3, and ≥ 4), class of DIM (6 classes: 5 to 10 days, 11 to 15 days, 16 to 20 days, 21 to 25 days, 26 to 30 days, and 31 to 35 days), season of calving (4 classes: December to February, March to May, June to August, and September to November), and herd (n = 1 to 634); a was the

vector of solutions for random additive genetic effect of the animal; *w* was the vector of permanent environmental effect of the cow; *e* was the vector of random residuals; and **X**, **Z** and **W** were incidence matrices relating the corresponding effects to the dependent variable. Random effects were assumed to be normally distributed with null means and variance-covariance structures of additive genetic, permanent environmental and residual effects equal to $\mathbf{G} \otimes \mathbf{A}$, $\mathbf{P} \otimes \mathbf{I}$ and $\mathbf{R} \otimes \mathbf{I}$, respectively, where **G**, **P** and **R** were the additive genetic, permanent environmental and the residual (co)variance matrices, **A** was the additive genetic relationship matrix and **I** was an identity matrix of appropriate order.

The same model, excluding the fixed effect of DIM class, was used to estimate genetic parameters of data within each class of DIM.

Estimates of heritability (h^2) and genetic correlations (r_a) were computed as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2} \text{ and } r_a = \frac{\sigma_{12}}{\sqrt{\sigma_{a1}^2 * \sigma_{a2}^2}},$$

where σ^2_a and σ^2_e were the additive genetic and residual variances of the trait, σ_{12} was the additive genetic covariance between trait 1 and trait 2, and σ^2_{a1} and σ^2_{a2} were the additive genetic variances of traits 1 and 2, respectively.

Coefficients of phenotypic variation (CV_p) of each studied trait was computed as the ratio of the phenotypic standard deviation to the mean of the trait, and coefficient of additive genetic variation (CV_a) was calculated as the ratio of the additive genetic standard deviation to the mean of the trait. Pearson's correlations (r_p) between the traits were assessed using the CORR procedure of SAS 9.4 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Descriptive Statistics

Concentrations of BHB and NEFA averaged 0.66±0.24 mmol/L and 0.41±0.21 mmol/L, respectively, with mean values across DIM depicted in Figure 1. Concerning BHB, the greatest values were in the window from 5 to 15 DIM (0.68 mmol/L) with a slight reduction until 35 DIM, whereas NEFA showed a linear and evident decreasing trend moving from the class 5 to 10 DIM (0.54±0.23 mmol/L) to the class 31 to 35 DIM (0.32±0.16 mmol/L). The CV_p of the class between 31 and 35 DIM was the greatest for NEFA and the lowest for BHB (Figure 1). Moving from 5 to 35 DIM, fat percentage, protein percentage and SCS reduced by 15%, 19% and 28%, respectively (Table 1). On the other hand, milk yield increased from 31.19±6.78 kg/d (5 to 10 DIM) to 35.17±7.50 kg/d (31 to 35 DIM; Table 1). Lactose percentage and MUN content increased with DIM and, on average, F/P did not show a clear tendency, peaking in the DIM class between 21 and 25 DIM and decreasing thereafter (Table 1).

Table 1. Mean and standard deviation of MIR-predicted blood metabolites, milk yield and quality traits across days in milk

Trait[1]	Days in milk						
	5 to 35	5 to 10	11 to 15	16 to 20	21 to 25	26 to 30	31 to 35
N of records	24,894	3,783	4,051	4,221	4,303	4,368	4,168
BHB, mmol/L	0.66±0.24	0.68±0.25	0.68±0.26	0.67±0.24	0.66±0.23	0.65±0.22	0.65±0.21
NEFA, mmol/L	0.41±0.21	0.54±0.23	0.46±0.21	0.42±0.19	0.38±0.18	0.35±0.17	0.32±0.16
Milk yield, kg/d	33.90±7.48	31.19±6.78	32.77±7.13	34.02±7.32	34.73±7.53	35.13±7.68	35.17±7.50
Fat, %	4.10±0.76	4.52±0.78	4.27±0.75	4.10±0.73	4.01±0.73	3.91±0.70	3.84±0.69
Protein, %	3.16±0.35	3.61±0.31	3.31±0.28	3.13±0.26	3.03±0.25	2.98±0.26	2.95±0.26
F/P	1.30±0.24	1.26±0.23	1.30±0.24	1.32±0.24	1.33±0.25	1.32±0.25	1.31±0.24
Lactose, %	4.78±0.17	4.65±0.16	4.76±0.16	4.81±0.15	4.82±0.16	4.83±0.16	4.83±0.16
MUN, mg/dL	16.95±6.55	15.92±6.60	16.59±6.38	16.72±6.43	16.89±6.56	17.46±6.63	18.00±6.49
SCS	2.19±1.90	2.70±1.78	2.36±1.84	2.14±1.86	2.05±1.90	2.01±1.93	1.93±1.96

[1]N = number; F/P = fat-to-protein ratio; MUN = milk urea nitrogen; SCS = somatic cell score.

Genetic variation and heritability

Although BHB exhibited lower CV_a than NEFA (Figure 1), it was generally more heritable within the first month of lactation (Figure 1), with overall h^2 of 0.22±0.01 vs. 0.17±0.01. For both metabolites the greatest h^2 was estimated between 5 and 10 DIM, and the lowest between 11 and 15 DIM. Moreover, the lowest CV_a for BHB (7.78%) was observed in correspondence of the greatest CV_a for NEFA (21.67%), i.e. from 31 to 35 DIM (Figure 1).

Estimates of h^2 of milk traits are summarised in Table 2. Focusing on the entire time window (5 to 35 DIM), the minimum h^2 was observed for milk yield and SCS (0.10±0.01) and the maximum for lactose percentage (0.39±0.02); on the other hand, CV_a ranged from 1.95% (lactose percentage) to 26.97% (SCS). In all DIM classes, the lowest h^2 were obtained for milk yield and SCS and the greatest for lactose percentage.

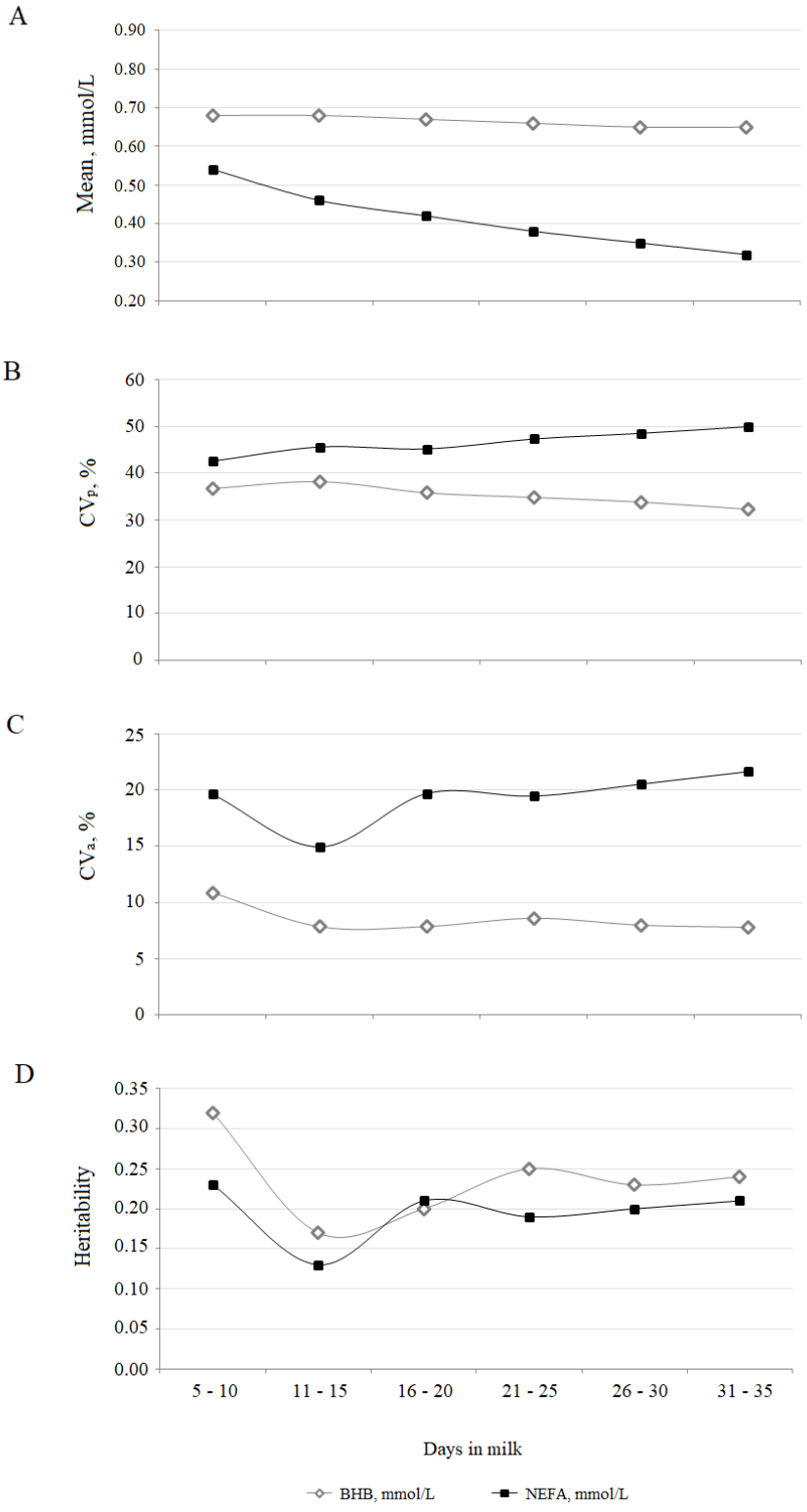

Figure 1. (A) Mean, (B) coefficient of phenotypic variation (CV_p), (C) coefficient of additive genetic variation (CV_a) and (D) heritability of mid-infrared predicted blood β-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) across days in milk.

Table 2. Heritability[1] (h^2) and coefficient of additive genetic variation (CV_a, %) of milk yield and quality traits across days in milk

Trait[2]	Days in milk													
	5 to 35		5 to 10		11 to 15		16 to 20		21 to 25		26 to 30		31 to 35	
	h^2	CV_a	h^2	CV_a	h^2	CV_a	h^2	CV_a	h^2	CV_a	h^2	CV_a	h^2	CV_a
Milk yield, kg/d	0.10	5.39	0.16	6.89	0.14	6.39	0.15	6.37	0.09	5.10	0.11	5.51	0.17	6.85
Fat, %	0.16	6.49	0.20	7.15	0.15	6.19	0.25	8.20	0.14	6.00	0.16	6.49	0.15	6.32
Protein,%	0.25	4.08	0.24	4.04	0.29	4.36	0.25	4.02	0.35	4.75	0.27	4.27	0.36	4.99
F/P	0.11	5.65	0.15	6.51	0.10	5.26	0.20	7.68	0.11	5.79	0.09	5.19	0.15	6.59
Lactose, %	0.39	1.95	0.35	1.98	0.45	2.12	0.49	2.11	0.49	2.18	0.48	2.13	0.51	2.18
MUN, mg/dL	0.11	11.67	0.20	17.22	0.13	12.97	0.14	13.23	0.18	14.93	0.13	12.55	0.04	6.75
SCS	0.10	26.97	0.13	23.76	0.14	28.76	0.15	33.01	0.17	37.77	0.14	35.38	0.08	28.41

[1]Standard errors of heritability estimates ranged from 0.01 to 0.04.

[2]F/P = fat-to-protein ratio; MUN = milk urea nitrogen; SCS = somatic cell score.

Correlations between predicted blood metabolites and milk traits

The r_p and r_a of BHB and NEFA with milk yield and composition traits during the whole observational period (5 to 35 DIM) are presented in Table 3. In general, for BHB the magnitude and direction of r_p and r_a with NEFA, protein percentage, lactose percentage and MUN content were similar. Conversely, r_p and r_a between NEFA and fat percentage (0.27 and -0.42±0.06, respectively) and between NEFA and F/P (0.41 and -0.14±0.07) had opposite directions. Moreover, except for the correlations with milk yield (r_p = 0.09; r_a = 0.63±0.06), both r_p and r_a of NEFA with other milk traits were negative.

Table 4 summarises the r_a between BHB and NEFA estimated in the different DIM classes. The strongest (0.69±0.06) and weakest (0.29±0.14) relationships were assessed from 5 to 10 DIM and 11 to 15 DIM, respectively. The r_a of BHB and NEFA with milk yield and composition traits in the different DIM classes are depicted in Figure 2. The pattern of r_a between BHB and milk yield fluctuated across DIM classes, whereas a linear decrease was observed between NEFA and milk yield. Both r_a of BHB and NEFA with fat percentage had a minimum between 11 and 15 DIM and peaked in the subsequent DIM class; however, the r_a between NEFA and fat percentage had a more persistent trend. The pattern of r_a of protein and lactose percentage with BHB and NEFA were almost identical. The across DIM-pattern of r_a between BHB and F/P resembled that of NEFA and F/P, but overall, the r_a between BHB and F/P were positive whereas they were negative between NEFA and F/P. The r_a between MUN and BHB ranged from 0.09±0.15 to -0.23±0.11 and between MUN and NEFA from -0.51±0.14 to -0.03±0.15. In both cases, the maximum r_a was reached in the class from 16 to 20 DIM. As regards SCS, trends and magnitude of r_a with BHB

and NEFA were comparable until 30 DIM, whereas opposite directions were observed in the last DIM class.

Bulls' estimated breeding values (EBV) of blood NEFA and BHB were significantly correlated (0.59, $P < 0.001$). When only bulls with a reliability ≥ 0.50 and born after 1994 were selected (n = 230), Pearson's correlation between EBV of the two blood metabolites was 0.54. Figure 3 depicts the correlations of BHB and NEFA EBV with those of milk yield, composition traits, SCS and MUN. The strongest and weakest associations were between NEFA and milk yield (0.48, $P < 0.001$) and between BHB and MUN (0.01, $P = 0.051$), respectively. In fact, all correlations involving MUN were almost close to zero, while those including SCS were negative. In this study, both protein and lactose percentage were negatively correlated with NEFA and BHB at EBV level; instead, the correlations of NEFA and BHB with fat percentage had opposite direction (Figure 3), being -0.139 ($P < 0.001$) and 0.120 ($P < 0.001$), respectively.

Table 3. Pearson's (above diagonal) and genetic correlations[1] (below diagonal) between mid-infrared predicted blood metabolites and milk yield and quality traits

Trait[2]	BHB	NEFA	Milk yield	Fat	Protein	F/P	Lactose	MUN	SCS
BHB, mmol/L	-	0.56***	0.09***	0.27***	-0.22***	0.41***	-0.25***	-0.03***	-0.02*
NEFA, mmol/L	0.52	-	0.04***	0.27***	-0.09***	0.33***	-0.32***	-0.18***	0.05***
Milk yield, kg/d	0.25	0.63	-	-0.05***	-0.20***	0.06***	0.01*	-0.01	-0.09***
Fat, %	0.02	-0.42	-0.38	-	0.31***	0.82***	-0.23***	0.01	0.10***
Protein, %	-0.20	-0.45	-0.45	0.53	-	-0.28***	-0.18***	-0.06***	0.15***
F/P	0.18	-0.14	-0.08	0.78	-0.11	-	-0.13***	0.04***	0.02**
Lactose, %	-0.20	-0.17	-0.21	0.08	0.22	-0.10	-	0.09***	-0.20***
MUN, mg/dL	-0.07	-0.15	0.02	0.21	0.12	0.13	-0.09	-	-0.06***
SCS	-0.15	-0.15	-0.20	-0.07	-0.08	-0.02	-0.17	-0.14	

[1]Standard errors of estimates of genetic correlations ranged from 0.03 to 0.09.

[2]F/P = fat-to-protein ratio; MUN = milk urea nitrogen; SCS = somatic cell score.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 4. Genetic correlation (standard error) between mid-infrared predicted blood β-hydroxybutyrate and nonesterified fatty acids across days in milk

Days in milk	Genetic correlation
5 to 10	0.69 (0.06)
11 to 15	0.29 (0.14)
16 to 20	0.45 (0.10)
21 to 25	0.54 (0.08)
26 to 30	0.38 (0.10)
31 to 35	0.64 (0.08)

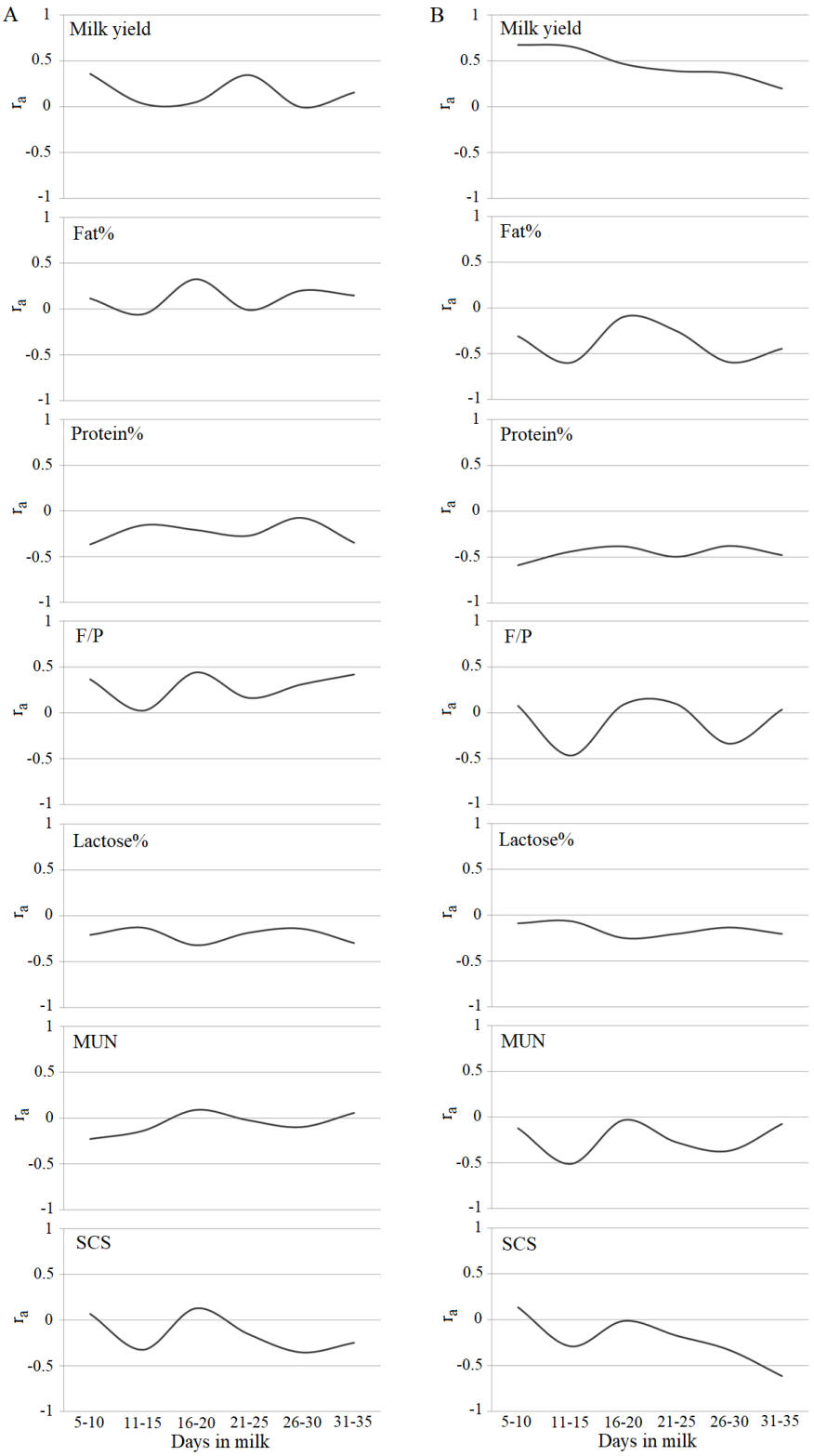

Figure 2. Genetic correlations (r_a) of mid-infrared predicted blood (A) β-hydroxybutyrate and (B) nonesterified fatty acids with milk traits across days in milk (F/P = fat-to-protein ratio; MUN = milk urea nitrogen; SCS = somatic cell score).

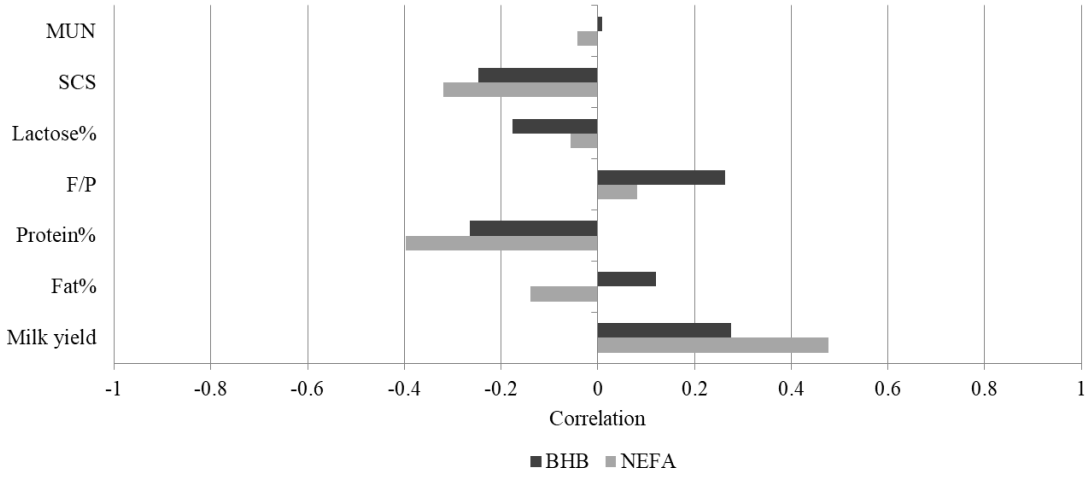

Figure 3. Correlations between estimated breeding values of mid-infrared predicted blood β-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) with milk traits (F/P = fat-to-protein ratio; MUN = milk urea nitrogen; SCS = somatic cell score). All correlations were significant ($P \leq 0.05$).

DISCUSSION

Phenotypic overview

The current study focused on early-lactation cows. A decreasing concentration of blood BHB and NEFA across DIM was somehow expected (McArt et al., 2013). However, the overall mean BHB was generally lower and exhibited a smaller decrease across DIM compared with previous studies in Holstein (van der Drift et al., 2012) and Norwegian Red cows (Belay et al., 2017b). The BHB trend was however more similar to that observed in primparous Holstein cows (Oikonomou et al., 2008). Conversely, NEFA concentrations were in line with those recently observed in the first three weeks of lactation of Nordic Red cows (Mäntysaari et al., 2019).

Concerning milk traits, the decrease of fat and protein percentage and the increase of milk yield and lactose percentage from 5 to 35 DIM were reported also in other studies (Miglior et al., 2006; Abdullahpour et al., 2013; Haile-Mariam and Pryce, 2017; Costa et al., 2019a). The trend for F/P was similar to that observed in Canadian Holsteins (Koeck et al., 2014). Moreover, in agreement with results of the present study, average fat (4.15%), protein (3.22%), lactose (4.84%), MUN (18.10 mg/dL) and F/P (1.30) at first test-day in early lactation (8 to 49 DIM) have been observed in Austrian Fleckvieh cows (Ederer et al., 2014).

Genetic variance

Overall, h^2 estimates of BHB and NEFA were consistent with those recently obtained from MIR predictions in Holstein cows (Hammami et al., 2017). As observed in the literature (Oikonomou et al., 2008), the greatest h^2 for BHB and NEFA were observed from 5 to 10 DIM. Despite this, in the present study the h^2 of both metabolites slightly decreased in the subsequent weeks.

The observed patterns of h^2 for milk yield and fat and protein percentage were similar to those reported for Holstein cows in Iran (Abdullahpour et al., 2013). Heritability of lactose exhibited an increasing trend along DIM as previously reported (Haile-Mariam and Pryce, 2017). Concerning F/P, MUN and SCS, h^2 estimates of the whole first month of lactation agreed with previous findings in Holstein (Negussie et al., 2008; Koeck et al., 2014; Hammami et al., 2017) and Austrian Fleckvieh cows (Ederer et al., 2014).

Correlations

The overall positive correlations of milk yield with BHB and NEFA (Table 3 and Figure 3) indicated that the best sires for milk production were those with offspring exhibiting on average greater blood BHB and NEFA in the first 35 DIM. This also supported the general idea that, in dairy cattle, genetic selection only focused on production has detrimental effects on health and fitness across generations (van der Werf et al., 2009; Stefani et al., 2018). In fact, high-producing dairy cows are subjected to homeorhesis, i.e. all metabolic pathways are addressed to mammary gland and are intended to milk synthesis (Baumann and Currie, 1980; Costa et al., 2019b). Therefore, the greater the energy requirements for milk synthesis, the greater the circulating blood NEFA and ketone bodies due to fat reserves mobilisation (McArt et al., 2013). Blood BHB and NEFA were negatively related to SCS in the first 35 DIM (Table 3 and Figure 3), suggesting that there may not be an indirect (desired) selection for udder health by acting on metabolic traits. In addition, this highlighted that different selection strategies and criteria should be adopted in order to simultaneously reduce milk SCS and enhance resistance to metabolic diseases in the Italian Holstein population in early lactation. However, it is worth highlighting that

from the current study it cannot be excluded that blood BHB and NEFA could be positively related to SCS in the rest of lactation. In fact, several studies estimated a positive correlation between ketosis and mastitis (Pfeiffer et al., 2015; Pryce et al., 2016; Costa et al., 2019c). Moreover, although SCS is the most adopted indicator of udder health, the r_a between SCS and mastitis may vary from 0 to 0.80 in dairy cows (Coffey et al., 1986; Lund et al., 1994), and this justifies the inclusion of both traits in some indexes for mastitis resistance and/or udder health (Heringstad et al., 2000). As expected, blood BHB and NEFA negatively correlated with lactose percentage (Table 3 and Figure 3), that is negatively genetically related to ketosis in cattle (Costa et al., 2019c). However, the correlation between NEFA EBV and lactose percentage EBV (-0.06) was weaker than the r_a between these two traits. The opposite associations of EBV for NEFA and BHB with EBV of fat percentage suggested different dependencies of the two blood metabolites with this trait in early lactation (≤ 35 DIM) at population level. In particular, the difference could be explained by the change of fat synthesis during and after lipomobilisation, when the peak of NEFA and BHB occurs, respectively. As regards protein content, the negative r_a with BHB confirmed recent findings (Belay et al., 2017a), whereas the negative r_p with NEFA was in contrast with the estimate (0.12) obtained in Nordic Red cows (Mäntysaari et al., 2019). According to the selection index theory, findings support the use of F/P as genetic indicator of ketosis resistance; in fact, F/P showed genetic variation, it is genetically correlated with the objective trait, and it is heritable (Klein et al., 2019).

Focusing on correlations across DIM, the non-linear trend of r_a between BHB and NEFA (Table 4) generally reflects the h^2 patterns of the two metabolites in the first month of lactation (Figure 1). In fact, between 11 and 15 DIM, both metabolites exhibited low CV_a and h^2 (Figure 1), as well as the lowest r_a. The low genetic

variance observed between 11 and 15 DIM may suggest that the potential of genetics in reducing susceptibility to ketosis in Italian Holstein population is not constant, fluctuating in the first 35 DIM. To our knowledge, this is the first study that assessed r_a of BHB and NEFA with milk traits specifically in the first month of lactation, thus it may be unfair to compare findings of current study with previous literature.

Population trend

The genetic trend of blood BHB and NEFA concentrations assessed using sires' EBV with reliability ≥ 50% (n = 230) is depicted in Figure 4. An increasing trend of EBV across year of birth of the sires was detected for both blood BHB and NEFA. The high pressure on milk production in the past, coupled with an unfavourable genetic association of milk yield with blood BHB and NEFA concentrations, are likely the main reasons to explain the worsening of cow metabolic status in early lactation at population level. In fact, EBV of blood metabolites similarly increased along years of birth and peaked in 2010. Overall, this suggest that bulls born in 2010 produce offspring more susceptible to negative energy balance at the beginning of lactation and thus to ketosis.

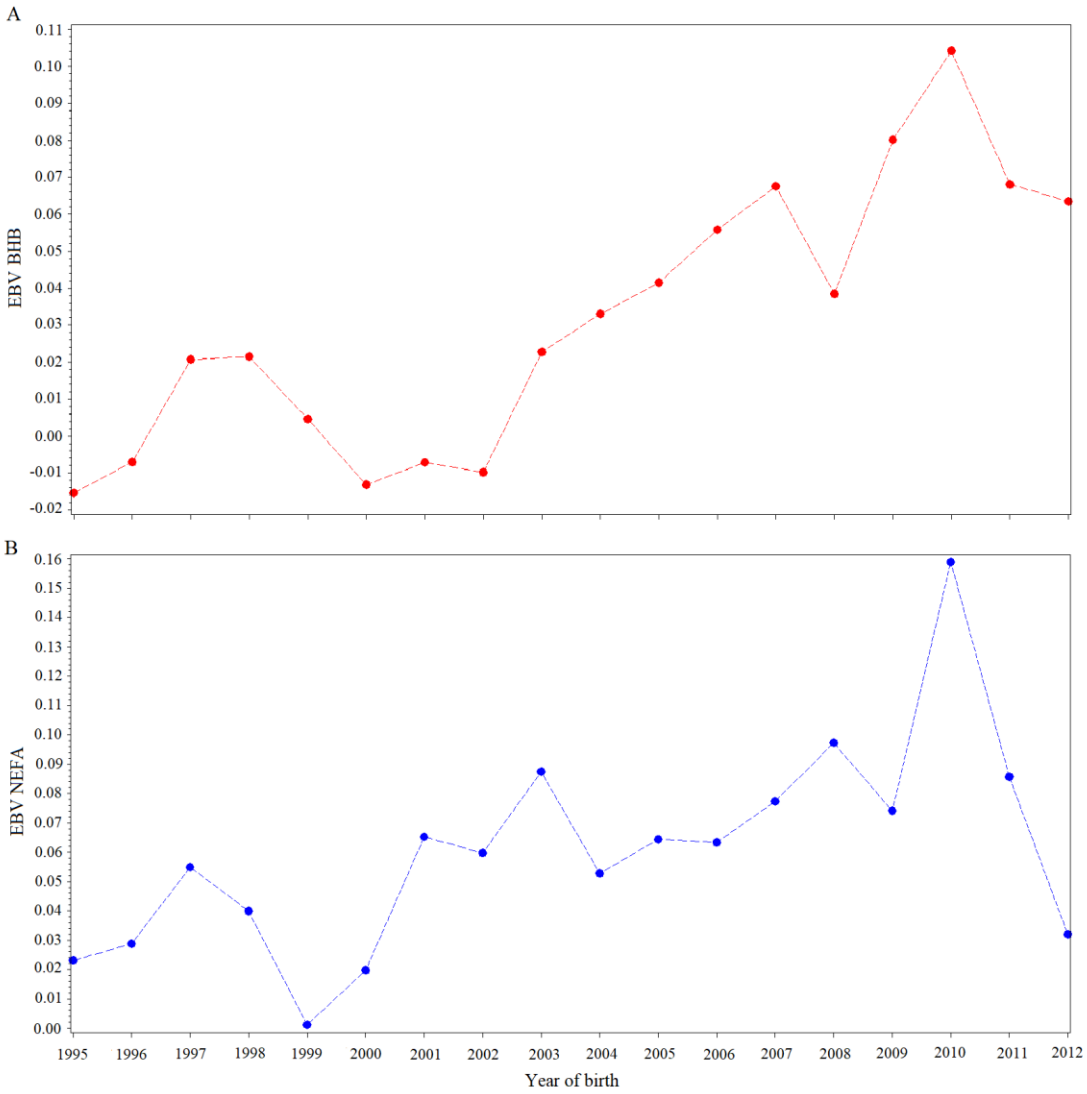

Figure 4. Trend of sires' (n = 230 bulls, with reliability ≥ 50%) estimated breeding values (EBV) for mid-infrared predicted blood (A) β-hydroxybutyrate (BHB, mmol/L) and (B) nonesterified fatty acids (NEFA, mmol/L) across year of birth.

CONCLUSIONS

In the current study we estimated h^2 of blood BHB and NEFA predicted from milk MIR spectra, and their genetic correlations with milk production and composition traits in the first 35 DIM. Both BHB and NEFA showed similar phenotypic and genetic variation across DIM, with the greatest concentrations and h^2 in the first 10 days after calving. Blood BHB and NEFA were genetically correlated (0.50 to 0.60). These findings suggest that BHB and NEFA should be taken into account in case of selection against metabolic issues. Blood BHB and NEFA were positively correlated with milk yield, suggesting that selection for milk production had detrimental effects on cow metabolic status. A negative genetic correlation of SCS, indicator of udder health, with both BHB and NEFA was estimated, making a simultaneous selection for both udder health and ketosis challenging. On average, genetic correlations of BHB and NEFA with MUN content, protein percentage and lactose percentage were similar, whereas opposite associations were observed with fat percentage and F/P. Using MIR-predicted blood metabolites has allowed to exploit phenotypes for large scale screening and genetic purposes. However, further investigations including ketosis data should be conducted to validate BHB and NEFA as proper indicators of ketosis resistance.

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General conclusions

Metabolic disorders and their related indicators are becoming increasingly important in the dairy sector. Due to its negative consequences on cow health and herd profitability, HYK has been widely studied in recent years, and methods to predict and monitor its occurrence has been investigated in several studies. The opportunity of using milk MIR spectra to predict blood metabolic indicators leads to the possibility of collecting routine information on the metabolic status of early-lactation cows at population level. In fact, prediction of blood indicators is more useful than milk metabolic indicators to monitor metabolic problems, as demonstrated for blood and milk BHB tested for HYK detection.

However, considering their moderate performance, developed MIR prediction models are still not accurate enough to be considered a diagnostic method, but they can be used to predict and investigate phenotypic and genetic variation of blood metabolites and related disorders on a large scale in dairy cattle. Differences between the most important Italian dairy breeds showed that Holstein-Friesian had the greatest concentration of BHB and NEFA, and the lowest blood urea, which may underline a more altered energy and nutritional status than Brown Swiss and Simmental cows in early lactation. For specialized dairy breeds, blood BHB and NEFA declined, whereas urea increased during the first month of lactation. In all breeds, BHB and NEFA concentrations increased with parity and reached a peak in spring and early summer, whereas blood urea increased from spring to winter.

Genetic analysis of predicted blood BHB and NEFA showed that these two metabolites were heritable, especially in the first 10 days after calving, and genetically correlated. Blood BHB and NEFA were positively genetically correlated with milk yield, suggesting that selection for milk production had detrimental effects on cow

metabolic status. On average, genetic correlations of BHB and NEFA with milk traits were similar, with the exception of those with fat percentage and F/P, for which opposite correlations were observed.

In conclusion, using MIR prediction models to determine blood metabolites has shown several advantages for large scale phenotypic investigations and genetic purposes. However, further analyses should be conducted in order to improve the accuracy of the developed models and to provide a feasible tool in the screening for metabolic problems at cow level.

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