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CICLO XXIX

RELATIONSHIPS BETWEEN FERTILITY OF COWS AND THEIR MILK YIELD, COMPOSITION AND INFRARED SPECTRA

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To my wife Lesly, and my son Santiago,

for being my inspiration, and join me in this adventure.

CONTENTS

RIASSUNTO

La produzione di latte ha un forte effetto sulla fertilità delle bovine e allo stesso tempo la riproduzione influisce sulla composizione del latte. Negli ultimi decenni, si è sviluppato un particolare interesse riguardo lo studio della composizione del latte e del suo rapporto con la salute, l'efficienza e la fertilità. Pertanto l'obiettivo principale di questa tesi è stato quello di valutare i rapporti tra la fertilità delle bovine da latte e la loro produzione di latte, la composizione e gli spettri a infrarossi del latte prodotto.

I dati utilizzati sono stati raccolti dalla Federazione Allevatori dell' Alto Adige / Südtirol di Bolzano / Bozen in Italia. I dati relativi al latte comprendono la produzione, la composizione e le proprietà fisiche. Per la fertilità, sono state considerate tutte le date di fecondazione e il giorno di parto. I campioni di latte raccolti sono stati analizzati utilizzando un MilkoScanTM FT + 6000 (Foss Electric, Hillerød, Danimarca), e lo spettro ricopriva 1,060 lunghezze d'onda, da 5,010 a 925 cm^{-1}. Sono state utilizzate quattro razze: le razze specializzate Frisona e Bruna, e le razze a duplice attitudine Pezzata Rossa e Grigio Alpina.

Nel secondo capitolo sono stati studiati gli effetti della razza e la sua interazione con la produzione di latte a livello di allevamento (Herd-L) e a livello individuale (di vacca entro allevamento) (Cow-L) sui caratteri di fertilità nelle bovine da latte. Per stabilire i livelli di produttività delle varie aziende e delle singole vacche, in base alla produzione di latte, è stato utilizzato un modello misto. L'intervallo dal parto alla prima inseminazione (iCF), l'intervallo dalla prima inseminazione al concepimento (iFC) e l'intervallo parto concepimento (DO) sono stati analizzati utilizzando un modello di rischio proporzionale di Cox. Il tasso di non ritorno a 56 giorni dopo il primo servizio (NRR), il tasso di gravidanza al primo servizio (PRF) e il numero di inseminazioni (INS) sono stati analizzati utilizzando una regressione logistica. Per tutti i caratteri è stata osservata una forte interazione tra la razza e la classe di

produttività, sia a livello di allevamento che a livello individuale. Le razze a duplice attitudine Pezzata Rossa e Grigio Alpina hanno una migliore fertilità rispetto alle vacche da latte specializzate di razza Frisona e Bruna, anche a parità di produzione, e gli effetti della produttività aziendale e individuale differivano tra loro e tra le razze. In conclusione, una maggiore produttività dell' azienda può determinare una maggiore fertilità nelle vacche, mentre una maggiore produzione di latte delle singole vacche all'interno di una azienda può risultare in una minore fertilità. Questi effetti, sia livello di allevamento che a livello individuale, hanno un andamento curvilineo e sono più forti nelle razze a duplice attitudine, essendo più evidenti passando da una produttività bassa a una intermedia, piuttosto che passando dal livello intermedio alle classi di produttività elevata.

Nel terzo capitolo sono state valutate le relazioni tra le fasi dell'estro nei bovini da latte e la composizione, gli indicatori fisici e gli acidi grassi del latte. I giorni di campionamento attorno alla prima inseminazione dopo il parto, nell'intervallo da -10 a +10 giorni, sono stati selezionati e classificati in 5 fasi: diestro-alto progesterone (Diestrus-HP) da -10 a -4 giorni; proestro da -3 a -1 giorni; giorno di estro 0 (giorno di inseminazione); metestro da 1 a 2 giorni; e diestro-progesterone in aumento (Diestrus-IP) da 3 a 10 giorni. Per analizzare i componenti del latte e gli indicatori fisici delle proprietà del latte è stato usato un modello misto, includendo l'effetto dell'a fase estrale, e abbiamo stimato i contrasti tra di essi. La composizione del latte ha mostrato un'elevata variabilità tra le fasi diverse dell'estro, e i caratteri maggiormente influenzati sono stati il grasso, la proteina e il lattosio. Anche il profilo acidico e gli indicatori fisici sono stati notevolmente influenzati, indicando importanti differenze causate dalle modifiche ormonali e comportamentali delle bovine in estro.

Nel quarto capitolo è stata valutata l'abilità di predizione dello stato di gravidanza delle vacche (PS) utilizzando grasso, proteina, caseina, lattosio e gli spettri FTIR . Per predire lo stato di gravidanza sono stati utilizzati modelli lineari generalizzati utilizzando grasso, proteina, lattosio, caseina e le singole lunghezze d'onda FTIR. È stato inoltre fittato un modello Bayesiano di selezione di variabile per predire lo stato di gravidanza utilizzando lo spettro FTIR completo . L'accuratezza di predizione è stata valutata utilizzando uno studio di validazione incrociata ripetuto 10 volte e calcolando l'area sotto a la curva del -receiver operating characteristic- (CV-AUC) basata sulle predizioni fenotipiche e sulle osservazioni. Nel complesso, le migliori accuratezze di predizione sono state ottenute per un modello che includeva i dati spettrali FTIR completi. Le vacche Grigio alpine hanno ottenuto il più alto CV-AUC (0.645), Brune e Pezzate Rosse hanno ottenuto risultati simili (0.630 e 0.628 rispettivamente), mentre le Frisone hanno ottenuto il valore più basso per gli spettri FTIR (0.607) completi. Per le singole analisi di lunghezza d'onda, picchi importanti sono stati rilevati: da wn 2,973 a wn 2,882 cm^{-1} corrispondente al filtro Fat-B delle analisi con monocromatore; wn 1,773 cm^{-1} dove è posizionato il filtro grasso-A; wn 1,546 cm^{-1} dove è posizionato il filtro della proteina; wn 1,468 cm^{-1} che è associato a urea e grasso; wn 1,399 cm^{-1} e wn 1,245 cm^{-1} associati con l'acetone; da wn 1,025 cm^{-1} fino a 1,013 x cm^{-1} dove è posizionato il filtro del lattosio. Questa ricerca fornisce nuove conoscenze riguardo a strategie alternative per lo screening dello stato di gravidanza dei bovini da latte.

ABSTRACT

Milk production has a strong effect on fertility and at the same time reproduction affects the milk composition. In the last decade, special interest has arisen in studying milk composition and its relation with health, efficiency, and fertility. Therefore the principal objective of this thesis was to assess the relationships between the fertility of dairy cows and their milk yield, composition, and infrared spectra.

Data used were collected by the Breeders Federation of Alto Adige/Südtirol from the northeastern Italian province of Bolzano/Bozen. The milk's data comprises production, composition and physical properties. For fertility, all the insemination dates were available as well as the calving date. The milk samples collected were analyzed using a MilkoScanTM FT+ 6000 (Foss Electric, Hillerød, Denmark), the spectrum covered 1,060 wavenumbers (wn) from 5,010 to 925 cm^{-1}. Four breeds were studied: the specialized dairy breeds Holstein and Brown Swiss and the double purpose breeds Simmental and Alpine Grey.

The effects of breed and its interaction with milk productivity at the herd level (Herd-L) and at cow-within-herd level (Cow-L) on fertility traits in dairy cattle were studied in the second chapter. A mixed model was fitted to establish milk production levels of the various herds and individual cows according to milk yield. The interval calving-first service (iCF), interval first service-conception (iFC) and days open (DO) traits were analyzed using a Cox's proportional hazards model. The non-return rate at 56d after first service (NRR), pregnancy rate at first service (PRF) and the number of inseminations (INS) traits were analyzed using a logistic regression. It was observed a strong interaction between breed and productivity class at both Herd-L and Cow-L on all traits. The dual-purpose Simmental and Alpine Grey breeds had better fertility than the specialized Holstein and Brown Swiss dairy cows, also at the same daily milk yield, and the effects of herd and cow productivity differed from each other and

differed among breeds. In conclusion, greater herd productivity can result in higher fertility in cows, while a higher milk yield of individual cows within a herd results in lower fertility. These effects at both Herd-L and Cow-L are curvilinear and stronger in the dual-purpose breeds, being more evident from the low to the intermediate levels than moving from the intermediate to the high productivity classes.

The relationships between the estrous cycle phases in dairy cattle to milk composition, milk physical indicators and milk fatty acids were assessed in the third chapter. The test days around the first insemination after calving in the range from -10 to +10 days were selected and classified in 5 phases: Diestrus high-progesterone (Diestrus-HP) from -10 to -4 d; Proestrus from -3 to -1 d; Estrus day 0 (insemination day); Metestrus from 1 to 2 d; and Diestrus increasing-progesterone (Diestrus-IP) from 3 to 10 d. A mixed model was fitted to analyze the milk components and the milk physical indicator properties, including the effect of the estrous phases and we estimated the contrasts among them. The milk composition showed high variability among the estrous phases, affecting principally the fat, protein and lactose. The fatty acid profile and the physical indicators were also highly affected indicating important differences occasioned by the hormonal and behavioral changes of cows in estrus.

The predictive abilities of fat, protein, lactose, casein, and FTIR spectral data to predict cow's pregnancy status (PS) were assessed in the fourth chapter. We used generalized linear models to predict PS using fat, protein, lactose, casein and single FTIR spectral bands. We also fitted a Bayesian variable selection model to predict PS using the complete FTIR spectrum. Prediction accuracy was evaluated using a 10 fold cross-validation study and calculating the area under a receiver operating characteristic curve (CV-AUC) based on phenotypic predictions and observations. Overall, the most prediction accuracies were obtained for a model that included the complete FTIR spectral data. Alpine Grey cows had the highest CV-AUC (0.645), while Brown Swiss and Simmental had similar results (0.630 and 0.628 respectively) and Holsteins had the lowest value for FTIR Spectra (0.607). For the single wavelength analyses, important peaks were detected at: from wn 2,973 to wn 2,872 cm^{-1} where Fat-B is usually filtered; wn 1,773 cm^{-1} where Fat-A is filtered; wn 1,546 cm^{-1} where protein is filtered; wn 1468 cm^{-1} associated with urea and fat; wn 1,399 cm^{-1} and wn 1,245 cm^{-1} associated with acetone; from wn 1,025 cm^{-1} to wn 1,013 x cm^{-1} where lactose is filtered. This research provides new insights to alternative strategies for pregnancy status screening on dairy cattle.

CHAPTER I

General Introduction

MILK PRODUCTION

More than 6 billions people worldwide consume milk and milk products; the majority of these people live in developing countries (FAO, 2017a). Milk is considered fundamental for the human nutrition especially in childhood, since it is an important source of dietary energy, protein and fat, contributing on average 134 kcal of energy/capita per day, 8 g of protein/capita per day and 7.3 g of fat/capita per day (FAO, 2013). Per capita milk consumption vary considerably among regions; in Argentina, Armenia, Australia, Costa Rica, Europe, Israel, Kyrgyzstan, North America and Pakistan the consumption is high (> 150 kg/capita/year); in India, Islamic Republic of Iran, Japan, Kenya, Mexico, Mongolia, New Zealand, North and Southern Africa, most of the Near East and most of Latin America and the Caribbean the consumption is medium (30 to 150 kg/capita/year); in Viet Nam, Senegal, most of Central Africa and most of East and Southeast Asia the consumption is low (< 30 kg/capita/year) (FAO, 2017a). In the western societies, the consumption of milk has decreased during the last decades. This trend may partly be explained by the claimed negative health effects that have been attributed to milk and milk products. This criticism has arisen especially because milk fat contains a high fraction of saturated fatty acids assumed to contribute to heart diseases, weight gain and obesity (Haug et al., 2007). In the other hand milk production worldwide has increased substantially, from 313 million tons produced in 1961 to 655 million tons in 2014 (FAO, 2017b). India is the world's largest milk producer, with 18% of global production, followed by the United States of America (12%), China and Brazil (5% each). However, there is a milk deficit in several countries and milk demand is growing rapidly and is expected that this demand will continue worldwide in the next decades (FAO, 2017c).

MILK COMPOSITION

Milk for human consumption is obtained from several species, however, the *Bos taurus* and *Bos indicus* predominate in milk production (FAO, 2017c). The bovine milk composition and hence the properties vary with several factors, especially breed, stage of lactation, health, nutrition and individuality of the animal (Fox, 2011).

The average milk composition is: water ~87%, lactose ~4.8 %, fat ~3-6%, proteins ~3.5%, minerals ~0.8% and vitamins ~0.1%. Lactose is a disaccharide consisting of glucose and galactose, is the most consistent component of milk and is the major osmoregulatory component in milk. It is responsible for drawing water into the intracellular secretory vesicles and thereby determines milk volume (Stelwagen, 2016).

Milk fat consists of lipids that are mainly present in microscopic globules as an oil-in-water emulsion. The milk fat composition consists mainly of triglycerides (~98%), diacylglycerol (~2%), cholesterol (<0.5%), phospholipids (~1%), free fatty acids and fat-soluble vitamins (A, D, E, K) (Månsson, 2008). The size of the milk fat globule (MFG) increases with increasing fat content in the milk probably because of a limitation in the production of MFGM (Wiking et al., 2004). The amount of MFG is approximately 10^{10} per ml with a total average area of 700 cm^2 per ml of milk. The MFG is very important on the stability and technological properties of milk (Walstra et al., 2006).

The milk fat contains over 400 different fatty acids (FA), varying in chain length and number, position and geometry of double bonds, for this reason, milk fat is the most complex of all natural fats (Jensen, 2002). Fatty acids are carboxylic acids with a long aliphatic chain, which is either saturated or unsaturated. The aliphatic chain is usually linear and his length, in association with the number of bonds it contains, determines the physical and chemical properties of the specific FA. According to the length of the carbon chain the FA can be classified as short-chain fatty acids (SCFA) if they contain 6 or fewer carbon atoms in the aliphatic chain, medium-chain fatty acids (MCFA) if the number of carbon atoms is between 7 and 12, and long-chain fatty acids (LCFA) if the carbon atoms are greater than 12. According to the degree of unsaturation of the carbon chain, the FA are classified as saturated fatty acids (SFA) without double bond or unsaturated fatty acids (UFA) with one or more double bonds. At the same time, the UFA can be classified into monounsaturated fatty acids (MUFA) with a single double bond and polyunsaturated fatty acids (PUFA) with more than one double bond. In addition, the two carbon atoms in the chain that are bound next to either side of the double bond can occur in a *cis* or *trans* configuration: a *cis* double bond causes the hydrocarbon chain to bend and restricts the conformational freedom of the fatty acid, in the *trans* conformation this does not happen.

From the total milk FA, ~70% are SFA and the most important fatty acid from a quantitative viewpoint is palmitic acid (16:0), which accounts for approximately 30% by weight of the total fatty acids, myristic acid (14:0) and stearic acid (18:0) make up 11 and 12% by weight, respectively. The remaining ~30% are UFA (MUFA 25% and PUFA 5%), the oleic acid (C18:1) accounts for 23% of the total fatty acids (Månsson, 2008; Markiewicz-Kęszycka et al., 2013).

There are two pathways of FA synthesis: SCFA and MCFA (C4:0 to C14:0) and ~50% of C16:0 (palmitic acid) are synthesized *de novo* from acetate and β-hydroxybutyrate. Acetate and butyric acid are produced in the rumen by fermentation of feed components. The butyric acid is converted to β-hydroxybutyrate during absorption through the rumen epithelium. The remaining ~50% of C16:0 and the LCFA originate from dietary lipids and from lipolysis of adipose tissue triacylglycerols (Parodi, 2004). The pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are synthesized by the bacterial flora in the rumen.

MCFA and LCFA, but mainly C18:0 may be desaturated in the mammary gland to form the corresponding monounsaturated acids (Månsson, 2008). Fatty acids both synthesized de novo as well as derived from the diet may be used by the mammary gland and adipose tissue for the production of triglycerides and phospholipids.

Bovine milk contains ~3.5% protein, but this level varies substantially with breed, individuality, stage of lactation, and health and nutritional status of the animal. The technological properties of milk, indeed the very existence of most dairy products, are determined mainly by the unique properties of some of its proteins (Fox, 2011). About ~80% of the protein consists of casein, actually a mixture of four proteins: α_{s1}-casein (38%), α_{s2}-casein (10%), β-casein (35%), and κ-casein (12%). The caseins are typical for milk and have some rather specific properties, supply amino acids to the neonate, and also supply calcium and phosphorus, which are essential for the rapidly growing neonate: they are to some extent phosphorylated and have little or no secondary structure. The remainder consists, for the most part, of whey proteins (WP), the four principal serum proteins are β-lactoglobulin (~60% of total WP), α-lactoalbumin (~20% of total WP), blood serum albumin (~10% of total WP), and immunoglobulins (~10% of total WP). Moreover, milk contains numerous minor proteins, including ~60 indigenous enzymes (Walstra et al., 2006; Fox, 2011).

REPRODUCTION OF COWS

In order to overcome the milk deficit, several approaches have been established like better cattle management practices, better nutrition, and intense genetic selection. However, an increase of cow's milk production and larger herd size has been associated with a loss in reproductive efficiency (Lucy, 2001), reporting a negative genetic correlation between milk production and fertility traits (Pryce et al., 2004). The cow's days open have been lengthened with a decrease in fertility rates, and consequently an increase in involuntary culling. This has aroused much interest in investigating causes and solutions to improve fertility in specialized dairy cows (Walsh et al., 2011; López-Gatius, 2012). An intensive selection on a narrow breeding goal typically reduces population genetic diversity, leading to increasing inbreeding that negatively impacts animal health, fertility and survival (Mc Parland et al., 2007).

To address the fertility loss, several models and methodologies to include fertility in the genetic evaluations have been proposed and numerous countries have included fertility traits into their total merit indices and genetic evaluations (VanRaden et al., 2004; Huang et al., 2007; Egger-Danner et al., 2015). Crossbreeding has been used as an alternative leading to improvements in fertility traits in dairy cattle (Weigel and Barlass, 2003; Madalena and Toledo-Alvarado, 2016). Different cross-breed combinations have led to differences in reproduction and other production traits (Heins et al., 2006; Malchiodi et al., 2014; Toledo-Alvarado et al., 2015). Therefore, it is essential the study of fertility traits of individual dairy breeds in order to propose solutions in genetic programs. In addition, there is an increase in fertility-related diagnoses and other tools aimed at improving selection for reproductive health (Egger-Danner et al., 2015; Roelofs et al., 2015), for example, the heat detection of dairy cows, is usually detected by behavioral signs, usually a cow that "stands" to be mounted is on estrus, yet there are several tools to help the farmer, like pedometers, neck-mounted collars to detect physical activity, pressure sensing devices and tail temperature detectors (Roelofs et al., 2015; Miura et al., 2017).

The fertility of cows has been defined as the ability of the cow to establish ovarian function postpartum, to show overt estrus, or to conceive and maintain a pregnancy when served at the appropriate time in relation to ovulation (Darwash et al., 1997). Conception and maintenance of pregnancy in cattle involve several management effects and physiological processes. Management issues usually include heat detection, insemination time and nutrition. The physiological issues include the production of an ovum capable of being fertilized and a uterus capable of carrying on the gestation (Darwash et al., 1999; Pryce et al., 2004).

The normal estrous cycle in cattle is 18 to 24 days, divided in two phases: the luteal phase (14 to 18 days) and the follicular phase (4 to 6 days). The puberty in heifers usually occurs between 6 to 24 months of age. The estrous cycle ceases during pregnancy due to high levels of progesterone from the corpus luteum, then after parturition, the estrous cycle re-start after an anestrus (Crowe, 2016).

Cow's fertility traits are calculated usually from the services and calving dates recorded by the milk recording organizations. The fertility measures for females usually are divided into fertility scores, interval traits, and age at specific reproduction event (Table 1) (Pryce et al., 2004; ICAR, 2016). Other traits registered and related to female fertility are calving easy and prolificacy or number of calves per gestation. In the case of bulls or male fertility, it can be assessed by traits measured in the bull itself (semen production and libido) or by the outcome of breeding recorded in mates (conception rate). The bull's semen collected can be examined and score several criteria like the volume of ejaculate, spermatozoa concentration, the proportion of live spermatozoa, the sperm percent of forward motility, etc. (ICAR, 2016).

FTIR-SPECTROSCOPY

Fourier transform infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption of a solid, liquid or gas. The term Fourier transform infrared spectroscopy originates from the fact that a Fourier transform (A complex mathematical function that converts an interferogram into a spectrum) is required to convert the raw data into the actual spectrum. Contrary to filter-based instruments, which measure the absorption at specific wavelengths, the FTIR equipment determine the full spectrum of the sample within the same period of time (Andersen et al., 2002). The basic principle of spectroscopy is based on the ability of each chemical compound to absorb, reflect or transmit energy generating vibrational motions defined as stretching (symmetric or asymmetric) and bending (Derrick et al., 2000). The major regions of the electromagnetic spectrum and FTIR spectrum of cow milk measured versus water background are shown in Figure 1.

The FTIR spectrometers for milk work with transmittance, measuring the radiation that the sample does not absorb or reflect. Following the Beer-Lambert law, the transmittance of the material is related to its optical depth and can be defined as

$$Transmittance = \Phi_e^t / \Phi_e^i ,$$

where Φ_e^t is the radiant flux transmitted by the material sample and Φ_e^i is the radiant flux received by that material sample. For a given wavelength or frequency of infrared (IR) radiation striking a sample, the transmittance is inversely related to the absorbance through the following equation: $Absorbance = \log(1/Transmittance)$ (Derrick et al., 2000).

The IR region extends from the red end of the visible spectrum to the microwave region (Figure 1), wavenumbers from about 14,000 to 20 cm^{-1} (wavelengths 0.7 to 500 μm). The IR region is usually divided into three regions for application and instrumentation reasons. The near-IR (NIR, NIRS) region extends from the visible region 14,000 cm^{-1} (0.7 μm) to the mid-IR region 4,000 cm^{-1} (2.5 μm). NIR instruments are often combined with UV-Vis spectrometers, NIR analysis is applied in agriculture for determining the quality of forages, grains, fats, dairy products, eggs, meat, etc. It is widely used to quantify the composition of agricultural products because it meets the criteria of being accurate, reliable, rapid, non-destructive, and inexpensive. The mid-IR (MIR) region covers the frequency range from 4,000 cm^{-1} (2.5 μm) to 500 cm^{-1} (20 μm). In this region the fingerprint region is located at 1,300-1,500 cm^{-1} (8.0-20 μm). The main absorption bands may be assigned to vibrational modes corresponding to individual functional groups (NH-OH, C-H stretch, carbonyl), both the presence and absence of these characteristic group frequency bands are useful for characterizing molecular structure. Multiple absorptions in this region make it difficult to assign individual bands, but the overall combined pattern is characteristic and useful for composition identification. The MIR spectrum has been often used for qualitative analyses of organic substances and due to relatively simple sample preparation procedures, it has been especially popular (Derrick et al., 2000). In the dairy industry, the use of MIR has been very important to measure the composition or properties of milk and dairy products and enables the dairy organizations to pay the farmer for the milk on a fair basis, and to manufacture products of consistent quality (Andersen et al., 2002). The far-IR (FIR) region expands from 500 to 20 cm^{-1} (20-500 μm). In this region, the molecules are involved in low-frequencies bending and torsional motions, such as lattice vibrations in crystals. For example, the FIR bands of isomers and LCFA can be differentiated in solid-state materials (Derrick et al., 2000).

Since 1993 when the first purpose-built MIR based on the FTIR was marketed, the FTIR spectrocopy has been the most widespread method used for compositional and quality analysis in the dairy industry (Andersen et al., 2002). The milk components and properties included in the routine analysis for milk include: fat, protein, casein, lactose, total solids, urea, citric acid, free fatty acids, some individual fatty acids and groups, freezing point, pH, ketosis screening, lactic acid, specific sugars, salt, density, adulteration screening, homogenizer efficiency, phospholipids and calcium (Andersen et al., 2002). The FTIR spectroscopy has also been used to predict many other detailed phenotypes as protein fraction compositions, fatty acid profiles, free amino acids and milk coagulation properties (De Marchi et al., 2014). In addition, other phenotypes having direct relationships with milk composition have been also studied with FTIR spectroscopy, such as feed intake, energy intake, and body energy status (McParland and Berry, 2016). The use of FTIR spectroscopy as an indicator of health and fertility has also been studied, associating the acetone and β-hydroxybutyrate to ketosis, and various fatty acids (e.g., C18:1 cis-9 and C10:0) to fertility (Bastin et al., 2016). Moreover, a direct influence of pregnancy on milk composition and FTIR spectrum has been reported, in specific it was observed an effect on the absorbance 212 wavenumbers in the MIR in early pregnancy (Lainé et al., 2017).

TABLES

Table 1. Description of reproduction traits in cattle

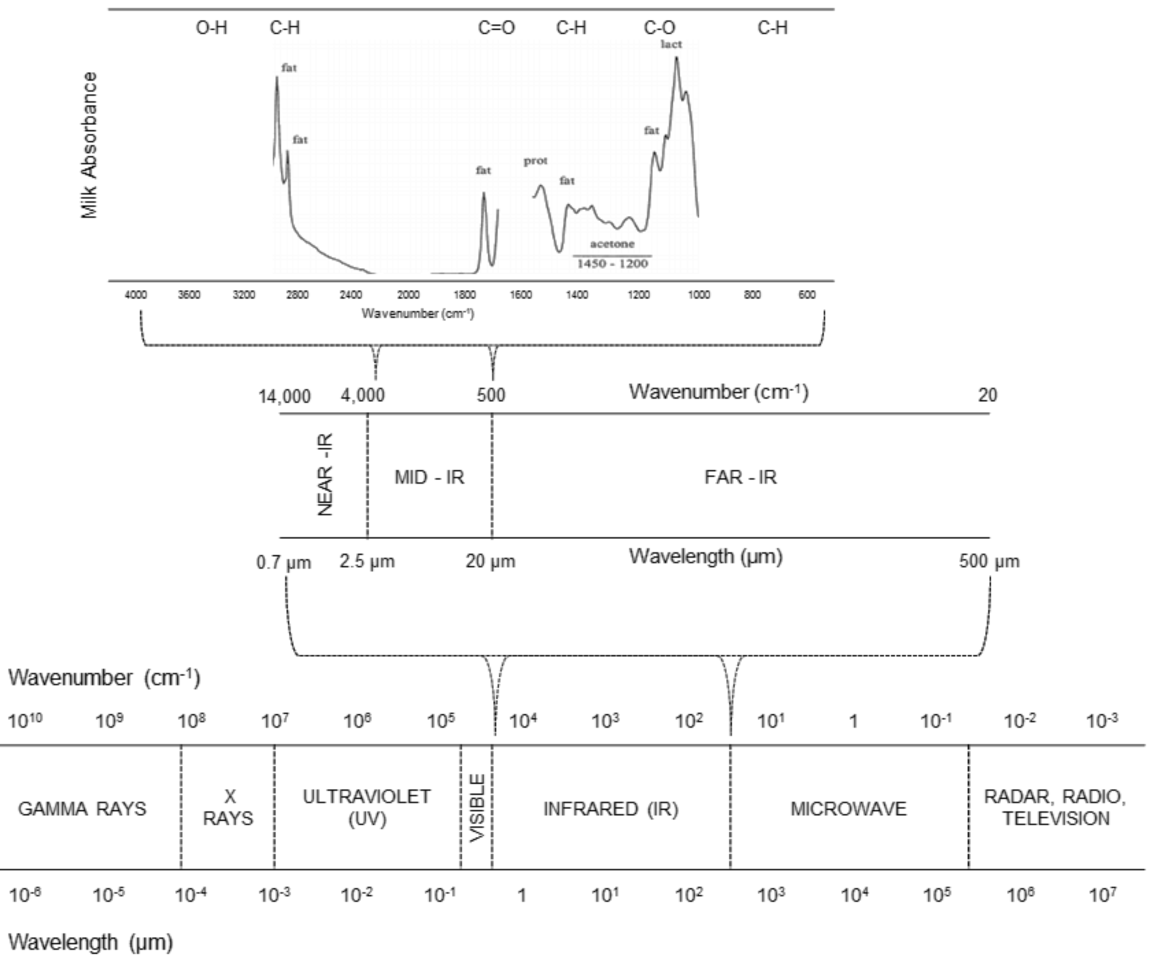
Trait	Description
Success fertility traits	
Non-return rate at *n* days (NRR*n*)	NRR is based on the observation that a bred/mated cow has not returned for another service within a defined number of days (*n*), usually 56, 60 or 90 days; Binary [0,1]
Conception rate (CR)	The outcome of an insemination validated by calving date; Binary [0,1]
Number of inseminations to conception (INS)	The number of inseminations to achieve pregnancy; Count [1, 2, 3…n]
Interval fertility traits	
Interval from parity to first heat (iPH)	The number of days from calving to the first heat detected; Continuous (days)
Voluntary waiting period (VWP)	The number of days intentionally in during early lactation in which cows are willingly not inseminated even if they display estrus; Continuous (days)
Interval between calving and first insemination (iCF)	The number of days from calving to the first service; Continuous (days)
Interval from first insemination to conception (iFC)	The number of days from the first to the successful service (or last service); Continuous (days)
Interval between services	The number of days between two consecutive inseminations; Continuous (days)
Days open (DO)	The number of days between calving to the successful insemination (or last service); Continuous (days)
Calving interval (CI)	The number of days between two consecutive calvings; Continuous (days)
Gestation length (GL)	The number of days between known conception date and subsequent calving date. In case of several consecutive breeding the last one is considered to be the conception date; Continuous (days)
Ages at reproductive events	
Age at puberty	The age at which heifers reach puberty and start cycling; Continuous (days)
Age at first breeding	The age at which heifers receive their first service; Continuous (days)
Age at first calving	The age at which heifers have their first calving; Continuous (days)

Table 2. Pregnancy diagnosis, recording of the result of a breeding in female (ICAR, 2016)

Method	Period
Observation of failure to return to oestrus in a specified return interval	Between 18 and 24 days after breeding
Palpation of ovaries, persistence of the corpus luteum	From day 18 to24
Progesterone essay	At day 24
Palpation of amniotic vesicle	From day 30-60
Ultrasonic method to detect the embryo	From about day 20
Calf birth	

FIGURES

Figure 1. Spectral regions of electromagnetic radiation with an expansion of infrared region and FTIR spectrum of cow milk measured versus water background. Typical absorption of milk fat (fat), milk protein (prot), milk lactose (lact), and milk acetone are indicated



REFERENCES

Andersen, S.K., P.W. Hansen, and H.V. Andersen. 2002. Vibrational spectroscopy in the analysis of dairy products and wine. Handb. Vib. Spectrosc.

Bastin, C., L. Théron, A. Lainé, and N. Gengler. 2016. On the role of mid-infrared predicted phenotypes in fertility and health dairy breeding programs.. J. Dairy Sci. 99:4080–94. doi:10.3168/jds.2015-10087.

Crowe, M.A. 2016. Reproduction, Events and Management: Estrous Cycles: Characteristics. Elsevier.

Darwash, A.O., G.E. Lamming, and J. Woolliams. 1999. The potential for identifying heritable endocrine parameters associated with fertility in post-partum dairy cows. Anim. Sci. 68:333–347.

Darwash, A.O.O., G.E.E. Lamming, and J.A.A. Woolliams. 1997. Estimation of genetic variation in the interval from calving to postpartum ovulation of dairy cows.. J. Dairy Sci. 80:1227–1234. doi:10.3168/jds.S0022-0302(97)76051-X.

Derrick, M.R., D. Stulik, and J.M. Landry. 2000. Infrared Spectroscopy in Conservation Science. Getty Publications.

Egger-Danner, C., J.B. Cole, J.E. Pryce, N. Gengler, B. Heringstad, A. Bradley, and K.F. Stock. 2015. Invited review : overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. Animal 9:191–207. doi:10.1017/S1751731114002614.

FAO. 2013. Milk and Dairy Products in Human Nutrition. E. Muehlhof, A. Bennett, and D. McMahon, ed. Rome, Italy.

FAO. 2017a. Dairy Production and Products: Milk and Milk Products. Accessed May 4, 2017. http://www.fao.org/agriculture/dairy-gateway/milk-and-milk-products/en/.

FAO. 2017b. FAOSTATS. Food Agric. Organ. United Nations.

FAO. 2017c. Dairy Production and Products: Milk Production. Accessed May 4, 2017. http://www.fao.org/agriculture/dairy-gateway/milk-production/en/.

Fox, P.F. 2011. Milk | Bovine Milk in: Encyclopedia of Dairy Sciences. Second Edi. J.W. Fuquay, ed. Elsevier, San Diego.

Haug, A., A.T. Høstmark, and O.M. Harstad. 2007. Bovine Milk in Human Nutrition – a Review. Lipids Health Dis. 6:25. doi:10.1186/1476-511X-6-25.

Heins, B.J., L.B. Hansen, and A.J. Seykora. 2006. Fertility and survival of pure Holsteins versus crossbreds of Holstein with Normande, Montbeliarde, and Scandinavian Red.. J. Dairy Sci. 89:4944–51. doi:10.3168/jds.S0022-0302(06)72545-0.

Huang, C., I. Misztal, S. Tsuruta, and T.J. Lawlor. 2007. Methodology of Evaluation for Female Fertility. Interbull Bull. 156–160.

ICAR (International Committee for Animal Recording). 2016. International Agreement of Recording Practices - Recording Guidelines.

Jensen, R.G. 2002. The composition of bovine milk lipids: January 1995 to December 2000.. J. Dairy Sci. 85:295–350. doi:10.3168/jds.S0022-0302(02)74079-4.

Lainé, A., C. Bastin, C. Grelet, H. Hammami, F.G. Colinet, L.M. Dale, A. Gillon, J. Vandenplas, F. Dehareng, and N. Gengler. 2017. Assessing the effect of pregnancy stage on milk composition of dairy cows using mid-infrared spectra. J. Dairy Sci. 1–14.

doi:10.3168/jds.2016-11736.

López-Gatius, F. 2012. Factors of a noninfectious nature affecting fertility after artificial insemination in lactating dairy cows. A review. Theriogenology 77:1029–1041. doi:10.1016/j.theriogenology.2011.10.014.

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: where will it end?. J. Dairy Sci. 84:1277–93. doi:10.3168/jds.S0022-0302(01)70158-0.

Madalena, F.E., and H. Toledo-Alvarado. 2016. Animals that Produce Dairy Foods: Bos indicus Breeds and Bos indicus×Bos taurus Crosses. First Edit. Elsevier.

Malchiodi, F., a. Cecchinato, and G. Bittante. 2014. Fertility traits of purebred Holsteins and 2- and 3-breed crossbred heifers and cows obtained from Swedish Red, Montbéliarde, and Brown Swiss sires. J. Dairy Sci. 97:7916–7926. doi:10.3168/jds.2014-8156.

Månsson, H.L. 2008. Fatty acids in bovine milk fat. Food Nutr. Res. 52. doi:10.3402/fnr.v52i0.1821.

De Marchi, M., V. Toffanin, M. Cassandro, and M. Penasa. 2014. Invited review: Mid-infrared spectroscopy as phenotyping tool for milk traits. J. Dairy Sci. 97:1171–1186. doi:10.3168/jds.2013-6799.

Markiewicz-Kęszycka, M., G. Czyżak-Runowska, P. Lipińska, and J. Wójtowski. 2013. Fatty acid profile of milk - a Review. Bull. Vet. Inst. Pulawy 57:135–139. doi:10.2478/bvip-2013-0026.

Mc Parland, S., J.F. Kearney, M. Rath, and D.P. Berry. 2007. Inbreeding effects on milk production, calving performance, fertility, and conformation in Irish Holstein-Friesians.. J. Dairy Sci. 90:4411–4419. doi:10.3168/jds.2007-0227.

McParland, S., and D.P. Berry. 2016. The potential of Fourier transform infrared spectroscopy of milk samples to predict energy intake and efficiency in dairy cows. J. Dairy Sci. 99:4056–4070. doi:10.3168/jds.2015-10051.

Miura, R., K. Yoshioka, T. Miyamoto, H. Nogami, H. Okada, and T. Itoh. 2017. Estrous detection by monitoring ventral tail base surface temperature using a wearable wireless sensor in cattle. Anim. Reprod. Sci.. doi:10.1016/j.anireprosci.2017.03.002.

Parodi, P.W. 2004. Milk fat in human nutrition. Aust. J. dairy Technol. 59:3–59.

Pryce, J.E., M.D. Royal, P.C. Garnsworthy, and I.L. Mao. 2004. Fertility in the high-producing dairy cow. Livest. Prod. Sci. 86:125–135. doi:10.1016/S0301-6226(03)00145-3.

Roelofs, J.B., E. van Erp-van der Kooij, and E. van Erp-van der Kooij. 2015. Estrus detection tools and their applicability in cattle: recent and perspectival situation. Anim. Reprod 12:498–504.

Stelwagen, K. 2016. Mammary Gland, Milk Biosynthesis and Secretion: Lactose.

Toledo-Alvarado, H., J. Berruecos-Villalobos, and C. Vázquez-Peláez. 2015. Bos indicus x Bos taurus gene proportion over weaning weight and age at first parturition in a multi-breed population. Rev. Mex. Ciencias Pecu. 6:1–13.

VanRaden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn, and G.R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285–92. doi:10.3168/jds.S0022-0302(04)70049-1.

Walsh, S.W.W., E.J.J. Williams, and A.C.O.C.O. Evans. 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. 123:127–38.

doi:10.1016/j.anireprosci.2010.12.001.

Walstra, P., J. Wouters, and T. Geurts. 2006. Dairy Science and Technology. 2nd ed. CRC Press, New York.

Weigel, K.A., and K.A. Barlass. 2003. Results of a producer survey regarding crossbreeding on US dairy farms. J. Dairy Sci. 86:4148–54. doi:10.3168/jds.S0022-0302(03)74029-6.

Wiking, L., J. Stagsted, L. Björck, and J.H. Nielsen. 2004. Milk fat globule size is affected by fat production in dairy cows. Int. Dairy J. 14:909–913. doi:10.1016/j.idairyj.2004.03.005.

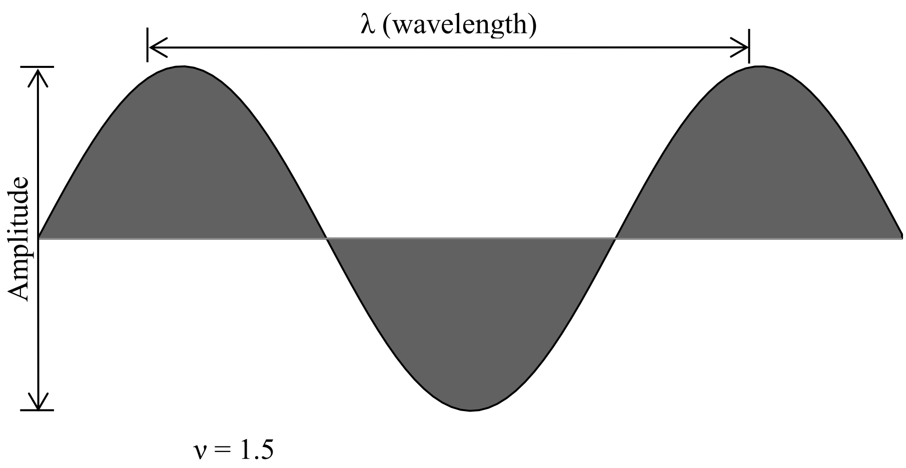
APPENDIX

Wave theory

All energies of the electromagnetic spectrum can be considered to be waves that move at the speed of light, with the types of radiation differing in amplitude, frequency, and wavelength. The amplitude is the height of the wave, the frequency (v) is the number of waves per unit time (cycles per second). The wavelength (λ), is the distance between two successive maxima or minima of a wave (length of one wave). The electromagnetic radiation can also be characterized by the number of waves per unit length, which is the wavenumber:

$$\bar{v} = {}^{1}/_{\lambda}$$

The frequency of electromagnetic waves at 1 second interval



AIMS OF THE THESIS

The principal objective of this thesis was to assess the relationships between the fertility of dairy cows and their milk yield, composition, and infrared spectra.

The specific objectives were:

→ Assess the effect of breed of cow and the interaction of breed and milk productivity measured at herd level and at cow-within-herd level on interval fertility traits, fertility success traits, and number of inseminations per cow in Holstein, Brown Swiss, Simmental, and Alpine Grey breeds.

→ To investigate the variations of milk constituents, physical indicators of milk and milk fatty acids composition within the estrous phases on Holsteins, Brown Swiss, Simmental and Alpine Grey cows.

→ Assess and to compare the prediction accuracies of a reproductive outcome (pregnancy status) that can be achieved using milk components derived from spectra data (fat, protein, casein and lactose) as well as single-band and whole-spectrum FTIR data. Our study is based on data generated within the Italian milk recording systems of Holstein, Brown Swiss, Simmental and Alpine Grey cattle breeds.

CHAPTER II

FERTILITY ACROSS BREEDS AND MILK PRODUCTION

Fertility traits of Holstein, Brown Swiss, Simmental and Alpine Grey cows are differently affected by herd productivity and milk yield of individual cows.

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INTERPRETIVE SUMMARY

Fertility traits of Holstein, Brown Swiss, Simmental and Alpine Grey cows are differently affected by herd productivity and milk yield of individual cows. *By Toledo-Alvarado et al.*

We assessed the effects of breed and the interaction between breed and level of milk production measured at the herd and the cow-within-herd levels on fertility traits in dairy cattle. The traits analyzed were the interval between calving and first insemination, the interval between first service and conception, the days open, the non-return rate at 56d after first service, the pregnancy rate at first service, and number of inseminations. We found that reproductive traits are greatly affected by the level of milk production and the effects of herd and cow production environments differ from each other, and differ among breeds.

ABSTRACT

Milk yield has a strong effect on fertility, but it may vary across different herds and individual cows. Therefore the aim of this study was to assess the effects of breed and its interaction with level of milk production at the herd level (Herd-L) and at a cow-within-herd level (Cow-L) on fertility traits in dairy cattle. Data were gathered from Holstein (n = 17,688), Brown Swiss (n = 32,697), Simmental (n = 27,791) and Alpine Grey (n = 13,689) cows in north-eastern, Italy. The analysis was based on records from the first 3 lactations on the years 2011 to 2014. A mixed model was fitted to establish milk production levels of the various herds (Herd-L) and individual cows (Cow-L) using milk as a response variable. The interval fertility traits were: interval from calving to first service (iCF), interval from first service to conception (iFC) and number of days open (DO). The success traits were: non-return rate at 56d after first service (NRR), pregnancy rate at first service (PRF) and the number of inseminations (INS). The iCF, iFC and DO traits were analyzed using a Cox's proportional hazards model. The NRR, PRF and INS traits were analyzed using logistic regression. There was a strong interaction between breed and productivity class at both Herd-L and Cow-L on all traits. The effects of herd and cow productivity differed from each other, and differed among breeds. The dual-purpose Simmental and Alpine Grey breeds had better fertility than the specialized Holstein and Brown Swiss dairy cows, and this difference is only partly due to different milk yields. Greater herd productivity can result in higher fertility in cows, while higher milk yield of individual cows within a herd results in lower fertility. These effects at both Herd-L and Cow-L are curvilinear and are stronger in dual-purpose breeds, which was more evident from low to intermediate milk yield levels than moving from central to high productivity classes. Disentangling the effects of milk productivity on fertility at Herd-L and at Cow-L, and taking the non-linearity of response into account could lead to better modeling of populations, within breed. It could also help with management e.g. in precision dairy farming of dairy and dual-purpose cattle. Moreover, assessing the fertility of various breeds and their different responses to herd and individual productivity levels could be useful in devising more profitable crossbreeding programs in different dairy systems.

Key words: fertility, survival analysis, milk production, G×E interaction.

INTRODUCTION

The reduction in fertility rate, alongside the rise in milk production in dairy cattle over recent decades (Lucy, 2001) has raised much interest in investigating its causes and in seeking solutions (Walsh et al., 2011; López-Gatius, 2012). Several studies have reported a negative genetic correlation between milk production and fertility traits (Pryce et al., 2004; Tiezzi et al., 2011, 2012), while others have found reproductive loss in dairy cattle to be associated with increased herd sizes, higher rates of inbreeding, changes in reproductive physiology, and worsening the body condition (Lucy, 2001; Walsh et al., 2011; Tiezzi et al., 2013). As a consequence, the number of days open has increased, pregnancy rates have decreased, and there has been an increase in the level of involuntary culling. However, caution should be exercised in interpreting these negative relationships, as the effects on reproductive performance associated with individual cows may be confounded with those at a herd level, which could lead to errors in interpretation. A more comprehensive assessment drawing on expertise from multiple scientific disciplines is needed to study the causes and effects of fertility loss (Bello et al., 2012). The diverse native characteristics of different breeds, and the different genetic improvement schemes among breeds and in different countries, mean that dairy cattle populations around the world have different genetic levels of fertility (Nilforooshan et al., 2009).

In order to address the problem, several countries have incorporated fertility traits into their genetic evaluations, and different models and methodologies have been proposed (VanRaden et al., 2004; Huang et al., 2007). A review by Egger-Danner et al. (2015) found that fifteen countries around the world with high levels of milk production include fertility in their total merit indices. It has been suggested that survival analysis may be a better option than linear methods, especially for event-time censored traits, as it allows partial records to be used in the analysis (Schneider et al., 2005). Phuong et al. (2015) proposed an extended lifetime performance model that incorporates the impact of variations in milk yield, energy balance and body condition on the reproductive success of individual cows. The model therefore successfully simulates the reproductive performance of different cow genotypes across feeding systems.

Crossbreeding of dairy cattle has been used as an alternative to pure breeding, and has led to improvements in various traits, including fertility (Weigel and Barlass, 2003). Different breed combinations have resulted in differences in fertility traits (Weigel and Barlass, 2003; Heins et al., 2006; Malchiodi et al., 2014). This means that a better understanding of the characteristics of individual breeds with respect to these traits is needed in order to design more profitable crossbreeding programs.

Vargas et al. (1998) studied interval fertility traits using event-time techniques of different breeds and crossbreeds, and reported that heifers in herds with lower milk yields were more likely to be bred. They found a significant difference between the effects of the milk yield of primiparous cows on iCF and on DO. They also reported a significant effect of heifer weight on age at first calving: herds and heifers with heavier body weights at 390 d had a higher probability of calving. Bello et al., (2012) point out that the associations between productivity and fertility may have been overlooked in the past due to confounding factors and inappropriate statistical analyses, the results of which may have been misinterpreted. According to these authors, lack of a clear distinction between herd level (**Herd-L**) and cow-within-herd level (**Cow-L**) in the modeling, and between the effects of different dairy production systems may also contribute to misleading conclusions being drawn. LeBlanc (2010) investigated the association between milk production rate and reproductive performance at both Herd-L and Cow-L using pregnancy, insemination, and calving rates as indicators of fertility. They found a positive association between pregnancy rate and earlier first insemination in high-yielding herds and cows. He reported that a high milk yield in cattle may be compatible with good reproductive performance, and also remarked on the complexity of fertility, and the danger of assessing it with only one indicator (pregnancy rate).

In this article, we assess the effect of breed of cow and the interaction of breed and milk productivity measured at Herd-L and at Cow-L on interval fertility traits, fertility success traits, and number of inseminations per cow in various breeds of dairy cattle (Holstein, Brown Swiss, Simmental, and Alpine Grey).

MATERIALS AND METHODS

Field Data

Female fertility and milk production data were collected by the Breeders Federation of Alto Adige/Südtirol (Associazione Provinciale delle Organizzazioni Zootecniche Altoatesine / Vereinigung der Südtiroler Tierzuchtverbände, Bolzano/Bozen, Italy) from the northeast of Bolzano/Bozen province in Italy. The region is mountainous and its farms are mainly small operating various farming systems, from the very traditional (small to medium herd sizes, old buildings, tied animals, with lactating cows moved to mountain pastures during the summer) to the more modern (large herd sizes, recent buildings with milking parlors and free animals, high levels of milk production, total mixed ration feeding system) (Sturaro et al., 2013). The test days cover the period from 2011 to 2015. Only records from the first 3 lactations and calvings of each cow from the years 2011 to 2014 were analyzed in order to exclude cows with fertility events in progress. Lactation period was divided into 11 categories of days in milk (DIM), each of 30 d but with the last an open category of more than 300 DIM. Breeds with few data and crossbred animals were excluded from the analysis.

Trait Definition and Data Editing

The interval fertility traits were defined as: the interval (d) between calving and first recorded insemination (**iCF**), the interval between first service and conception (**iFC**), and the number of days open (**DO**). The success traits analyzed were: the non-return rate at 56d after first service (**NRR**), and pregnancy rate at first service (**PRF**). NRR and PRF were coded as binary variables (0, 1), where 1 indicated a cow that did not have a second insemination registered within 56d of the first service (for NRR), or a cow that became pregnant at the first service (for PRF). Number of inseminations (**INS**) was considered an ordinal variable with 5 levels, the fifth being an open class of 5 or more inseminations. Pregnancy status was positively confirmed by a subsequent calving; otherwise it was set to unknown. Cows without a subsequent calving after the last service were penalized by the addition of a penalty insemination. Gestation length was required to be within 30 d of the average for each breed (about ±5 % within the average gestation length for all the breeds average), and if the pregnancy was outside this limit the record was excluded. Calving interval, iCF, iFC, and DO were required to be lower than the average + 3 SD (733, 243, 476, and 403 d, respectively). Data above the upper limits were replaced with the upper limit value and the record was considered censored. The lower limit for iCF and iFC was 0 d, while for DO it was 20 d. If there was no confirmation of pregnancy, the record was considered censored. After editing ~12% of the original data was eliminated, and the final dataset comprised 11,442 Holstein, 21,043 Brown Swiss, 16,727 Simmental, and 8,237 Alpine Grey cows distributed across 4,013 herds, many of which (47% of the total) were multi-breed herds.

Statistical Analysis

Herd-L and Cow-L predictions. There were large variations in the size, breed composition, and level of infrastructure of the herds. In order to establish the milk production levels of the different herds (Herd-L) and the individual cows within the herds (Cow-L), a mixed model was fitted using the MIXED procedure in the SAS/STAT software (SAS Institute, 2012) and with REML as the estimation method. The mixed model for Herd-L was:

$$y_{ijklmnop} = \mu + D_i + C_j + b_1H_k + b_2B_l + b_3S_m + b_4G_n + R_o + e_{ijklmnop}$$

where $y_{ijklmnop}$ is the milk production for the test day; μ is the general mean; D_i is the category of DIM (i = 11 categories); C_j is the year of the test day (j = 2011 to 2015); H_k, B_l, S_m, G_n are the percentages, respectively, of Holstein, Brown Swiss, Simmental, and Alpine Grey cows in the herd; b_1, b_2, b_3, b_4 are the linear regression coefficients for H_k, B_l, S_m, G_n, respectively; R_o is the random effect of herd (o = 4,013 herds); $e_{ijklmnop}$ is the random experiment error $(DNI \sim (0, \sigma_e^2))$. The herds' solutions were used to classify them into five milk productivity levels (Herd-L = HerdL-1, HerdL-2, HerdL-3, HerdL-4, HerdL-5). The mixed model for Cow-L for each breed was:

$$y_{ijklmn} = \mu + D_i + C_j + H_k + L_l + A_m + e_{ijklmn}$$

where y_{ijklmn} is the milk production for the test day; μ is the general mean; D_i is the category of DIM (*i* = 11 categories); C_j is the year of the test day (*j* = 2011 to 2015); H_k is the Herd-L (*k* = 5 herd levels); L_l is the number of the lactation (*l* = 1 to 3); A_m is the random effect of the animal (*m* = 57,449 cows)$(NID \sim (0, \sigma_a^2))$.; e_{ijklmn} is the random experiment error $(NID \sim (0, \sigma_e^2))$. The cows' solutions were used to classify them into five milk productivity levels (Cow-L = CowL-A, CowL-B, CowL-C, CowL-D, CowL-E). The central classes (HerdL-3, CowL-C), representing the majority of the herds (HerdL-3: n = 1,576, 39.3% of all herds) and cows (CowL-C: n = 23,132, 40.4% of all cows), were used as reference values in the subsequent analysis of fertility traits.

Analysis of Fertility Traits. The analysis was carried out using the PHREG procedure in the SAS/STAT software (SAS Institute, 2012) with a proportional hazard model (Cox, 1972) fitted for interval fertility traits (iCF, iFC and DO). The model was as follows:

$$\lambda_i(t|X_i) = \lambda_0(t)e^{(x_i'\beta)},$$

where $\lambda_i(t|X_i)$ is the hazard (Hazard Ratio: **HR**) of either receiving the first service after calving at time *t* for iCF, becoming pregnant after the first insemination at time *t* for iFC, or becoming pregnant after calving at time *t* for DO; $\lambda_0(t)$ is the baseline hazard function; β is an unknown vector of regression coefficients for the fixed effects; x' is a vector for the fixed effects of the number of the lactation (1 to 3), the year of calving (2011 to 2014), and breed interacting with either herd (20 levels) or cow-within-herd (20 levels). The hazard or risk in this context does not have a negative meaning. In fact, it refers to the probability of the occurrence of the reproductive event.

The variables NRR and PRF were analyzed by logistic regression using the LOGISTIC procedure in the SAS/STAT software (SAS Institute, 2012) and with a binary logit model with the form:

$$logit(\pi) \equiv log\left(\frac{\pi}{1-\pi}\right) = \alpha + \beta' x$$

where $\pi = Pr(Y = 1|x),$ which is the response probability (odds ratio: **OR**) of becoming pregnant for NRR and PRF; α is the intercept of the parameter; $\beta = (\beta_1, \ldots, \beta_i)$ is the vector of *i* slope parameters; and *x* is a vector for the fixed effects of the number of the lactation (1 to 3), the year of calving (2011 to 2014) and breed interacting with either herd (20 levels) or cow-within-herd (20 levels).

The variable INS was analyzed by logistic regression using the LOGISTIC procedure in the SAS/STAT software (SAS Institute, 2012) and with a cumulative logit model, a parallel lines regression model based on cumulative probabilities, with the form:

$$g\big(Pr(Y \leq i|x)\big) = \alpha_i + \beta' x, \qquad i = 1,2,3,4,5$$

where $g\big(Pr(Y \leq i|x)\big)$ is the probability (OR) of requiring fewer inseminations to become pregnant; $\alpha_1, \ldots, \alpha_5$ are the intercept parameters for the first five inseminations after calving; $\beta = (\beta_1, \ldots, \beta_i)$ is the vector of the *i* slope parameters; and *x* is the vector for the fixed effects of the number of the lactation (1 to 3), the year of calving (2011 to 2014) and breed interacting with either herd (20 levels) or cow-within-herd (20 levels).

The hazards ratio (HR) and odds ratio (OR) estimates together with their confidence intervals for each breed were used to plot these across the different Herd-L or Cow-L. It was then estimated linear and quadratic contrasts for each breed across Herd-L and Cow-L. A significant ($P < 0.05$) higher-order contrast was used to plot a linear or quadratic tendency.

RESULTS

Herd-L and Cow-L according to the Milk Production

Solution values were used to classify herds and individual cows into five categories: Herd-L (1 to 5), Cow-L (A to E). Figure 1 shows the frequencies of the herd and cow-within-herd categories obtained from the mixed model analysis. The distribution of the observations of herds and cows across the five classes ($< -1.5\sigma$; -1.5σ to -0.5σ; -0.5σ to $+0.5\sigma$; $+0.5\sigma$ to $+1.5\sigma$; $> +1.5\sigma$) was centered to 0 ± SD of daily milk production.

The average milk production levels of each breed within each Herd-L and each Cow-L class are presented in Figure 2. Holsteins had the highest average daily milk production in each Herd-L class (21.9, 24.0, 26.7, 28.9 and 31.6 kg/d in HerdL-1 to HerdL-5, respectively), followed by herds comprising dual-purpose Simmental and Brown Swiss cows, which produced about 4 kg/d less milk per cow in each of the 5 Herd-L classes. The average milk production of herds of local dual-purpose Alpine Greys was about 10 kg/d lower than the Holsteins in each Herd-L class. These differences are consistent with the four breeds' different genetic background for milk yield, and with the different herd characteristics in terms of geographical area, size, facilities, management, feeding, health, etc. Holsteins are often reared on modern dairy farms using loose housing, milking parlors and total mixed rations, local breeds are often kept on very traditional farms (tied cows, hay feed with some concentrates, etc.), while Brown Swiss and Simmental cows may be kept in both types of dairy system (Sturaro et al., 2013). Recent research carried out in the same area on multi-breed herds (Stocco et al., 2016) found lower within-herd differences in milk productivity (Holsteins produced about 3 kg/d more than the Brown Swiss and Simmentals, and 7 kg/d more than the Alpine Greys).

Average milk yield values of the Cow-L classes were slightly greater than the corresponding Herd-L classes because there were more cows in the more productive Herd-L classes than in the less productive classes. As a consequence the average milk yields of the cows of the 4 breeds studied were greater than the average milk yields of herds of the same breed. The SD of the Cow-L was similar to that of the Herd-L of the same breed, but the pattern of Cow-L averages was slightly curvilinear because the distribution of individual cows is slightly skewed due to the different numbers of cows in the two extreme classes (Figure 1). The classification shows milk production of the lowest category of Holstein, whether HerdL-1 (21.9 kg/d) or CowL-A (21.1 kg/d), was similar to the highest categories of Alpine Grey (21.1 kg/d for HerdL-5, 20.8 kg/d for CowL-5), whereas the categories of the Brown Swiss and Simmental herds and cows partly overlap with both the Holstein and Alpine Grey categories.

Descriptive Statistics

The descriptive statistics for iCF, iFC, and DO are reported in Table 1. On average, cows with uncensored records were inseminated 84.7 days after calving across all breeds, conception was reported to be successful 31.5 days later, and the interval between calving to conception was 117.1 days. The largest number of records was obtained from the Brown Swiss breed (32,697), and the lowest from the Alpine Greys (13,689). The distribution of records across levels of production (Herd-L, Cow-L) was similar for all breeds.

The percentages of censored records ranged from 22% for the dual-purpose breeds to 29% for the specialized dairy breeds. These proportions of censored data suggest that the different breeds have different culling rates and highlight the importance of the inclusion of these data in the analysis in order to decrease bias. The high proportions of censored data in our study justified the use of survival analysis to study time-dependent traits. Vargas et al. (1998) reported a rate of 10% censored records for DO in primiparous Holstein and Jersey cows in Costa Rica, while Malchiodi et al. (2014) reported rates of 7.5%, 24.8%, and 18.7% for iCF, DO, and iFC, respectively, in Holstein cows in Italy. In addition Tiezzi et al. (2011), reported 16.9% censored records for DO and iFC, in a previous study with Brown Swiss cows in the same northeast Italy region.

The estimated means for Holstein cows were the highest values across breeds for these traits. Brown Swiss cows exhibited slightly lower (more favorable) values, while those of the dual-purpose Simmental and Alpine Grey cows were the lowest. The values for censored records were much higher than those for uncensored data: on average + 20%, + 109%, and + 41% for iCF, iFC, and DO, respectively.

The number of records and the percentages of success events for NRR and PRF are shown in Table 2: here, too, the better results were from the dual-purpose breeds. The difference between the two extremes (Holstein and Alpine Grey) was about 9% for NRR, but increased to 27% after confirmation with calving (PRF), a figure that also reflects the higher culling rate of specialized dairy breeds. Similar values (0.71 for NRR and 0.45 for PRF) were obtained for Brown Swiss cows in the same Italian mountain region (Tiezzi et al., 2011), whereas Holstein cows reared on intensive dairy farms on the plains had much lower success rates (0.40 for NRR and 0.34 for PRF; Malchiodi et al., 2014) than in the present study. It should be remembered that only primiparous cows were included in those studies, whereas our estimates included cows in their first 3 parities. Norman et al. (2009) reported ranges of 45 – 48% for NRR70 and 24 – 34% for PRF in Holstein cows, compared with ranges of 51 – 54% for NRR70 and 33 – 41% for PRF in Jersey cows with several parities in the USA. Variations in milk production levels, nutrition, management, genetics and herd size may explain the different rates in the various studies.

Descriptive statistics for INS are reported in Table 3. Again, the specialized dairy breeds required the highest number of inseminations to get pregnant, the dual-purpose cows the lowest, with a difference of 22% between the two extremes (Holstein and Alpine Grey). Comparable results for INS were reported for Holstein primiparous cows reared on the plains, with 2.53 inseminations (Malchiodi et al., 2014), while a value of 1.74 inseminations was reported for Brown Swiss cows in the mountains (Tiezzi et al., 2011).

Kaplan-Meier Survival Functions

Kaplan-Meier estimates of the survival function for iCF, iFC, and DO are presented in Figure 3 (a, b, and c, respectively). It shows clearly the differences between the specialized dairy (Holstein and Brown Swiss) and dual-purpose breeds (Simmental and Alpine Grey) for all the interval fertility traits examined. With respect to iCF, at 100d from calving only 59% of the Holstein and 62% of the Brown Swiss cows were inseminated, against 73% of Alpine Greys and 75% of Simmentals. This could indicate a shorter puerperium, earlier heat detection, and/or a shorter voluntary waiting period for dual-purpose than for specialized dairy breeds (Pryce et al., 2004; Malchiodi et al., 2014).

The Holstein and Brown Swiss also differed from the Simmental and Alpine Grey in iFC. The risk of becoming pregnant 21d after first insemination was 41% for Holstein cows and 42% for Brown Swiss, versus 51% for Simmentals and 53% for Alpine Greys. There is an increment in risk approximately every 21d, corresponding to the natural estrus cycles.

Regarding DO, at 116d after calving the Holstein cows had a 44% risk and the Brown Swiss a 48% risk of becoming pregnant, compared with 63% for Simmental and 64% for Alpine Grey cows. Malchiodi et al. (2014) reported Kaplan-Meier curves showing Holsteins as having a 49% risk of becoming pregnant at 100d, similar to our results for the same period. Vargas et al. (1998) observed a difference in the survival curves for DO after 100d between Holstein and Jersey primiparous cows with different milk yields; cows with the lowest milk yields had the lowest risk.

Since the differences among the four breeds examined in the present study due to milk productivity level and/or environmental conditions cannot be ascertained from Kaplan-Meyer curves, these factors will be analyzed in greater detail later. It is worth noting that, although Brown Swiss and Simmental cows have very similar milk production levels, their reproductive performances differ; the Brown Swiss are more similar to Holsteins (despite the latter having greater milk production), and Simmentals are more similar to Alpine Greys (despite the latter having lower milk production).

Hazard Ratios for iCF, iFC, and DO

As we observed an interaction between breed and class of productivity for all traits (at both Herd-L and Cow-L), HRs and their confidence intervals for the interval traits (iFC, iCF, and DO) of each breed and productivity class at Herd-L and Cow-L were estimated and are presented in Figure 4. The HRs are plotted against the average daily milk yields of the corresponding breed at Herd-L and Cow-L. This representation allows us to compare breeds while simultaneously taking into account their different levels of production. In each figure, the reference value (HR = 1.00) is the central class of milk productivity of Holsteins at the herd level (HerdL-3) and the cow-within-herd level (CowL-C).

A first result to be noted is that the different breeds have different HR estimates for all the interval fertility traits studied, with a few exceptions. Moreover, the various productivity classes (both Herd-L and Cow-L) affect the interval reproduction traits, and this effect also differs according to breed (effect of the breed-productivity interaction).

Looking, firstly at the iCF of Holsteins, we observed that both Herd-L and Cow-L moderately affected the interval between calving and first insemination almost linearly, but with opposite signs. In fact, an increase in herd productivity had a favorable effect on this reproductive trait (i.e., it increased the risk of a given calving-insemination interval), whereas an increase in the milk yield of an individual cow negatively affected its reproduction rate. A negative energy balance at the beginning of lactation, which is directly related to a high nutrient demand in order to produce milk, conflicts with the expression of estrous behavior, and is more evident in cows with a higher milk yield (Harrison et al., 1990).

With regards to the Brown Swiss, which is other specialized dairy breed, we found that, on average, the HRs at both Herd-L and Cow-L were greater than the corresponding values for the Holstein breed. Comparing the central classes, at HerdL-3 and CowL-C the Brown Swiss were 1.09 and 1.15 times, respectively, more likely of being inseminated at a given time from calving. The effects of productivity class were also slightly different in the two breeds. With respect to Herd-L, we found the favorable effect of productivity in Brown Swiss herds to be more than double that of the Holstein herds. It can also be noted that the pattern was curvilinear as there was a large improvement in the trait moving from the lowest to the central Herd-L, and a smaller improvement moving from the central to the highest Herd-L. With respect to Cow-L, the effect of increasing the productivity of Brown Swiss cows was also curvilinear, but with the opposite sign. Like the Holsteins, the trait worsened moving from low- to mid-producing cows, whereas there was a much smaller change (improvement) moving from mid- to high-producing cows. The authors are unaware of any scientific literature regarding the effects of herd and individual productivity on the fertility traits of Brown Swiss cows.

On average, both dual-purpose breeds had a much higher risk of being inseminated at a given time from calving (i.e., of having a shorter calving-first insemination interval). Comparison of the central classes shows that compared with Holsteins the Simmentals and Alpine Greys were more likely to being inseminated at a given time from calving 1.54 and 1.45 time , respectively, at Herd-L, and 1.66 and 1.58 times, respectively, at Cow-L. Both breeds exhibited a large effect of Herd-L and a moderate effect of Cow-L. The higher the

Herd-L the shorter the iCF, with the exception of the highest Herd-L of the Simmental herds (quadratic response). At Cow-L, both breeds exhibited a slightly curvilinear pattern, although it should be noted that the confidence intervals of the HR estimates in these cases were rather high.

Moving on to the iFC, we noted a small curvilinear effect of milk productivity at both Herd-L and Cow-L, although with opposite signs (Alpine Greys excluded). Moreover, the two dual-purpose breeds had a greater risk of being pregnant at a given interval from first insemination than the specialized dairy breeds at every production level, and the two dairy breeds overlap, as do the two dual-purpose breeds.

The DO is the sum of iCF and iFC, and here the dual-purpose breeds had even greater average HR values than the specialized breeds at every production level. The pattern of productivity effects, at both Herd-L and Cow-L, is influenced more by iCF than by iFC traits. Overall, the Holstein Herd-L had a minor effect on the DO HR, while Cow-L tended to have a negative effect going from the lowest CowL-A (HR=1.16) to the central CowL-C (Reference Value, HR = 1.00), although this negative effect was not evident at the highest CowL-E (HR = 1.04). The pattern of productivity effects on the DO of Brown Swiss cows was similar to that of Holsteins but more accentuated, while the average HR values were slightly higher. The Herd-L HRs values of the Brown Swiss ranged from 0.89 (HerdL-1) to 1.14 (HerdL-5), while the Cow-L values ranged from 1.41 (CowL-A) to 1.14 (CowL-E). We found a much greater effect of milk productivity on the dual-purpose breeds. The effect was positive at Herd-L (1.52 to + 1.86 for Alpine Grey, and 1.36 to 1.70 for Simmental) and negative at Cow-L (1.98 to 1.76 for Alpine Grey, and 2.03 to 1.70 for Simmental), especially moving from the low- to mid-production levels. Vargas et al., (1998) reported differences between Jersey and Brown Swiss crosses for DO, with hazard ratios of 1.52 and 1.42 respectively, compared with Holsteins. They also described non-linear effect of milk yield on days open with hazard ratios from 0.78 (low milk yield) to 0.92 (high milk yield), compared to intermediate milk yield (HR = 1.00).

Odds Ratios for PRF, NRR and INS

The OR estimates and their confidence intervals for success traits (NRR, PRF, and INS) at Herd-L and Cow-L for the various breeds are presented in Figure 5. Unlike the reproductive interval traits, there was a much greater overlap among the different breeds with respect to NRR at 56 d after first insemination at both Herd-L and at Cow-L. The differences among the average ORs of the breeds seem, therefore, to depend more on differences in the average milk yield than on differences in fertility at the same milk production level. The second general observation, common to all three success traits, is that the sign of the effect of productivity on fertility is roughly the same at both Herd-L and Cow-L (except with Holsteins). There appears to be a clear negative effect of increased production on NRR at both Herd-L and Cow-L up to a milk yield of about 25 kg/d, while thereafter the effect is not so clear. This explains why productivity effects tend to be significant in dual-purpose breeds but not in dairy breeds. In interpreting these results, it must be taken into account that the first insemination occurs on average at a shorter iCF in dual-purpose breeds than in dairy breeds. Our results are not consistent with the study carried out by LeBlanc, (2010), who found a positive association between pregnancy rates and high-producing cows and herds, although the environmental conditions, management systems and herd sizes differed in the two studies.

In the case of the ORs for PRF, the main finding is that at Herd-L the dual-purpose breeds are clearly more fertile than the dairy breeds at every production level. The difference between the ORs of NRR and PRF in the two groups of breeds is explained by the difference between the average NRR and PRF of the breeds. As seen in Table 2, PRF is always lower than NRR: by 17% in Holstein and Brown Swiss cows, by 12% in Simmentals, and by 10% in

Alpine Greys; i.e., it was more often the case that pregnancy status was not confirmed with subsequent parturition, as predicted by their non-return in estrus within 56d of insemination, in dairy cows than in dual-purpose cows. This could be due to different incidences of estrus detection, abortions, or the culling or selling of cows. With respect to PRF, the dairy breeds did not seem to be much affected by productivity at Herd-L, while there was a curvilinear effect with dual-purpose breeds. The negative effect of productivity is evident in all breeds at Cow-L (at least till 25 kg/d), but there is a greater overlap in the breed estimates and only Simmental tended to be more fertile than the other breeds.

Regarding INS, the two dual-purpose breeds had a much greater risk of undergoing fewer inseminations per conception than the dairy breeds, and the effect of productivity was lower than other traits, especially for dairy breeds at Herd-L.

CONCLUSIONS

Our results show that there are important differences among the breeds studied with respect to interval and success fertility traits. It is also clear that reproductive traits are greatly affected by level of milk production, and that this is the case for herds with different milk production levels and for cows with different milk yields in similar production environments. The effects of common (herd) and individual (cow) production environments are clearly different from each other and also differ according to breed. The dual-purpose breeds (Alpine Grey and Simmental) have a greater reproductive potential than the dairy breeds (Holstein and Brown Swiss), a difference that is only partly due to different production levels. These results indicate that exists a tendency to improve the reproductive intervals and to decrease the success fertility rates from lower production herds to higher production herds, at least up to a milk yield of about 25 kg/d. Bearing in mind that the survival analysis used in the present study also took into account censored data and their different proportions in different breeds/productivity classes, DO may be considered an overall indicator of fertility. The DO, in fact, depends on the interval to first insemination, success of first insemination, number of inseminations, and interval from first insemination to conception. This trait clearly shows that herd productivity has an opposite effect to individual productivity. A better production environment could lead to better overall fertility responses, while an increase in the milk yield of individual cows within a herd leads to worsening fertility. These associations between fertility and milk production levels are non-linear at both Herd-L and Cow-L, but are more evident moving from low to medium milk yields than moving from medium to high milk yields, and they, therefore, affect the dual-purpose more than the dairy breeds, particularly the Holsteins.

Within breed, disentangling the effects of milk productivity on fertility at the herd and the cow levels, and taking non-linearity of response into account could contribute to improving the design of population modeling, helping in management purposes e.g. in precision dairy farming of fertility in dairy and dual-purpose cattle.

A better understanding of the fertility rates of different breeds, and their different responses to herd and individual productivity levels could provide a useful basis for designing more profitable crossbreeding programs in different dairy systems.

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TABLES

Table 1. Descriptive statistics for interval from calving to first service (iCF), interval from first service to conception (iFC) and days open (DO)

			Uncensored records		Censored Records	
Trait	n	Censored, %	Mean	SD	Mean	SD
iCF, d						
Holstein	17,688	29.9	93.7	40.8	114.7	58.2
Brown Swiss	32,697	29.1	87.3	37.7	107.0	55.8
Simmental	27,791	23.4	77.5	32.3	92.4	46.8
Alpine Grey	13,689	22.3	80.6	31.7	92.8	46.9
iFC, d						
Holstein	17,688	28.6	40.4	63.7	81.0	100.3
Brown Swiss	32,697	28.3	38.3	62.6	79.4	102.0
Simmental	27,791	23.1	25.0	47.7	52.7	78.4
Alpine Grey	13,689	22.0	22.5	46.2	48.9	77.2
DO, d						
Holstein	17,688	29.2	135.5	72.8	193.8	103.7
Brown Swiss	32,697	28.6	126.6	71.5	183.6	103.6
Simmental	27,791	23.2	102.9	57.3	145.2	87.6
Alpine Grey	13,689	22.1	103.5	55.3	141.7	96.9

Table 2. Number of records and percentages of success events for pregnancy rate at first service (PRF) and non-return rate at 56 days (NRR)

Trait	n	%
NRR		
Holstein	17,688	65
Brown Swiss	32,697	66
Simmental	27,791	70
Alpine Grey	13,689	71
PRF		
Holstein	17,688	48
Brown Swiss	32,697	49
Simmental	27,791	58
Alpine Grey	13,689	61

Table 3. Descriptive statistics for number of inseminations to conception (INS)

Trait	n	Mean	SD
INS			
Holstein	17,688	2.23	1.30
Brown Swiss	32,697	2.19	1.28
Simmental	27,791	1.89	1.10
Alpine Grey	13,689	1.83	1.07

FIGURES

Figure 1. Number of herds in each Herd-Level class (HerdL-1, HerdL-2, HerdL-3, HerdL-4, HerdL-5) and number of cows in each Cow-Level class (CowL-A, CowL-B, CowL-C, CowL-D, CowL-E) according to their solutions for milk production.

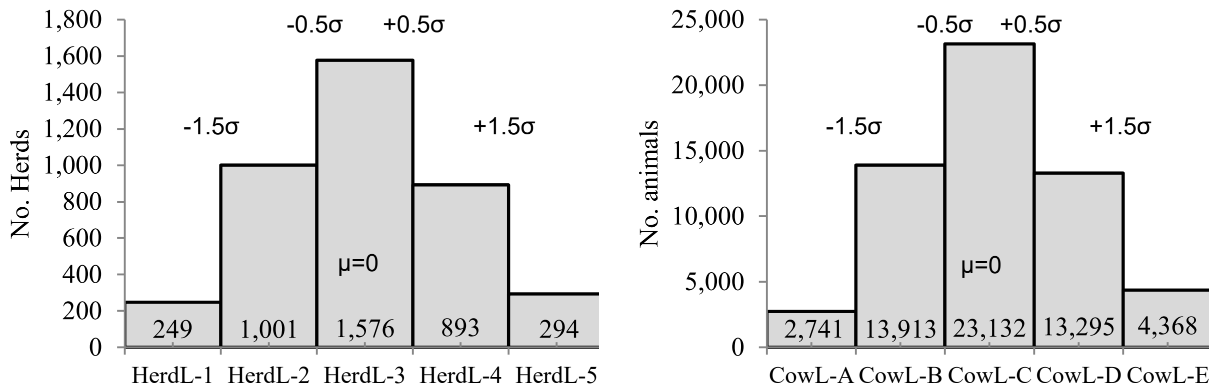


Figure 2. Means for the milk yield of cows of the four breeds at the Herd Level and Cow Level.

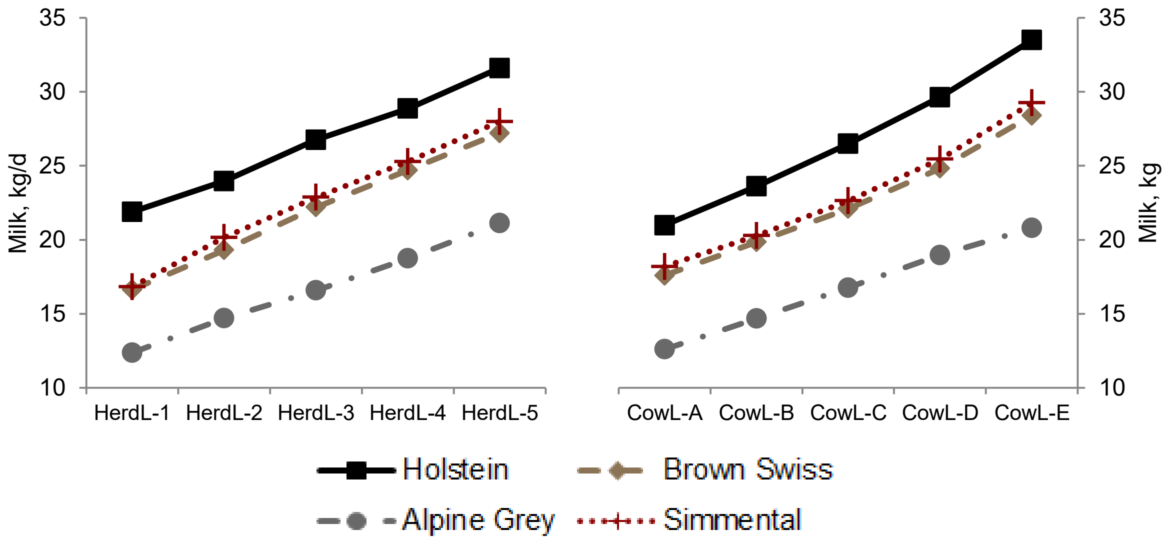


Figure 3. Kaplan-Meier estimates of the survival function for: a) interval from calving to first service (iCF); b) interval from first service to conception (iFC); c) number of days open (DO).

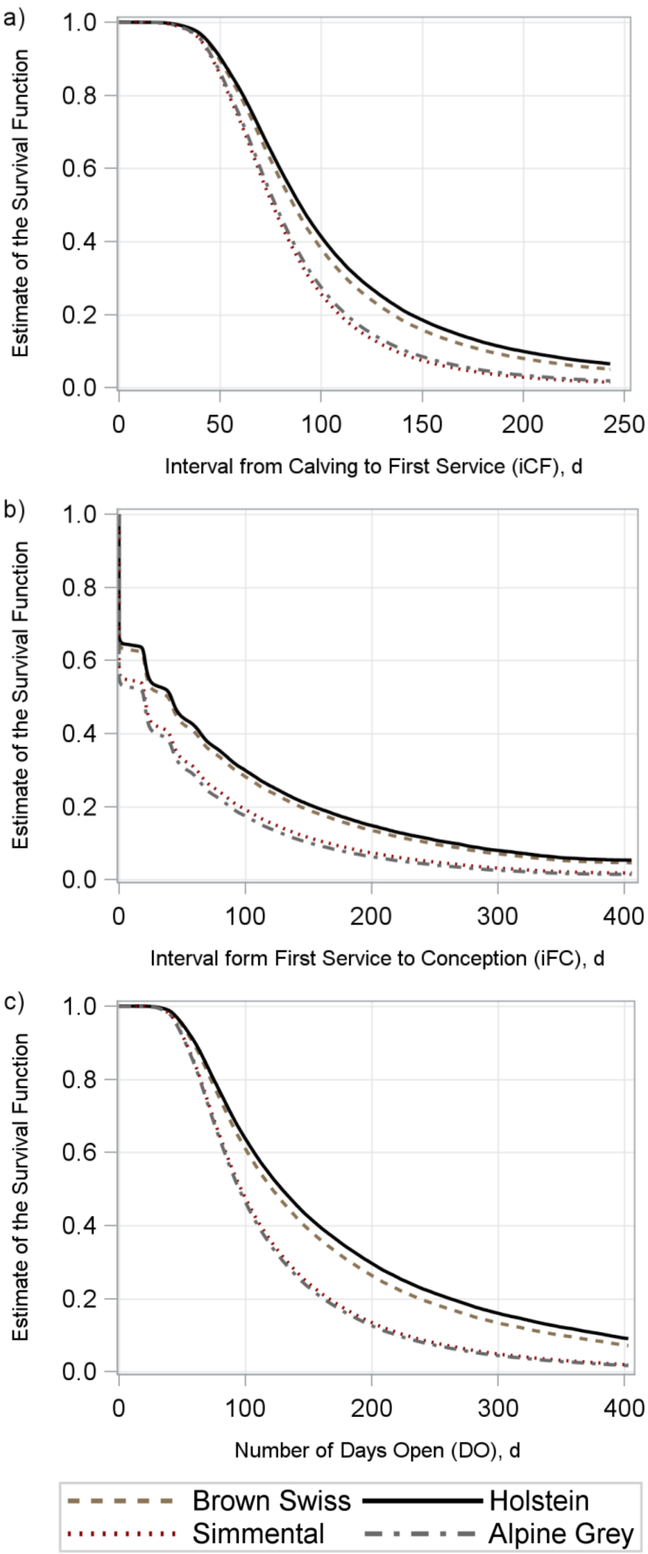


Figure 4. Hazards ratios estimates and their confidence intervals at different levels of milk production for the interval from calving to first service (iCF), the interval from first service to conception (iFC) and number of days open (DO), at the Herd Level and Cow Level. rv = reference value

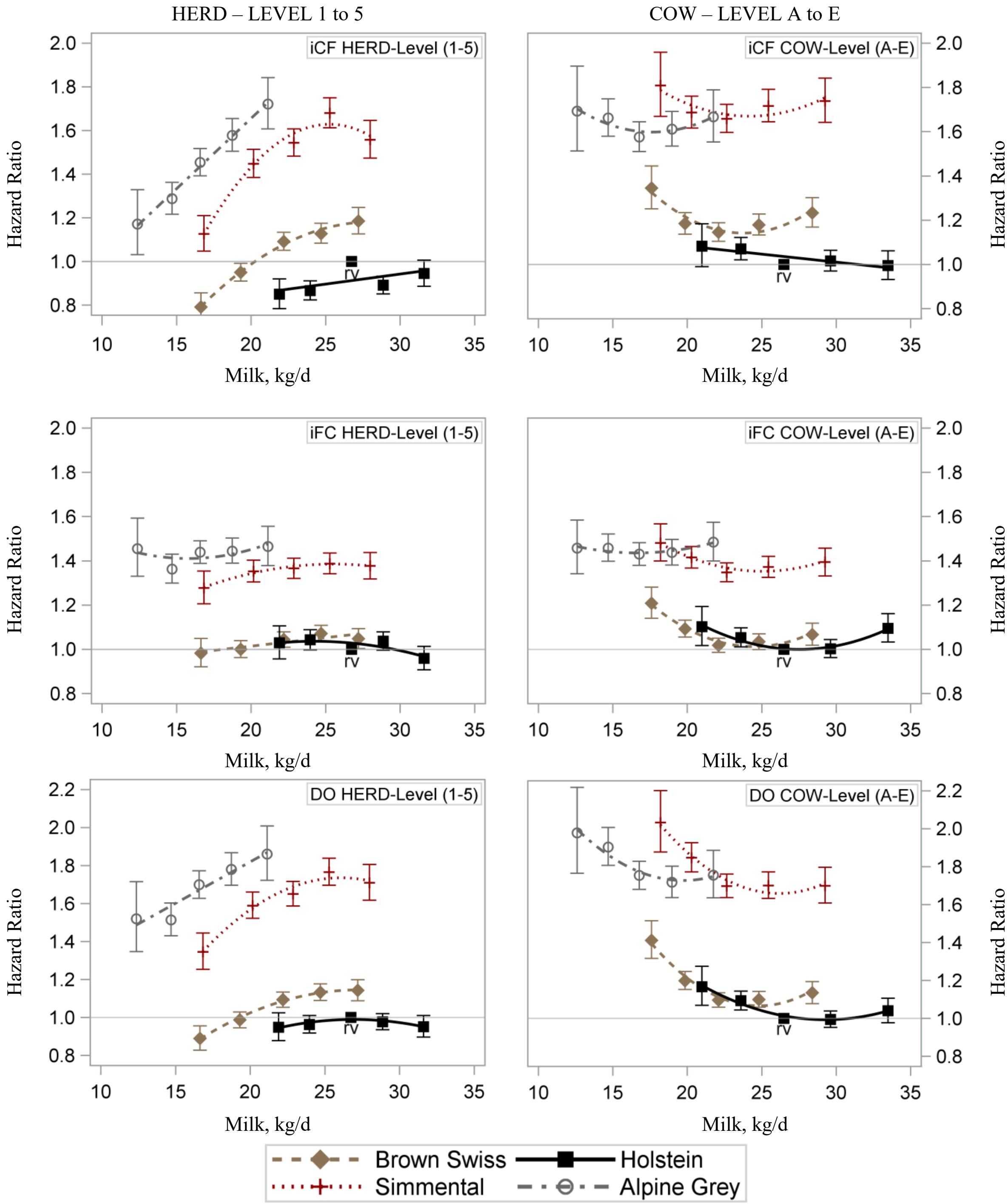
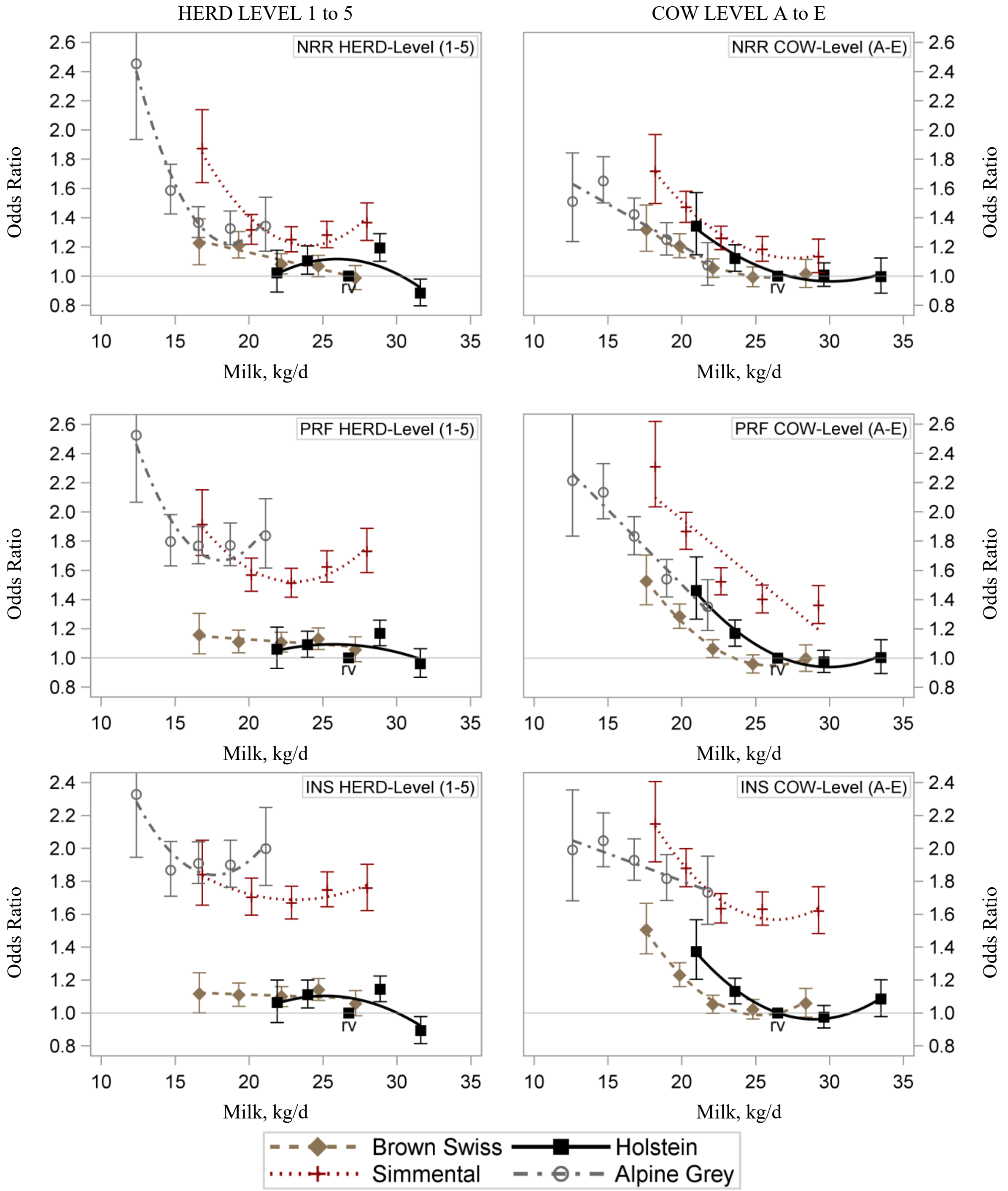


Figure 5. Odds ratios estimates and their confidence intervals for the pregnancy rate at first service (PRF), non-return rate after 56 days (NRR) and number of inseminations to conception (INS), at the Herd Level and Cow Level. rv = reference value



REFERENCES

Bello, N.M., J.S. Stevenson, and R.J. Tempelman. 2012. Invited review: milk production and reproductive performance: modern interdisciplinary insights into an enduring axiom.. J. Dairy Sci. 95:5461–75. doi:10.3168/jds.2012-5564.

Cox, D.R. 1972. Regression Models and Life-Tables. J. R. Stat. Soc. Ser. B 34:187–220.

Egger-Danner, C., J.B. Cole, J.E. Pryce, N. Gengler, B. Heringstad, A. Bradley, and K.F. Stock. 2015. Invited review : overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. Animal 9:191–207. doi:10.1017/S1751731114002614.

Harrison, R.O., S.P. Ford, J.W. Young, A.J. Conley, and A.E. Freeman. 1990. Increased Milk Production Versus Reproductive and Energy Status of High Producing Dairy Cows. J. Dairy Sci. 73:2749–2758. doi:10.3168/jds.S0022-0302(90)78960-6.

Heins, B.J., L.B. Hansen, and A.J. Seykora. 2006. Fertility and survival of pure Holsteins versus crossbreds of Holstein with Normande, Montbeliarde, and Scandinavian Red.. J. Dairy Sci. 89:4944–51. doi:10.3168/jds.S0022-0302(06)72545-0.

Huang, C., I. Misztal, S. Tsuruta, and T.J. Lawlor. 2007. Methodology of Evaluation for Female Fertility. Interbull Bull. 156–160.

LeBlanc, S. 2010. Assessing the association of the level of milk production with reproductive performance in dairy cattle. J. Reprod. Dev. 56 Suppl:S1–S7. doi:10.1262/jrd.1056S01.

López-Gatius, F. 2012. Factors of a noninfectious nature affecting fertility after artificial insemination in lactating dairy cows. A review. Theriogenology 77:1029–1041. doi:10.1016/j.theriogenology.2011.10.014.

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: where will it end?. J. Dairy Sci. 84:1277–93. doi:10.3168/jds.S0022-0302(01)70158-0.

Malchiodi, F., a. Cecchinato, and G. Bittante. 2014. Fertility traits of purebred Holsteins and 2- and 3-breed crossbred heifers and cows obtained from Swedish Red, Montbéliarde, and Brown Swiss sires. J. Dairy Sci. 97:7916–7926. doi:10.3168/jds.2014-8156.

Nilforooshan, M.A., J.H. Jakobsen, F.W. Fikse, B. Berglund, and H. Jorjani. 2009. International genetic evaluations of fertility traits using multi-trait MACE. Pages 99–102 in Interbull Technical Workshop on Genomic Information in Genetic Evaluations, Uppsala, January 27 - 28.

Norman, H.D., J.R. Wright, S.M. Hubbard, R.H. Miller, and J.L. Hutchison. 2009. Reproductive status of Holstein and Jersey cows in the United States. J. Dairy Sci. 92:3517–28. doi:10.3168/jds.2008-1768.

Phuong, H.N., P. Blavy, O. Martin, P. Schmidely, and N.C. Friggens. 2015. Modelling impacts of performance on the probability of reproducing, and thereby on productive lifespan, allow prediction of lifetime efficiency in dairy cows. Animal 1–11. doi:10.1017/S1751731115001718.

Pryce, J.E., M.D. Royal, P.C. Garnsworthy, and I.L. Mao. 2004. Fertility in the high-producing dairy cow. Livest. Prod. Sci. 86:125–135. doi:10.1016/S0301-6226(03)00145-3.

SAS Institute. 2012. SAS/STAT Software. Release 9.4. SAS Institute inc., Cary, NC.

Schneider, M. del P., E. Strandberg, V. Ducrocq, and A. Roth. 2005. Survival Analysis Applied to Genetic Evaluation for Female Fertility in Dairy Cattle. J. Dairy Sci. 88:2253–2259. doi:10.3168/jds.S0022-0302(05)72901-5.

Stocco, G., C. Cipolat-Gotet, T. Bobbo, A. Cecchinato, and G. Bittante. 2016. Breed of cow and herd productivity affect milk composition and modeling of coagulation, curd firming and syneresis. J. Dairy Sci. 129–145. doi:10.3168/jds.2016-11662.

Sturaro, E., E. Marchiori, G. Cocca, M. Penasa, M. Ramanzin, and G. Bittante. 2013. Dairy systems in mountainous areas: Farm animal biodiversity, milk production and destination, and land use. Livest. Sci. 158:157–168. doi:10.1016/j.livsci.2013.09.011.

Tiezzi, F., C. Maltecca, A. Cecchinato, M. Penasa, and G. Bittante. 2012. Genetic parameters for fertility of dairy heifers and cows at different parities and relationships with production traits in first lactation. J. Dairy Sci. 95:7355–62. doi:10.3168/jds.2012-5775.

Tiezzi, F., C. Maltecca, A. Cecchinato, M. Penasa, and G. Bittante. 2013. Thin and fat cows, and the nonlinear genetic relationship between body condition score and fertility. J. Dairy Sci. 96:6730–41. doi:10.3168/jds.2013-6863.

Tiezzi, F., C. Maltecca, M. Penasa, A. Cecchinato, Y.M. Chang, and G. Bittante. 2011. Genetic analysis of fertility in the Italian Brown Swiss population using different models and trait definitions. J. Dairy Sci. 94:6162–72. doi:10.3168/jds.2011-4661.

VanRaden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn, and G.R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285–92. doi:10.3168/jds.S0022-0302(04)70049-1.

Vargas, B., T. Van Der Lende, M. Baaijen, and J.A. Van Arendonk. 1998. Event-time analysis of reproductive traits of dairy heifers. J. Dairy Sci. 81:2881–9. doi:10.3168/jds.S0022-0302(98)75848-5.

Walsh, S.W., E.J. Williams, and A.C.O. Evans. 2011. A review of the causes of poor fertility in high milk producing dairy cows. Anim. Reprod. Sci. 123:127–38. doi:10.1016/j.anireprosci.2010.12.001.

Weigel, K.A., and K.A. Barlass. 2003. Results of a producer survey regarding crossbreeding on US dairy farms. J. Dairy Sci. 86:4148–54. doi:10.3168/jds.S0022-0302(03)74029-6.

CHAPTER III

ESTROUS CYCLE AND MILK COMPOSITION ON DAIRY CATTLE

Changes in milk characteristics and fatty acid profile during the estrous cycle in dairy cows

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INTERPRETIVE SUMMARY

Changes in milk characteristics and fatty acid profile during the estrous cycle in dairy cows. *By Toledo-Alvarado et al.*

We assess the effects of various estrous phases on milk yield, composition, physical traits, and fatty acid composition in dairy cows. Fat and lactose increased on estrous phase while protein parallel decreased. The C14:0 and C16:0 decreased on estrus phase with an analogous increment of the C18:0 and C18:1c9. In consequence, the fatty acid categories showed a similar behavior. The urea, SCS, freezing point, pH and homogenization index also varied on estrus indicating important differences occasioned by the hormonal and behavioral changes of cows in standing estrus.

ABSTRACT

The relationship of the estrous cycle to milk composition and milk physical properties was assessed using data on Holstein (10,696), Brown Swiss (20,501), Simmental (17,837), and Alpine Grey (8,595) cows reared in the north-eastern Italy. The first insemination after calving was selected for each cow and it was considered the day of estrus. Test days around the insemination were selected in the range from -10 to +10 d relative to the day of estrus and used to create 5 estrus phase categories: Diestrus high-progesterone (Diestrus-HP) from -10 to -4 d; Proestrus from -3 to -1 d; Estrus day 0; Metestrus from 1 to 2 d; and Diestrus increasing-progesterone (Diestrus-IP) from 3 to 10 d. Milk yield, milk components and milk physical properties were analyzed using a mixed model which included the random effect of the herd and the fixed effects of: year-month, parity number, linear and quadratic DIM nested in parity number, breed, pregnancy status, estrous phase, day nested in the estrous phases, and the interactions between; pregnancy status with estrous phase, and breed with estrous phase. Contrasts between breeds and estrus phases were performed. Milk composition, particularly fat, protein and lactose, showed high variability among the estrous phases. Fat increased 0.14% from diestrus HP to estrous phase while protein parallel decreased -0.03%. The lactose presented a constant value over the diestrus-HP and rising one day before the estrus following a gradual reduction over the next estrous phases. Fatty acids were also affected across the estrous phases: the C14:0 and C16:0 decreased (-0.34% and -0.48%) from proestrus to estrus phase with an analogous increment of the C18:0 and C18:1c9 (0.40% and 0.73%). In consequence, the fatty acid categories showed a similar behavior: the UFA, MUFA, PUFA, TFA, and LCFA increased in the estrous phase while the SFA, MCFA, and SCFA decreased. Finally, the urea, SCS, freezing point, pH and homogenization index were also affected indicating important differences occasioned by the hormonal and behavioral changes of cows in standing estrus. The variations on milk profiles of cows showing estrous should be taken into account on breeding programs and could be useful in precision dairy farming. Also, these milk composition variations could be used to identify of cows in estrus.

Key words: mammary gland activity, *de novo* fat synthesis, heat detection, milk quality, saturated fatty acids.

INTRODUCTION

The estrous cycle in dairy cattle has been widely studied, mainly for its importance for reproductive performance in dairy cattle. Opportune heat detection and the correct insemination timing and techniques are fundamental to have a good reproductive management program (Kaproth and Foote, 2011; Nebel et al., 2011). The negative correlation between milk production and fertility (Lucy, 2001; Pryce et al., 2004) has caused modifications in breeding programs, together with the inclusion of fertility traits into their genetic evaluations (VanRaden et al., 2004; Huang et al., 2007). In addition, the genomic selection has led to a positive genetic gain in pregnancy rates in the last decade, at least in North America (García-Ruiz et al., 2016). However, reproductive improvement in dairy cattle continues to be a priority with estrus detection being a particular concern (Roelofs et al., 2010). The goal of a good heat detection program should be to identify estrus positively and accurately, differentiating between cycling cows and the cows with irregular cycles (Nebel et al., 2011). Estrus is usually detected by behavioral signs, such as "standing" to be mounted; however, several innovative tools have been developed to detect estrus such as automated systems like neck-mounted collars to detect physical activity, pedometers, pressure sensing devices and tail temperature detectors. Nevertheless, these technologies require potentially burdensome investment in management and equipment (Roelofs et al., 2015; Miura et al., 2017).

Some studies have indicated a reduction of milk yield during the day of standing estrus (Lopez et al., 2004, 2005; Akdag et al., 2010). However, studies on the variation of milk yield and characteristics in relation to the phase of the estrous cycle have been scarce and variable in results, and often very old. For example, some studies have reported a positive effect of fat (Copeland, 1929; Erb et al., 1952), or a reduction in protein content (King, 1977) on estrus day while other researchers have not detected any such effects in Holsteins and Jerseys (Cowan and Larson, 1979; Akdag et al., 2010). Horrell et al., (1985) described a small increase in lactose contents in Holsteins during estrus whereas Akdag et al., (2010) did not find such effects in Jerseys. Some studies have reported no changes in somatic cell count (SCC) for cows showing estrus (Anderson et al., 1983; Horrell et al., 1985), whereas others have inferred an increase in SCC during estrus (King, 1977). In other dairy species, increases in SCC have been found for Nili-Ravi buffaloes in proestrus/estrus phase compared to metestrus and diestrus stages (Akhtar et al., 2008), whereas increases SCC at estrus have been determined in Saanen dairy goats and associated with increases in plasma estradiol (Moroni et al., 2007).

As the scientific information is scarce, contradictory, and often originating from dairy populations not representative of modern dairy breeds and farming conditions, a better understanding of the associations of milk characteristics with the phases of the estrous cycle is needed. Moreover, this study could improve our knowledge on mammary gland functions and lead to new on-farm indicators of reproductive changes of the cow. Therefore, the aim of this study was to investigate the effects of various estrous phases on milk yield, composition, physical traits, and fatty acid composition in Holsteins, Brown Swiss, Simmental and Alpine Grey cows.

MATERIALS AND METHODS

Data and Editing

Data used were collected between January 2011 and December 2016 during the milk recording of dairy cows by the Breeders Federation of Alto Adige/Südtirol (Associazione Provinciale delle Organizzazioni Zootecniche Altoatesine / Vereinigung der Südtiroler Tierzuchtverbände, Bolzano/Bozen, Italy) from the northeastern region of Bolzano/Bozen province in Italy. We extracted a total of 85,329 test-day (**TD**) records related to inseminations of 20,501 Brown Swiss, 10,696 Holsteins, 17,837 Simmentals and 8,595 Alpine Grey cows distributed across 4,071 herds. Parity numbers were grouped into 1^{st} (n = 25,820), 2^{nd} (n = 20,358), 3^{rd} (n = 15,114) and ≥ 4^{th} (n = 24,037). The TD retained ranged from 30 to 200 DIM. Pregnancy length was required to be within 30 d of the average for each breed and the calving interval was required to be within 300 to 700 d.

Milk Characteristics

Milk data included TD production, and characteristics routinely obtained during milk recording by the laboratory of the Federazione Latterie Alto Adige/Sennereiverband Südtirol (Bolzano/Bozen). The SCC were analyzed using a Fossomatic^{TM} (Foss Electric, Hillerød, Denmark) and logarithmically transformed to SCS. All the other milk characteristics were predicted on the basis of Fourier-Transform Infrared (FTIR) spectra. The milk samples were analyzed by a MilkoScan^{TM} (Foss Electric, Hillerød, Denmark) using the calibration equations pre-installed by the company. All the operations respected the methods and conditions of ICAR. The milk components analyzed were lactose, fat, protein, casein, and urea. The fat/protein (**F:P**) ratio was also calculated. The milk physical traits were: the Freezing Point Depression (**FPD**)(Arnvidarson et al., 1998) expressed in 10^{-2} ºC, and the Homogenization

Index (**HI**) reflecting the fat globule size (Sjaunja et al., 1994). The main fatty acids analyzed were: miristic acid (14:0), palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1cis9). The fatty acid categories studied were: Free Fatty Acids (**FFA**), SFA, MUFA, PUFA, UFA, Short-Chain Fatty Acids (**SCFA**), Medium-Chain Fatty Acids (**MCFA**), Long-Chain Fatty Acids (**LCFA**) and Trans Fatty Acids (**TFA**). For all milk traits, only data within the range of $\bar{x} \pm 5sd$ for each trait were kept.

Estrous Cycle Definition

All insemination dates were available as well as the calving date for each cow. The first insemination or service after calving was selected for each cow and was considered the day when the cow was in estrus. The interval between calving and first service was on average 85.3 ± 33 d. The TDs close to the first service dates were selected in the range from -10 to +10 d with the day of estrus as day zero. With this reference, we created 5 categories: Diestrus high-progesterone (**Diestrus-HP**) from -10 to -4 d (n = 27,574); Proestrus from -3 to -1 d (n = 12,302); Estrus day 0 (n = 4,144); Metestrus from 1 to 2 d (n = 8,275); and Diestrus increasing-progesterone (**Diestrus-IP**) from 3 to 10 d (n = 33,052). We use Figure 1 to further clarify the relative importance of key hormones during the various estrous phases as we defined above. Conception at first service was confirmed if the cow had not been serviced with a second insemination within 90 d after first service (n = 47,606) with a subsequent calving; otherwise, the cow was deemed to be nonpregnant (n = 37,723) (ICAR, 2016).

Statistical Analysis

A univariate mixed effects model was used for the analysis of milk yield, milk components, other traits, and individual fatty acids and fatty acid categories, for a total of 24 response variables. Data were analyzed using PROC MIXED of SAS (SAS Institute, 2012). For each trait the model was defined as follows:

$$y_{ijklmnrt} = \mu + YM_i + parity_j + breed_k + conception_l + estrous_m + day_n(estrous)_m + \beta_{1j}(dim_t) + \beta_{1j}(dim_t^2) + estrous_m \times conception_l + estrous_m \times breed_k + herd_r + \varepsilon_{ijklmnrt},$$

where $y_{ijklmnrt}$ is the trait (milk, lactose, fat, protein, casein, F:P, urea, SCS, pH, FPD, HI, C14:0, C14:16, C18:0, C18:1c9, SFA, UFA, MUFA, PUFA, TFA, SCFA, MCFA, LCFA, FFA); μ is the general mean; YM_i is the year-month date of the TD t (i = 1 to 72); $parity_j$ is the parity number of the cow (j = 1,2,3,4); $breed_k$ is the breed of the cow (k = Brown Swiss, Holstein, Simmental, Alpine Grey); $conception_l$ is the pregnancy success for the first insemination (l = 0, 1); $estrous_m$ is the estrous phase of the cow (m = Diestrus-HP, Proestrus, Estrus, Metestrus, Diestrus-IP); $day_n(estrous)_m$ is the distance of the TD in days relative to estrus nested within the estrous phase (n = -10 to 10); dim_t is the number of days in milk (DIM) at the TD t; $\beta_{1j}$and β_{2j} are the regression coefficients on linear and quadratic DIM nested within corresponding parity; $estrous_m \times conception_l$ is the interaction between the estrous phase and the conception success; $estrous_m \times breed_k$ is the interaction between the estrous phase and the breed of the cow; $herd_r$ is the random effect of the herd (r = 4,071) $\left(NID \sim (0, \sigma_h^2)\right)$; $\varepsilon_{ijklmnrt}$ is the random experiment error $\left(NID \sim (0, \sigma_e^2)\right)$. Contrasts were estimated between least squares means (**LSM**) of each trait for the effect of (1) estrous: (a) Estrus vs. Diestrus-HP, (b) Estrus vs. Proestrus, (c) Estrus vs. Metestrus, and (d) Estrus vs. Diestrus-IP; and (2) breed: (a) Brown Swiss and Holstein vs. Simmental and Alpine Grey, (b) Brown Swiss vs. Holstein, and (c) Simmental vs. Alpine Grey.

The results relative to the effects of year-season and DIM were similar to those found in literature so they are not presented in tables or discussed

RESULTS AND DISCUSSION

Descriptive Statistics and Main Causes of Variation of Milk Characteristics

Descriptive statistics for milk yield, milk components, other milk traits, and the fatty acid profile are reported in Table 1. The overall average of milk yield was 26.87 kg/d with a coefficient of variation (**CV**) of 26%, result of the differences among production systems and breeds. All milk components showed a small to medium variability. Fat showed more variability (CV = 18%) than protein and casein (CV = 9%), while lactose presented minor variability (CV = 3%). Urea averaged 20.92 ± 7.56 mg/100g (CV= 36%), and ranged from 4.90 to 41.20 mg/100g, thereby indicating the high variability of the feeding systems of the breeds, particularly with respect to the amount of crude protein in the diet. The SCS showed also medium variability (CV = 33%) pointing also the intrinsic variability of all the factors involved in the milking process. On the other hand, the pH (CV = 0.9) and the FPD (CV = 1.5) both used normally as quality indicator traits for freshness and adulteration of the milk, showed very small variability since there is a finely tuned system to ensure the quality of the milk. The HI given by the FOSS spectrometers is the degree of homogenization of the fat, and/or determines the homogenization index of the sample; in this context the HI indicates the mean diameter, calculated by volume, of the fat globules with the limits from 0 (bigger size of fat globules) to 1 (smaller size of the fat globules) (Sjaunja et al., 1994). The average HI was 0.67 (CV = 13%) with values within the range of 0.49 to 0.93. The mean values of the fat composition in terms of major FA and FA categories were similar to other publications (Månsson, 2008; Rutten et al., 2009; Gottardo et al., 2017). The CV for the fatty acids ranged from 9 to 17% while the variability of the fatty acids categories ranged from 5 to 27%, showing similar variability in comparison with other studies in the same region of Italy for the same breeds (Gottardo et al., 2017). The FFAs showed a high CV (54%).

The results of the statistical analysis of the 24 milk characteristics considered are summarized in Table 2. This large database allowed us to quantify the contemporary effect of several sources of variation such as breed, parity and days in milk of cows, the phase of the estrous cycle, the day within estrus cycle, and the subsequent pregnancy, and also the year-month and the herd of cows. Moreover, the main interactions have been included in the statistical model. The majority of these factors of variation and of their interactions were significant for all or large part of the traits analyzed. Many previous studies assessed some of these factors of variation, but none, we are aware of, studied contemporarily all these factors, so that direct comparisons are not possible.

The estrous phase and the day nested in the estrous phases influenced all the traits included in the study (Table 2). The conception outcome was significantly different for milk yield and almost all the principal components of milk, with the exception of fat. For the rest of the traits, the effect of conception outcome had variable results, important for SCS, TFA, MCFA and FFA, and not significant for the individual fatty acids and for the rest of the fatty acid categories. The interaction between estrous × conception outcome did not have an important effect for the majority of the traits (Table 2), with the exception of lactose and the FPD, and a low effect for F:P, HI, and some fatty acids categories (UFA, MUFA, LCFA, and FFA).

The breed effect had an important effect ($P < 0.001$) over all the traits, as a result of the natural differences in milk composition of the four breeds involved in the study. In the case of the interaction between estrous × breed, it had an influence on the milk yield and on the main components of milk (except lactose), these variations of the milk and milk components among breeds and estrous phases indicate that estrous phases affect differently the activity of mammary gland of cows of different breeds.

The effects of temporary individual factors like parity number, linear and quadratic DIM nested in parity number, and year-month of the test day were also highly significant (Table 2), showing high variability of milk composition across lactations, within lactation, and in different seasonal conditions.

The Effect of Estrous Cycle on Milk Yield and Quality

The LSM for the milk production and quality traits in the different estrous cycle phases is presented in Table 3. There was a small but significant increment of milk yield on the estrus day respect to the other previous or following phases of the estrous cycle.

The effect of the estrous cycle on the main components of milk (fat, protein, casein, lactose and F:P) is evident comparing the LSM of the different phases of the estrous cycle (Table 3) together with the results of the day by day variation within the estrous phases (Figure 2). We observed a clear effect of the estrus cycle on milk fat content that, respect to diestrus-HP, started to increase during proestrus, reached its zenith the day of estrus, begun to decrease during metestrus, to return to the basal value during the following diestrus-IP. The pattern of the fat content during estrous cycle has some similarity with the patterns usually characterizing estradiol, FSH and LH hormones in dairy cows (Figure 1). An increment of the fat content during estrus has been described in other studies in Jerseys (Copeland, 1929) and Holsteins cows (Erb et al., 1952).

In the case of protein and casein, the pattern is opposite respect to fat and much more attenuated, with a small decrease around the day of estrus. The F:P ratio had similar tendencies than fat in all the phases of estrous, indicating the major influence of fat over the protein on this trait. Our results coincide with King, (1977) who reported a positive effect of estrous on fat together with a negative effect of protein on the estrus day. The lactose presented a constant value over the diestrus-HP and rising one day before the estrus, then lactose remained constant on the metestrus phase, following a gradual reduction over the diestrus-IP. Akdag et al. (2010) found no differences for lactose, but the breed (Jerseys), and the sample number is very different.

The LSM for each day nested in the estrous phases for the other milk traits are plotted in Figure 3. The urea content of milk showed a less regular pattern decreasing from diestrus-HP to the estrus and then to the nadir value reached at the end of metestrus and recovering rapidly during the diestrus-IP. This tendency could reflect the variability on the nutrition and a reduction in food consumption around estrus. In dairy goats, the stress associated with estrus has been related with variation in milk urea content and correlated with feeding and metabolism changes, in spite of a non-significant association with cycle stage (Moroni et al., 2007). Studies about the variation of milk urea around the insemination day are not consistent, but extremes high or low values of milk's urea have been associated as a risk factor for conception failure in Holstein cows (Melendez et al., 2000; Albaaj et al., 2017).

The SCS showed a pattern similar to that characterizing fat content but slightly anticipated. In fact, it incremented gradually from the diestrus-HP to the proestrus and then gradually decreased in the estrus, metestrus and initial diestrus-IP phases. No significant differences were found between the estrus versus either proestrus or metestrus phases. This increase could be due to the increase of estrogens in the udder explaining the highest value of SCS one day before estrus coinciding with the estradiol peak (Zdunczyk et al., 2003). In addition, the SCS has been associated with an increase of FFA (discussed later) which also presented the highest contents in this same day (Bachman et al., 1988). King, (1977) reported a significant increase of SCC on estrus day respect to respect to metestrus in Ayrshire and Holstein cows. On the other hand, some publications have reported non-significant differences for cows showing estrus (Anderson et al., 1983; Horrell et al., 1985) respect to cows not showing estrus, independently of the phase of the estrous cycle. In other species variations in SCS has also been found, Akhtar et al., (2008) reported an increment of SCS in Nili-Ravi buffaloes in proestrus/estrus and Moroni et al., (2007) described also an increment of SCS at estrus phase in Saanen dairy goats. In both studies the authors associated these increments with high plasma estradiol.

The FPD showed a stable pattern from the diestrus-HP until the proestrus where dropped reaching the nadir value on the day of estrus and recovering gradually in the metestrus and diestrus-IP phases. This quality indicator is used normally to detect adulterated milk, yet, independently from adulteration, it is affected by several factors, environmental, physiological and intrinsic to milk composition. The principal factors affecting the FPD are the pH and the lactose concentration, and, to a lesser degree, potassium, chloride, sodium, citrates, and urea (Zagorska and Ciprovica, 2013). So the depression of the FPD in the estrus in comparison with other estrous phases could be explained by the increase of the lactose content in these same stages in combination with variations in the pH of the milk.

The pH is commonly associated with bacterial deterioration (low values) and mastitis (high values), and also adulterations with alkali such as detergents (Vassen, 2003). The tendency of the pH values over the estrous phases (Figure 3), independently from mastitis, adulteration or microbial deterioration of milk, showed a rapid increment in estrus and metestrus phases with a gradual reduction during the diestrus-IP. The variations of pH of milk in the different estrous phases can be attributed to the casein and protein variations (Rose, 1961; Ma and Barbano, 2003); nonetheless, contents of several milk constituents as chloride, sodium, potassium, lactose, calcium, and magnesium can affect directly the pH of milk (Luck and Smith, 1975; Vassen, 2003).

The HI index had markedly decreased since one day before estrus, remained low during estrus and metestrus to regain the initial value during all diestrus (IP and HP). This indicated that the fat globule sizes increased in the estrus and metestrus phases. The increase in fat content can explain this increase of fat globule size of the samples of cows showing estrus since high-fat contents have been related to an increase of globule sizes (Goulden and Phipps, 1964; Wiking et al., 2004) but not the maintenance of this value during metestrus. Nevertheless, changes in the feeding behavior has been described that also affect the fat globule sizes (Abeni et al., 2005; Couvreur and Hurtaud, 2007),

Figure 4 shows the LSM for the most important FAs of the milk: miristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1*c*9) for each day nested in the estrous cycle phases. It is evident that miristic acid presented a constant value during all diestrus (IP and HP) and proestrus, with a sudden reduction on the day of estrus and a gradual return to initial values during metestrus. Palmitic acid presented a similar sudden decrease the day of estrus, but remained stable at low values during the metestrus and diestrus-IP, to increase progressively its milk content during diestrus-HP to the zenith value reached at proestrus (day -2). In the other hand, the two long chain FAs, stearic and oleic acids, showed an opposite tendency with a rapid increase during proestrus and reaching the zenithal values on the day of estrus. Then in the metestrus, oleic acid has a rapid decrease to initial values, whereas stearic acid showed a smooth reduction during the diestrus-IP phase. As well known, there are two main routes for production of even milk fatty acids: (1) from the 4:0 to 14:0 and about half of the 16:0 they are synthesized *de novo* in the udder mainly from acetate and β-hydroxybutyrate, acetate and butyric acid being produced in the rumen by fermentation of feed components, and then the butyric acid is converted to β-hydroxybutyrate during absorption through the rumen epithelium; (2) the rest of the 16:0 and almost all of the LCFA are originated from dietary lipids absorbed by small intestine and from lipolysis of adipose tissue triacylglycerols (Grummer, 1991; Månsson, 2008). Therefore, the increase of stearic and oleic acids in the estrous phase indicates the release in the mammary gland of LCFA (Figure 6) from the mobilization of body fat reserves, concomitantly the contents of miristic and palmitic fatty acids tended to be lower because the high uptake of LCFA tends to inhibit the *de novo* synthesis of FA by the mammary gland tissue (Gross et al., 2011).

This dynamic relation is more evident for the fatty acid groups (Figure 5), where the SFA increase progressively until the proestrus phase with a sudden decrease on the day of estrus, and a subsequent increase gradually in the metestrus and diestrus-IP phases. Simultaneously the contrary pattern is observed for the UFA, MUFA, PUFA and also TFA, gradually decreasing in the diestrus-HP with a rapid increase from proestrus to estrus, and then progressively decreasing in the metestrus and diestrus-IP phases. The TFA are produced during biohydrogenation of PUFA and isomerization of MUFA in the rumen, and the most common in ruminant fat is Vaccenic acid (18:1 *trans*-11), accounting for 60-80% of total TFA (Vargas-Bello-Pérez and Garnsworthy, 2013). The increase of PUFA and TFA in milk on the day of estrus probably reflects a greater uptake also of dietary FAs by the mammary gland, probably as a result of the contemporary minor uptake by body fat depots.

Another way to represent the different proportions between mammary gland *de novo* synthesis on one hand and dietary sources combined with fat mobilization, on the other hand, is to observe the FAs grouped according to their carbon-chain length (Figure 6). Again for the SCFA, MCFA, and LCFA, the antagonistic pattern is obvious. The SCFA and the MCFA showed an increase during the proestrus followed by a significant reduction in the estrus phase, then the SCFA increased in the metestrus and stabilizes in the diestrus, while the MCFA remained low in the metestrus and augmented gradually in the diestrus-HP; simultaneously the LCFA decreased progressively during the diestrus-HP and substantially increased from the proestrus to the estrous day, then in the metestrus and diestrus-IP phases the LCFA decreased progressively.

The FFA, or non-esterified fatty acids, presented the maximum value on the proestrus time (Figure 6), exactly one day before estrus and slowly decreased during the estrus and the diestrus-IP phase. This effect can be associated with the estradiol peak in the proestrus, since it has been associated with an increase of FFA, due to a shift of lipoprotein lipase activity (Bachman et al., 1988). An elevated level of FFA is one of the indicators of negative energy balance, since the energy requirements of the cows are compensated by intensive lipolysis, and releasing fatty acids in the blood (Adewuyi et al., 2005). The increment of FFA mobilization has been described especially in periparturient cows explained by (1) the suppression of *de novo* synthesis or uptake, and then esterification of fatty acids, (2) promotion of lipolysis, (3) reduction of the intracellular re-esterification of fatty acids released by lipolysis and (4) some combination among these possibilities (Bell, 1995).

Differences in Milk Yield and Quality Between Cows that Conceived or Not Conceived at Estrus

It is evident that the differences between the milk traits of cows that conceived or not at the insemination carried out the day of estrus (estrus was defined as the day of insemination) during diestrus-HP and proestrus (i.e. during the 10 days before insemination) cannot depend on future pregnancy but reflect the differences between the cows initially more or less fertile. The differences observed during the 10 days following insemination (metestrus and diestrus-IP), on the other hand, could reflect both the effect of initial fertility of the cows (like in the previous phases) and also of a very initial stage of pregnancy. These two effects cannot be clearly distinguished.

A possible help to the interpretation of results could be offered by the analysis of the interaction between estrous phases and conception effects. The first case regards the traits with differences among the estrous cycle phases but without significant differences due to the eventual conception of cows and to the interaction between estrus phases and conception, and it could be interpreted as absence of effects of both initial fertility of the cows and of the following conception of these cows. This is the case of milk fat content, of the proportion of the 4 major FAs, of SFA, of PUFA, and of SCFA, but also of milk pH and urea content (Table 2).

The second case regards traits different in cows that conceived and in cows that did not conceive, but with no significant interaction between estrus phases and pregnancy, and it could be interpreted as traits reflecting the initial differences in the cow's fertility level, but probably not affected by the initial stage of pregnancy. This is the case of milk yield, of milk content of protein and casein, of SCS, and, among FAs, of TFA and of MCFA (Table 2). In fact, even though the differences are quantitatively not much relevant, the more fertile cows showed slightly lower milk yield and a slightly greater protein and casein contents (Table 3). Both these differences could be interpreted as a slightly more favorable energy balance for the more fertile cows (Patton et al., 2007; Olson et al., 2011) especially if considered that these differences derive from an analysis that corrects the LSMs for the herd, year and season, breed, parity and days in milk of the cows. The small difference in SCS could probably depend on a greater concentration per unit milk due to the lower milk produced daily than to a greater production of somatic cells per day by the udder. Lastly, the greater MCFA and lower TFA are both coherent with the hypothesis of a better energy balance.

The third case is represented by traits not affected by the main effect of pregnancy, but presenting a significant interaction with estrus phases. In this case, an interpretation is a possible effect induced by conception without initial differences due to fertility level of the cows. The traits in this situation are the two physical characteristics of milk (FPD and HI), and the MUFA, UFA, and LCFA, among the FA categories. Further studies are needed for the interpretation of these results.

Lastly, the fourth case is represented by traits affected by both the main effect of pregnancy and the interaction, which could be interpreted as traits different in a cow with initial differences in fertility level but also influenced by the establishing of pregnancy. However, in these last cases, it is not possible to exclude that the results observed could be simply the result of the different effect of initial fertility level in the different phases of the estrous cycle, independent from the effect of pregnancy. This is the case only of the content of lactose and FFA (Table 2).

Breed Effects

The least squares means of milk yield and quality traits of the four breeds and the significance level of their contrasts are presented in Table 4. First of all, it worth to note that the two specialized dairy breeds (Holstein and Brown Swiss) were different from the dual purpose breeds (Simmental and Alpine Grey) for all traits with the only exception of milk content of oleic acid and SCFA, whereas the two dairy breeds and the two dual purpose breeds were different from each other for all traits considered.

The milk yield was greater for Holsteins, followed by the two large-framed Alpine breeds (Brown Swiss and Simmental) and lastly by the medium-framed Alpine Grey. The variations across breeds are explained by genetic potential characterizing each breed, and also by environmental differences, especially related to the farming systems (Toledo-Alvarado et al., 2017). The Alpine Grey cows are mainly raised in small traditional farms with tied animals fed hay and some concentrate, while the Holstein cows are mainly present in more modern dairy systems with loose housing and total mixed rations. The Brown Swiss and Simmental cows are raised in both types of farming systems. A better description of the dairy systems in the Alps is given by Sturaro et al., (2013) and the different contribution of breed and farming systems is given by (Stocco et al., 2017a)

The Brown Swiss had the highest contents of the major components of milk (except lactose, higher in Alpine Grey), and Holstein the lowest values (except fat and fat:protein ratio, lower in Alpine Grey), with dual purpose breeds being intermediate; thus confirming results from previous surveys in same (Stocco et al., 2017a; b). Similar results are those characterizing the 4 breeds in relation to urea and FPD of milk. Also confirmed is the lower SCS value of milk from Simmental cows and the modest differences in terms of milk pH.

Whereas no information is available for HI, that was greater in Holsteins and smaller in Alpine Grey cows.

Moving to major FAs and FA categories (Table 4), in the large majority of cases, Holstein and Alpine Grey were the extreme breeds. The former was, in fact, characterized by greater proportion of the most represented milk FAs, palmitic and oleic acids, and conversely by the lower proportion of SCFA. These results are similar to those of Gottardo et al., (2017), relative to the same database but considering the entire lactation of cows and not the test-date closest to the first insemination (early lactation). Taking into consideration that Holsteins and Alpine Grey are the extreme breeds also for milk yield, while their milk fat content is not much different, we could argue that these differences are probably a consequence of the different proportions among FAs supplied by intestinal absorption, by fatty depot mobilization and by *de novo* udder synthesis. If the greater proportion of oleic acid characterize Holstein-Friesian also in comparisons with other breeds and different farming systems (Kelsey et al., 2017; Vanbergue et al., 2017) , caution should be used when large differences in milk-fat content characterizes the breeds compared, especially in the case of Jersey (Maurice-Van Eijndhoven et al., 2017; Poulsen et al., 2017).

The interactions between breed and estrus phases were significant for the majority of the traits analyzed (Table 2) but the differences among breeds were not relevant in terms of the general pattern during the subsequent phases of the estrous cycle. The results are presented in supplementary materials (Appendix 1 to 5) but are not discussed here.

Possible Use of Estrus Cycle Modification of Milk Traits

The results obtained here permit a better understanding of the effect of estrus cycle on milk characteristics and consequently on udder functions. The modifications observed, even though statistically significant, are not much relevant for a direct use by dairy industry. Nevertheless, they suggest a possible use for diagnoses of cows in relation to their reproductive activity. All the quality traits analyzed in this study, except SCS, were obtained from milk spectra through proper calibration. This means that absorbance of many wavelengths of the FTIR spectrum are affected by the estrus cycle of the cows and potentially could be used as diagnostic tools. In particular, FTIR spectrum could be used for a diagnosis of cows in heat or, better, approaching heat. It is clear that this use cannot be based on milk samples collected periodically within the milk recording activities and analyzed by some centralized laboratory, but could be interesting when infrared devices are installed in the milking parlor for a day to day (milking to milking) analyses of milk. If the entity of the effects of estrus phases are not useful for diagnose based on a single milk sample, the evolution of milk (FTIR spectrum) at each milking could be much more valuable.

For a possible diagnostic use of incoming or actual estrus, the choice of the traits (and/or corresponding FTIR wavelengths), more than on absolute level of differences with the previous days, should be based on the level of significance (*F*-value) of "Estrous" effect, or also of "Days within estrous" effect. From Table 2 it is possible to see that the most promising traits seem to be lactose content, fat:protein ratio, FPD, HI, miristic and stearic fatty acids and LCFA. Another criterion of evaluation of candidate traits/wavelengths for estrus diagnosis could be the time of maximum differentiation. From the figures plotting the day within estrus phases pattern of different traits, it is possible to see that MCFA exhibit a zenithal value 2 days before estrus and FFA 1 day before (Figure 6). It is also possible to see that TFA reach their nadir value 4 days and PUFA 2 days before estrus (Figure 5). An anticipation of incoming estrus could allow planning direct observation of cows and their insemination if estrus will be confirmed.

CONCLUSIONS

Milk composition showed high variability among the estrous phases: all the 24 milk traits studied were affected significantly. Among them, the fat, protein, casein and lactose were highly affected by the reproduction cycle of the cow and also the milk's FA profile showed important differences, probably induced by the hormonal and behavioral changes of the cows (dry matter intake, rumination time and activity). Moreover, the estrous cycle also affected the urea, SCS, FPD, pH and HI. The breed and environmental effects were important factors to explain the variation of the milk composition. Assessments of the relation of milk composition with estrous phases could lead to new on-farm low-cost indicators of reproductive changes of the cow and potentially improve the design of breeding programs in dairy cattle. The milk profile could perhaps be useful in automated management-systems to identify cows in estrus or predict cows with incoming estrus and should be taken into account for breeding purposes. However, further research is needed to study the prediction capability of the milk composition to discriminate cows showing estrus.

ACKNOWLEDGMENTS

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TABLES

Table 1. Descriptive statistics of milk yield, main components, other traits, and fatty acid (FA) profile

Trait	n	Cows	Mean	SD	P1	P99
Milk yield, kg/d	85,329	57,629	26.87	7.19	12.40	45.70
Main components, %						
Fat	85,329	57,629	3.88	0.70	2.28	5.96
Protein	85,329	57,629	3.26	0.32	2.61	4.07
Casein	85,329	57,629	2.57	0.25	2.04	3.19
Lactose	85,329	57,629	4.83	0.17	4.36	5.19
Fat:Protein	85,329	57,629	1.20	0.22	0.70	1.89
Other milk traits						
SCS, ln	85,329	57,629	4.07	1.35	1.61	7.62
pH	85,329	57,629	6.60	0.06	6.44	6.75
Urea, mg/100ml	85,315	57,620	20.92	7.56	4.90	41.10
FPD, 10^{-2} °C	85,329	57,629	-52.60	0.79	-54.40	-50.70
HI	85,329	57,629	0.67	0.09	0.49	0.93
Individual FA, % total FA						
Miristic acid (C14:0)	62,833	37,089	12.31	1.27	8.58	14.90
Palmitic acid (C16:0)	62,833	37,089	31.71	3.07	23.42	37.99
Stearic acid (C18:0)	62,833	37,089	10.41	1.55	7.02	14.46
Oleic acid (C18:1c9)	62,833	37,089	21.27	3.62	14.58	31.88
FA categories, % total FA						
SFA	62,833	37,089	70.09	3.46	59.92	76.52
UFA	62,833	37,089	29.09	3.87	22.29	40.87
MUFA	62,833	37,089	24.79	3.56	18.23	35.22
PUFA	62,833	37,089	3.09	0.62	1.83	4.77
TFA	62,833	37,089	2.17	0.59	0.84	3.61
SCFA	62,833	37,089	10.53	1.27	7.12	13.12
MCFA	62,833	37,089	42.85	7.14	25.07	59.01
LCFA	62,833	37,089	31.86	4.80	23.12	45.90
FFA	78,772	53,376	0.64	0.35	0.03	1.71

SCS: somatic cell score; FPD: freezing point depression; HI: homogenization index; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-unsaturated fatty acids; SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; FFA: free fatty acids.

Table 2. Results from ANOVA (*F*-Value and significance) for of milk yield, main components, other traits, and fatty acid (FA) profile

Trait	Estrous	Day (Estrous)	Conception	Estrous *x* Conception	Breed	Estrous *x* Breed	Parity	DIM (parity)	DIM^2 (parity)	Year-Month	RMSE
Milk yield, kg/d	3.9**	3.1***	157.0***	1.0	1,676.6***	2.6**	433.2***	156.6***	12.6***	50.9***	4.36
Main components, %											
Fat	93.8***	13.6***	0.7	2.0	341.9***	2.4**	6.4***	2.6*	6.4***	24.5	0.62
Protein	31.0***	3.9***	47.9***	1.3	1,391.5***	2.7**	11.7***	264.9***	14.5***	73.0***	0.24
Casein	38.4***	3.7***	46.5***	1.6	1,378.8***	3.1***	14.4***	233.3***	12.2***	92.5***	0.18
Lactose	273.7***	34.9***	17.4***	4.7***	176.2***	1.8	155.2***	31.4***	4.7***	33.7***	0.14
Fat:Protein	144.7***	16.4***	6.2*	2.6*	142.7***	4.0***	11.5***	49.5***	19.8***	5.9***	0.19
Other milk traits											
SCS, ln	41.8***	3.4***	18.3***	0.1	131.4***	0.7	30.2***	31.3***	4.2**	3.6***	1.22
pH	43.6***	4.9***	0.3	0.9	218.7***	0.9	0.3	92.8***	18.3***	181.5***	0.05
Urea, mg/100ml	11.0***	3.9***	1.4	0.4	240.7***	1.4	16.1***	30.7***	14.2***	96.4***	6.51
FPD, 10^{-2} °C	339.8***	29.8***	0.2	7.2***	149.5***	2.6**	31.7***	17.4***	2.9*	43.6***	0.67
HI	181.7***	16.7***	0.9	4.3**	264.0***	3.0***	56.3***	28.3***	12.3***	629.8***	0.07
Individual FA, % total FA											
Miristic acid (C14:0)	119.1***	3.6***	0.3	2.3	64.7***	1.3	20.7***	436.8***	281.2***	56.1***	1.08
Palmitic acid (C16:0)	76.1***	3.2***	0.9	1.6	202.2***	1.8*	16.0***	559.3***	300.5***	171.0***	2.51
Stearic acid (C18:0)	165.8***	8.4***	2.3	1.7	345.9***	0.8	28.3***	1,086.4***	480.0***	59.6***	1.24
Oleic acid (C18:1c9)	71.8***	4.6***	2.2	2.0	402.6***	2.0*	26.9***	461.3***	264.3***	172.1***	2.93
FA categories, % total FA											
SFA	97.7***	3.9***	0.0	2.2	92.5***	1.5	15.3***	360.9***	222.9***	146.1***	2.83
UFA	89.1***	3.2***	1.5	2.5**	100.7***	1.5	14.7***	483.5***	293.7***	108.9***	3.26
MUFA	90.2***	4.5***	1.8	2.8*	228.9***	2.6**	31.5***	315.5***	191.3***	195.2***	2.87
PUFA	13.8***	1.6*	0.0	0.7	217.2***	2.4**	36.1***	152.2***	73.4***	366.0***	0.47
TFA	103.6***	17.9***	7.5**	2.1	832.4***	2.3**	92.4***	27.6***	0.5	122.2***	0.45
SCFA	11.7***	2.8***	0.4	1.1	914.1***	1.7	17.8***	3.7**	4.2**	46.6***	1.03
MCFA	22.1***	6.5***	9.9**	1.6	592.6***	2.4**	8.4***	132.7***	37.2***	104.5***	6.06
LCFA	165.7***	9.2***	1.7	2.8*	184.6***	1.0	37.7***	735.6***	394.2***	287.3***	3.71
FFA	58.3***	6.9***	10.0**	2.6*	112.9***	3.1***	7.3***	79.6***	13.4***	80.9***	0.30

SCS: somatic cell score; FPD: freezing point depression; HI: homogenization index; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-unsaturated fatty acids; SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; FFA: free fatty acids. RMSE: root mean square error.

*P < 0.05; **P < 0.01; ***P < 0.001.

Table 3. Least square means of milk yield, main components, other traits, and fatty acid (FA) profile for the estrous cycle phases and for conception success averaged across all breeds

	Estrous cycle phase					Conception[2]	
Trait	Diestrus-HP[1]	Proestrus[1]	Estrus	Metestrus[1]	Diestrus-IP[1]	No	Yes
Milk yield, kg/d	25.78**	25.81**	26.03	25.82*	25.73***	26.09^{B}	25.57^{A}
Main components, %							
Fat	3.82***	3.87***	3.95	3.87***	3.78***	3.85	3.86
Protein	3.19***	3.18***	3.15	3.19***	3.20***	3.17^{A}	3.19^{B}
Casein	2.50***	2.50*	2.49	2.52***	2.52***	2.49^{A}	2.51^{B}
Lactose	4.81***	4.81***	4.86	4.86	4.83***	4.84^{B}	4.83^{A}
Fat:Protein	1.20***	1.22***	1.26	1.21***	1.19***	1.22^{b}	1.21^{a}
Other milk traits							
SCS, ln	4.04***	4.17	4.17	4.14	4.02***	4.08^{A}	4.13^{B}
pH	6.59***	6.59***	6.60	6.60	6.59***	6.59	6.59
Urea, mg/100ml	20.56	20.45	20.42	20.05**	20.60	20.45	20.38
FPD, 10^{-2} °C	-52.47***	-52.58***	-52.83	-52.73***	-52.57***	-52.64	-52.63
HI	0.69***	0.69***	0.67	0.67	0.68***	0.68	0.68
Individual FA, % total FA							
Miristic acid (C14:0)	12.41***	12.37***	12.02	12.13***	12.31***	12.24	12.25
Palmitic acid (C16:0)	31.75***	31.88***	31.40	31.48	31.40	31.56	31.59
Stearic acid (C18:0)	10.27***	10.30***	10.71	10.63*	10.49***	10.49	10.47
Oleic acid (C18:1c9)	21.50***	21.58***	22.31	22.09**	21.65***	21.85	21.80
FA categories, % total FA							
SFA	69.98***	69.99***	69.15	69.40***	69.61***	69.62	69.63
UFA	29.21***	29.23***	30.15	29.90**	29.56***	29.63	29.58
MUFA	24.94***	24.99***	25.72	25.63	25.21***	25.32	25.27
PUFA	3.12***	3.11***	3.17	3.15	3.14**	3.13	3.13
TFA	2.23***	2.24***	2.38	2.33***	2.29***	2.30^{B}	2.28^{A}
SCFA	10.31***	10.28***	10.18	10.32***	10.32***	10.28	10.28
MCFA	41.98**	42.41***	41.47	41.62	41.77*	41.74^{A}	41.95^{B}
LCFA	31.99***	32.08***	33.32	33.16*	32.58***	32.65	32.59
FFA	0.64**	0.65	0.65	0.63***	0.60***	0.63^{B}	0.62^{A}

[1]: asterisks regard the significance of the contrast of this phase with estrus: *P < 0.05; **P < 0.01; ***P < 0.001. [2]: means with superscript letters are significantly different ($^{a,\,b}$ $P < 0.05$; $^{A,\,B}$ $P < 0.01$) from each other. SCS: somatic cell score; FPD: freezing point depression; HI: homogenization index; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-unsaturated fatty acids; SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; FFA: free fatty acids.

Table 4. Least square means of milk yield, main components, other traits, and fatty acid (FA) profile for each breed: Holstein (Ho), Brown Swiss (BS), Simmental (Si), and Alpine Grey (AG)

Trait	Breed LSM:				Contrasts[1]:		
	Ho	BS	Si	AG	Ho+BS *vs* Si+AG	Ho *vs* BS	Si *vs* AG
Milk yield, kg/d	30.06	25.80	26.46	21.01	***	***	***
Main components, %							
Fat	3.81	4.05	3.92	3.66	***	***	***
Protein	3.03	3.33	3.20	3.18	*	***	**
Casein	2.39	2.62	2.51	2.50	*	***	*
Lactose	4.81	4.83	4.82	4.88	***	***	***
Fat:Protein	1.26	1.22	1.23	1.16	***	***	***
Other milk traits							
SCS, ln	4.31	4.22	3.91	3.99	***	***	**
pH	6.58	6.60	6.60	6.61	***	***	***
Urea, mg/100ml	18.76	21.86	19.86	21.18	*	***	***
FPD, 10^{-2} °C	-52.49	-52.75	-52.64	-52.65	*	***	
HI	0.70	0.67	0.69	0.66	***	***	***
Individual FA, % total FA							
Miristic acid (C14:0)	12.29	12.39	12.10	12.21	***	***	***
Palmitic acid (C16:0)	32.05	31.40	32.11	30.76	***	***	***
Stearic acid (C18:0)	9.91	10.50	10.69	10.83	***	***	***
Oleic acid (C18:1c9)	22.87	20.76	21.78	21.90		***	
FA categories, % total FA							
SFA	69.64	70.03	69.96	68.88	***	***	***
UFA	29.60	29.12	29.24	30.47	***	***	***
MUFA	25.77	24.41	25.25	25.77	***	***	***
PUFA	3.09	3.14	3.00	3.32	***	***	***
TFA	2.52	2.05	2.24	2.37	*	***	***
SCFA	9.72	10.84	10.08	10.50		***	***
MCFA	40.49	42.61	44.74	39.56	***	***	***
LCFA	32.99	31.55	32.68	33.28	***	***	***
FFA	0.70	0.65	0.63	0.55	***	***	***

[1]: asterisks regard the significance of the contrast: *P < 0.05; **P < 0.01; ***P < 0.001. SCS: somatic cell score; FPD: freezing point depression; HI: homogenization index; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-unsaturated fatty acids; SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; FFA: free fatty acids.

Table 5. Least square means of milk yield, main components, other traits, and fatty acid (FA) profile at different parities averaged across all breeds.

Trait	Parity			
	1st	2nd	3rd	4th
Milk yield, kg/d	22.33	25.69	27.40	27.92
Main components, %				
Fat	3.83	3.88	3.87	3.86
Protein	3.20	3.22	3.17	3.13
Casein	2.53	2.54	2.49	2.46
Lactose	4.91	4.84	4.81	4.78
Fat:Protein	1.20	1.21	1.23	1.23
Other milk traits				
SCS, ln	3.76	4.04	4.21	4.42
pH	6.59	6.59	6.60	6.60
Urea, mg/100ml	21.44	20.37	20.05	19.81
FPD, 10^{-2} °C	-52.92	-52.66	-52.53	-52.42
HI	0.65	0.68	0.69	0.70
Individual FA, % total FA				
Miristic acid (C14:0)	11.80	12.35	12.42	12.42
Palmitic acid (C16:0)	30.97	31.88	31.81	31.66
Stearic acid (C18:0)	10.66	10.33	10.43	10.51
Oleic acid (C18:1c9)	23.10	21.44	21.35	21.41
FA categories, % total FA				
SFA	68.50	69.87	70.07	70.06
UFA	30.48	29.23	29.25	29.47
MUFA	26.80	24.96	24.74	24.70
PUFA	3.37	3.08	3.04	3.05
TFA	2.61	2.26	2.19	2.12
SCFA	10.48	10.27	10.22	10.17
MCFA	41.78	42.43	41.81	41.37
LCFA	34.13	32.16	32.09	32.11
FFA	0.60	0.66	0.65	0.63

SCS: somatic cell score; FPD: freezing point depression; HI: homogenization index; SFA: saturated fatty acids; UFA: unsaturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-unsaturated fatty acids; SCFA: short-chain fatty acids; MCFA: medium-chain fatty acids; LCFA: long-chain fatty acids; FFA: free fatty acids.

FIGURES

Figure 1. Schematic depiction of the pattern of secretion of follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone, Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and estradiol, during the estrous cycle in cattle.

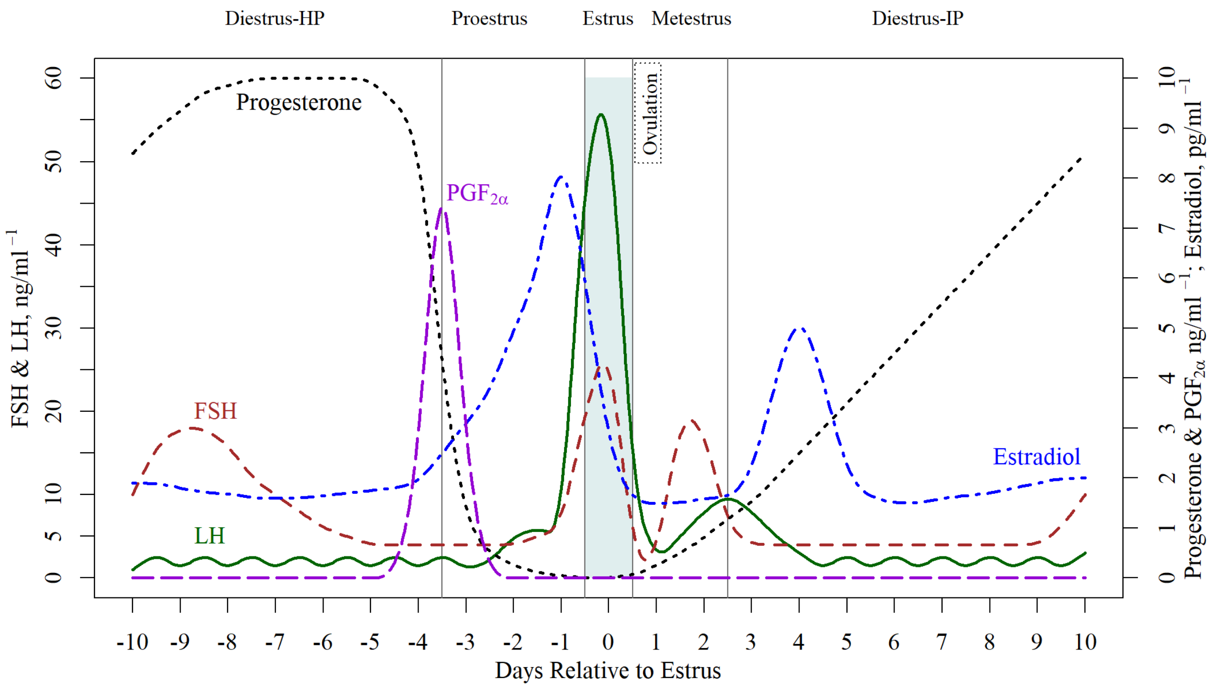


Figure 2. Least squares means of fat, protein, casein, lactose and fat:protein ratio for each day nested in the estrous cycle phases.

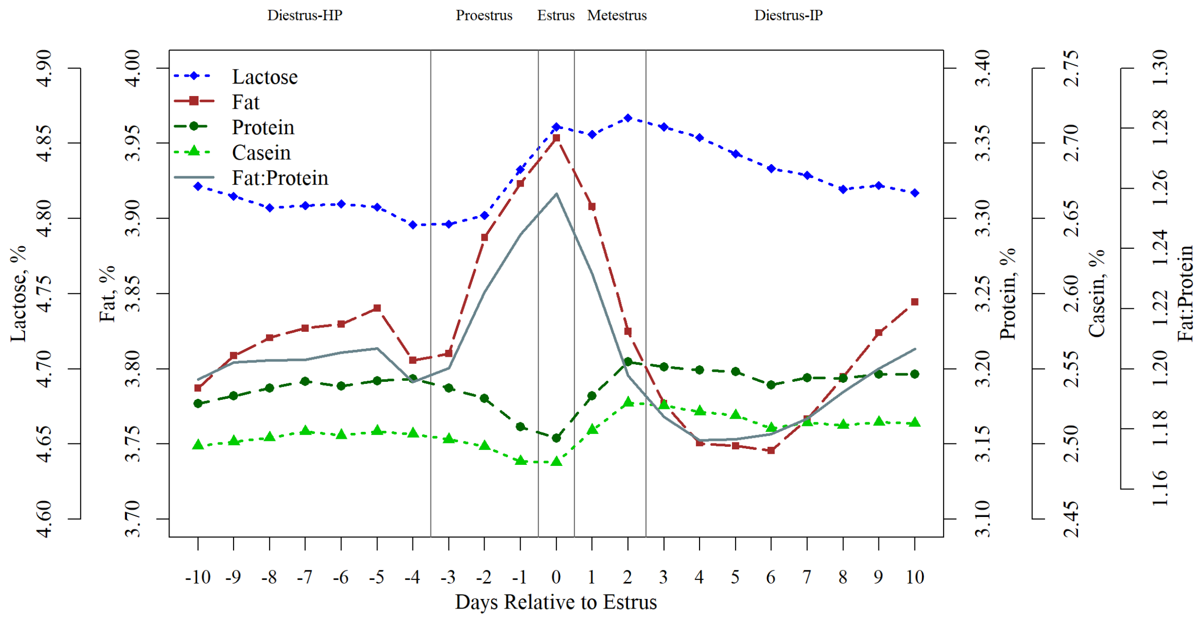


Figure 3. Least squares means of somatic cell score (SCS), pH, urea, freezing point depression (FPD), and homogenization index (HI) for each day nested in the estrous cycle phases.

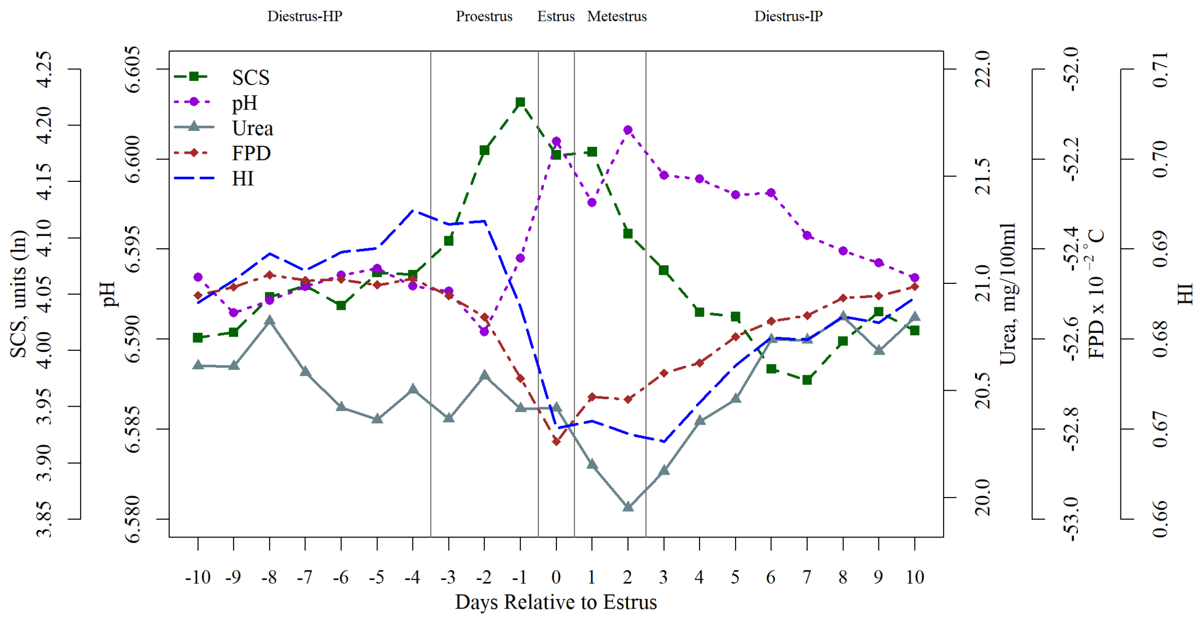


Figure 4. Least squares means of miristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1*c*9) for each day nested in the estrous cycle phases.

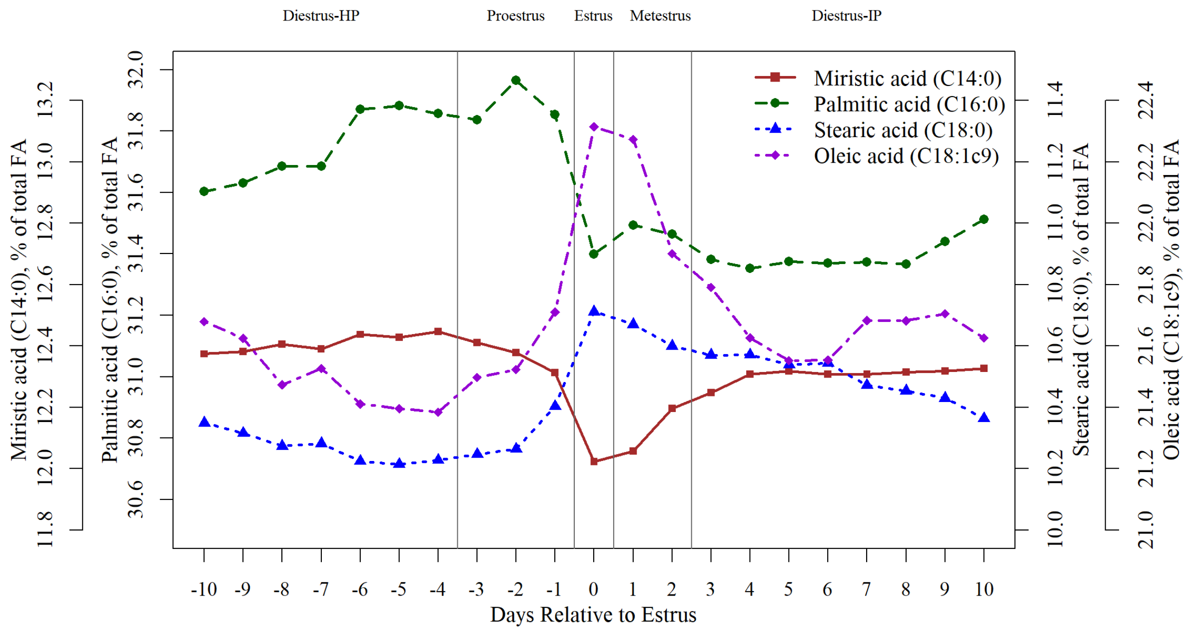


Figure 5. Least squares means of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and trans-unsaturated fatty acids (TFA) for each day nested in the estrous cycle phases.

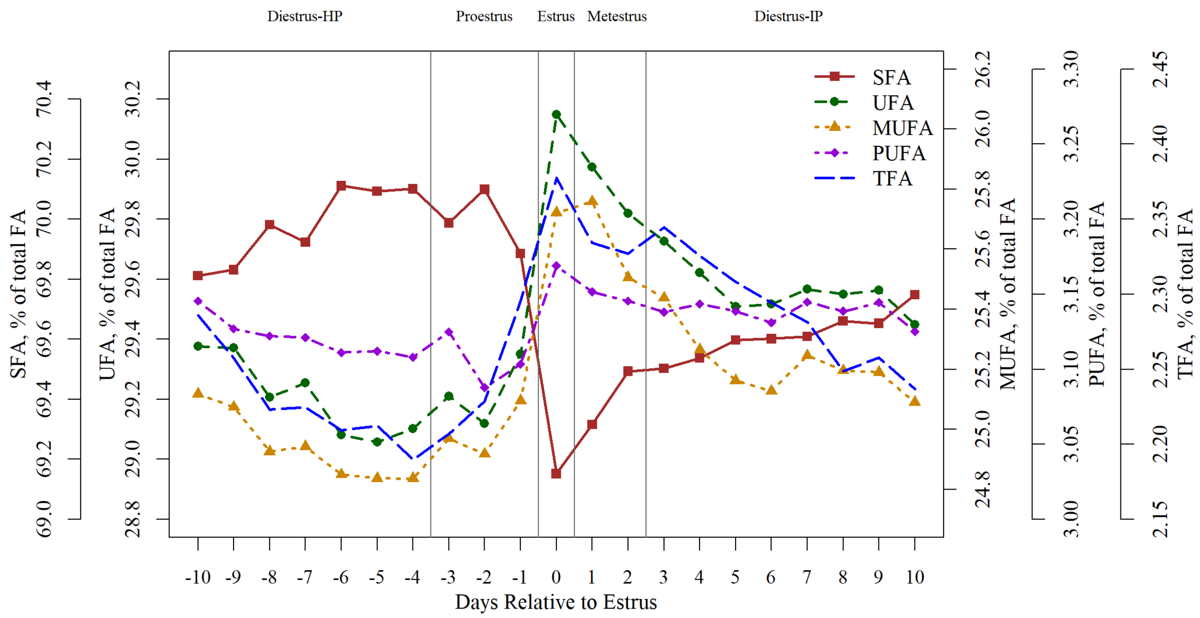
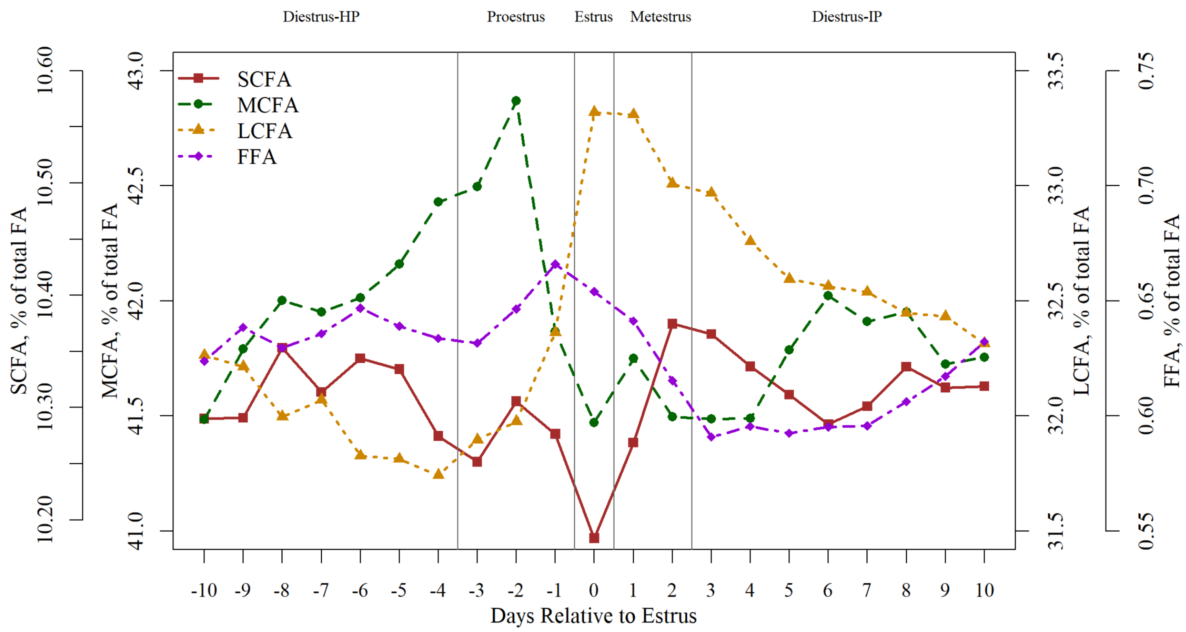


Figure 6. Least squares means of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA), and free fatty acids (FFA) for each day nested in the estrous cycle phases.



REFERENCES

Abeni, F., L. Degano, F. Calza, R. Giangiacomo, and G. Pirlo. 2005. Milk Quality and Automatic Milking: Fat Globule Size, Natural Creaming, and Lipolysis. J. Dairy Sci. 88:3519–3529. doi:10.3168/jds.S0022-0302(05)73037-X.

Adewuyi, A.A., E. Gruys, and F.J.C.M. van Eerdenburg. 2005. Non esterified fatty acids (NEFA) in dairy cattle. A review.. Vet. Q. 27:117–26. doi:10.1080/01652176.2005.9695192.

Akdag, F., O. Cadirci, and B. Siriken. 2010. Effect of estrus on milk yield and composition in jersey cows. Bulg. J. Agric. Sci. 16:783–787.

Akhtar, M.S., A.A. Farooq, M. Hussain, and M. Aziz. 2008. Contents in milk of Nili-Ravi buffaloes. Pak. J. Agri. Sci. 45:339–341.

Albaaj, A., G. Foucras, and D. Raboisson. 2017. Changes in milk urea around insemination are negatively associated with conception success in dairy cows. J. Dairy Sci. 100:1–9. doi:https://doi.org/10.3168/jds.2016-12080.

Anderson, K.L., A.R. Smith, S.L. Spahr, B.K. Gustafsson, J.E. Hixon, P.G. Weston, E.H. Jaster, R.D. Shanks, and H.L. Whitmore. 1983. Influence of the estrous cycle on selected biochemical and cytologic characteristics of milk of cows with subclinical mastitis.. Am. J. Vet. Res. 44:677–80.

Arnvidarson, B., L. Nygaard, and P.W. Hansen. 1998. Determination of extraneous water in milk samples, or the freezing point depression of milk samples. US Pat. No. Grant US5739034A.

Bachman, K.C., M.J. Hayen, D. Morse, and C.J. Wilcox. 1988. Effect of pregnancy, milk yield, and somatic cell count on bovine milk fat hydrolysis.. J. Dairy Sci. 71:925–931. doi:10.3168/jds.S0022-0302(88)79638-1.

Bell, A.W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation.. J. Anim. Sci. 73:2804–2819. doi:/1995.7392804x.

Copeland, L. 1929. Effect of Heat (Oestrum) on Butterfat Percentage and Milk Yield. J. Dairy Sci. 12:464–468. doi:10.3168/jds.S0022-0302(29)93598-0.

Couvreur, S., and C. Hurtaud. 2007. Le globule gras du lait: sécrétion, composition, fonctions et facteurs de variation. INRA Prod. Anim. 20:369–382.

Cowan, C.M., and L.L. Larson. 1979. Relationship of the Estrous Cycle to Milk Composition. J. Dairy Sci. 62:546–550. doi:10.3168/jds.S0022-0302(79)83288-9.

Erb, R.E., M.M. Goodwin, R.A. Morrison, and A.O. Shaw. 1952. Lactation Studies. II. Effect of Estrus. J. Dairy Sci. 35:234–244. doi:10.3168/jds.S0022-0302(52)93696-5.

García-Ruiz, A., J.B. Cole, P.M. VanRaden, G.R. Wiggans, F.J. Ruiz-López, and C.P. Van Tassell. 2016. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. Proc. Natl. Acad. Sci. 113:E3995–E4004. doi:10.1073/PNAS.1519061113.

Gottardo, P., M. Penasa, F. Righi, N. Lopez-Villalobos, M. Cassandro, and M. De Marchi. 2017. Fatty acid composition of milk from Holstein-Friesian, Brown Swiss, Simmental and Alpine Grey cows predicted by mid-infrared spectroscopy. Ital. J. Anim. Sci. 0:1–10. doi:10.1080/1828051X.2017.1298411.

Goulden, J.D.S., and L.W. Phipps. 1964. Factors affecting the fat globule sizes during the homogenization of milk and cream. J. Dairy Res. 195–200.

Gross, J., H.A. van Dorland, R.M. Bruckmaier, and F.J. Schwarz. 2011. Milk fatty acid profile related to energy balance in dairy cows. J. Dairy Res. 479–488. doi:doi:10.1017/S0022029911000550.

Grummer, R.R. 1991. Effect of feed on the composition of milk fat.. J. Dairy Sci. 74:3244–57. doi:10.3168/jds.S0022-0302(91)78510-X.

Horrell, R.I., K.L. Macmillan, R. Kilgour, and K. Bremner. 1985. Changes in milk yield and composition at oestrus in dairy cows. J. Dairy Res. 52:9–16. doi:10.1017/S0022029900023840.

Huang, C., I. Misztal, S. Tsuruta, and T.J. Lawlor. 2007. Methodology of Evaluation for Female Fertility. Interbull Bull. 156–160.

ICAR (International Committee for Animal Recording). 2016. International Agreement of Recording Practices - Recording Guidelines.

Kaproth, M.T., and R.H. Foote. 2011. Reproduction, Events and Management | Mating Management: Artificial Insemination, Utilization. Second Edi. J.W. Fuquay, ed. Academic Press, San Diego.

Kelsey, J.A., B.A. Corl, R.J. Collier, and D.E. Bauman. 2017. The Effect of Breed, Parity, and Stage of Lactation on Conjugated Linoleic Acid (CLA) in Milk Fat from Dairy Cows[1]. J. Dairy Sci. 86:2588–2597. doi:10.3168/jds.S0022-0302(03)73854-5.

King, J.O. 1977. The effect of oestrus on milk production in cows.. Vet. Rec. 101:107–8.

Lopez, H., D.Z. Caraviello, L.D. Satter, P.M. Fricke, and M.C. Wiltbank. 2005. Relationship

Between Level of Milk Production and Multiple Ovulations in Lactating Dairy Cows. J. Dairy Sci. 88:2783–2793. doi:http://dx.doi.org/10.3168/jds.S0022-0302(05)72958-1.

Lopez, H., L.. Satter, and M.. Wiltbank. 2004. Relationship between level of milk production and estrous behavior of lactating dairy cows. Anim. Reprod. Sci. 81:209–223. doi:10.1016/j.anireprosci.2003.10.009.

Luck, H., and A. Smith. 1975. Relationship between constituent concentrations and the pH value of mammary gland secretions. S Afr J Dairy Technol.

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: where will it end?. J. Dairy Sci. 84:1277–93. doi:10.3168/jds.S0022-0302(01)70158-0.

Ma, Y., and D.M. Barbano. 2003. Serum Protein and Casein Concentration: Effect on pH and Freezing Point of Milk with Added CO2. J. Dairy Sci. 86:1590–1600. doi:10.3168/jds.S0022-0302(03)73744-8.

Månsson, H.L. 2008. Fatty acids in bovine milk fat. Food Nutr. Res. 52. doi:10.3402/fnr.v52i0.1821.

Maurice-Van Eijndhoven, M.H.T., H. Bovenhuis, H. Soyeurt, and M.P.L. Calus. 2017. Differences in milk fat composition predicted by mid-infrared spectrometry among dairy cattle breeds in the Netherlands. J. Dairy Sci. 96:2570–2582. doi:10.3168/jds.2012-5793.

Melendez, P., A. Donovan, and J. Hernandez. 2000. Milk Urea Nitrogen and Infertility in Florida Holstein Cows. J. Dairy Sci. 83:459–463. doi:10.3168/jds.S0022-0302(00)74903-4.

Miura, R., K. Yoshioka, T. Miyamoto, H. Nogami, H. Okada, and T. Itoh. 2017. Estrous detection by monitoring ventral tail base surface temperature using a wearable wireless sensor in cattle. Anim. Reprod. Sci.. doi:10.1016/j.anireprosci.2017.03.002.

Moroni, P., G. Pisoni, G. Savoini, E. van Lier, S. Acuña, J.P. Damián, and A. Meikle. 2007. Influence of Estrus of Dairy Goats on Somatic Cell Count, Milk Traits, and Sex Steroid Receptors in the Mammary Gland. J. Dairy Sci. 90:790–797. doi:10.3168/jds.S0022-0302(07)71563-1.

Nebel, R.L., C.M. Jones, and Z. Roth. 2011. Reproduction, Events and Management | Mating Management: Detection of Estrus. Second Edi. J.W. Fuquay, ed. Academic Press, San Diego.

Olson, K.M., B.G. Cassell, M.D. Hanigan, and R.E. Pearson. 2011. Interaction of energy balance, feed efficiency, early lactation health events, and fertility in first-lactation Holstein, Jersey, and reciprocal F1 crossbred cows.. J. Dairy Sci. 94:507–11. doi:10.3168/jds.2010-3433.

Patton, J., D. a Kenny, S. McNamara, J.F. Mee, F.P. O'Mara, M.G. Diskin, and J.J. Murphy. 2007. Relationships among milk production, energy balance, plasma analytes, and reproduction in Holstein-Friesian cows. J. Dairy Sci. 90:649–658. doi:10.3168/jds.S0022-0302(07)71547-3.

Poulsen, N.A., F. Gustavsson, M. Glantz, M. Paulsson, L.B. Larsen, and M.K. Larsen. 2017. The influence of feed and herd on fatty acid composition in 3 dairy breeds (Danish Holstein, Danish Jersey, and Swedish Red). J. Dairy Sci. 95:6362–6371. doi:10.3168/jds.2012-5820.

Pryce, J.E., M.D. Royal, P.C. Garnsworthy, and I.L. Mao. 2004. Fertility in the high-producing dairy cow. Livest. Prod. Sci. 86:125–135. doi:10.1016/S0301-6226(03)00145-3.

Roelofs, J., F. López-Gatius, R.H.F. Hunter, F.J.C.M. van Eerdenburg, and C. Hanzen. 2010. When is a cow in estrus? Clinical and practical aspects. Theriogenology 74:327–344. doi:10.1016/j.theriogenology.2010.02.016.

Roelofs, J.B., E. van Erp-van der Kooij, and E. van Erp-van der Kooij. 2015. Estrus detection tools and their applicability in cattle: recent and perspectival situation. Anim. Reprod 12:498–504.

Rose, D. 1961. Factors affecting the pH-sensitivity of the heat stability of milk from individual cows. J. Dairy Sci. 44:1405–1413. doi:10.3168/jds.S0022-0302(61)89901-3.

Rutten, M.J.M., H. Bovenhuis, K. a Hettinga, H.J.F. van Valenberg, and J. a M. van Arendonk. 2009. Predicting bovine milk fat composition using infrared spectroscopy based on milk samples collected in winter and summer.. J. Dairy Sci. 92:6202–6209. doi:10.3168/jds.2009-2456.

SAS Institute. 2012. SAS/STAT Software. Release 9.4. SAS Institute inc., Cary, NC.

Sjaunja, L.-O., S.K. Andersen, B. Arnvidarson, N. Brems, T. Lapp, and L. Nygaard. 1994. Infrared attenuation measuring system. US Pat. No. Grant US5343044A.

Stocco, G., C. Cipolat-Gotet, T. Bobbo, A. Cecchinato, and G. Bittante. 2017a. Breed of cow and herd productivity affect milk composition and modeling of coagulation, curd firming, and syneresis. J. Dairy Sci. 100:129–145. doi:10.3168/jds.2016-11662.

Stocco, G., C. Cipolat-Gotet, V. Gasparotto, A. Cecchinato, and G. Bittante. 2017b. Breed of cow and herd productivity affect milk nutrient recovery in curd, and cheese yield, efficiency and daily production. animal 1–11. doi:10.1017/S1751731117001471.

Sturaro, E., E. Marchiori, G. Cocca, M. Penasa, M. Ramanzin, and G. Bittante. 2013. Dairy systems in mountainous areas: Farm animal biodiversity, milk production and destination, and land use. Livest. Sci. 158:157–168. doi:10.1016/j.livsci.2013.09.011.

Toledo-Alvarado, H., A. Cecchinato, and G. Bittante. 2017. Fertility traits of Holstein, Brown Swiss, Simmental and Alpine Grey cows are differently affected by herd productivity and milk yield of individual cows. J. Dairy Sci. in press.

Vanbergue, E., L. Delaby, J.L. Peyraud, S. Colette, Y. Gallard, and C. Hurtaud. 2017. Effects of breed, feeding system, and lactation stage on milk fat characteristics and spontaneous lipolysis in dairy cows. J. Dairy Sci. 100:4623–4636. doi:10.3168/jds.2016-12094.

VanRaden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn, and G.R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285–92. doi:10.3168/jds.S0022-0302(04)70049-1.

Vargas-Bello-Pérez, E., and P.C. Garnsworthy. 2013. Ácidos grasos trans y su rol en la leche de vacas lecheras. Cienc. e Investig. Agrar. 40:449–473. doi:10.4067/S0718-16202013000300001.

Vassen, P. 2003. The pH- and freezing point values of milk in the western southern cape and factors affecting these values. University of the Free State,.

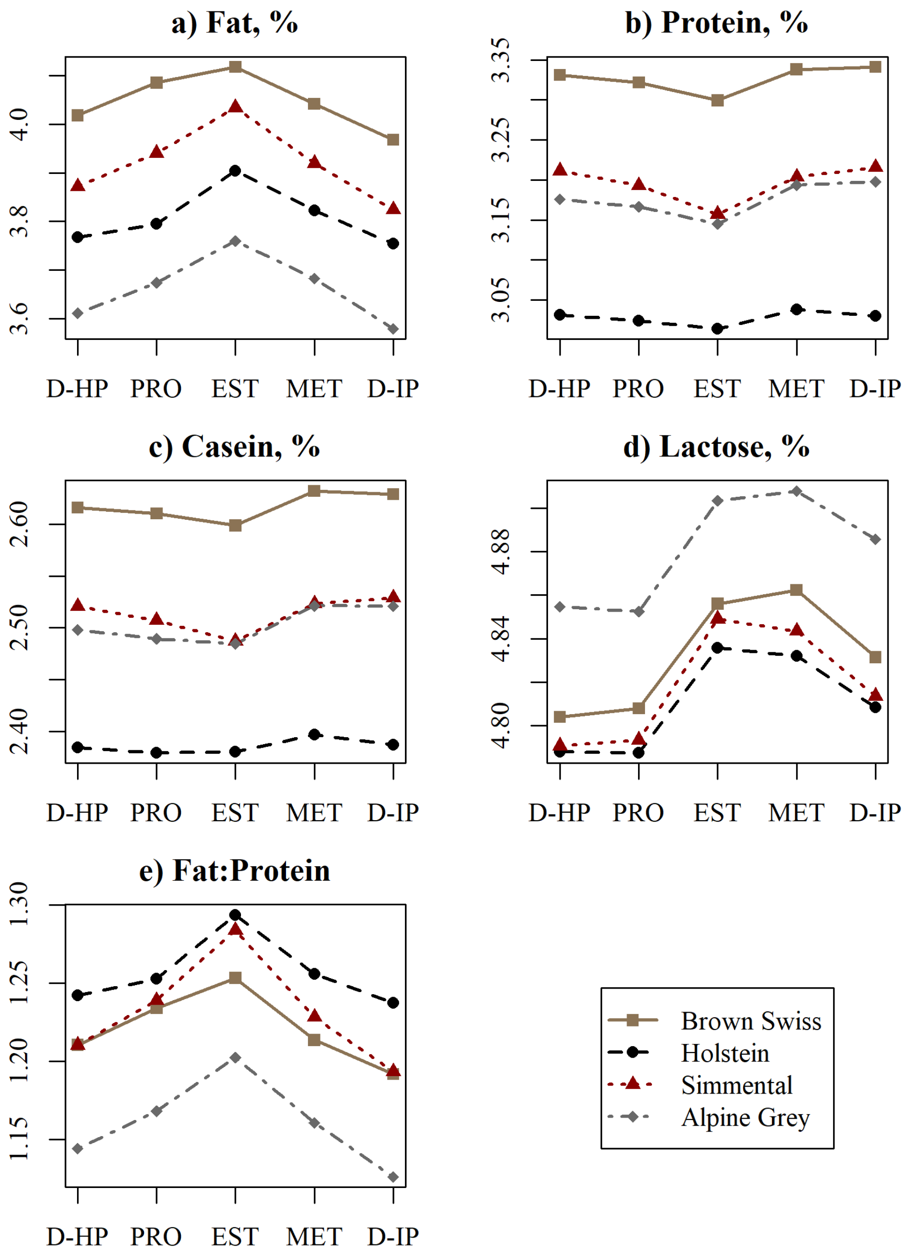
Wiking, L., J. Stagsted, L. Björck, and J.H. Nielsen. 2004. Milk fat globule size is affected by fat production in dairy cows. Int. Dairy J. 14:909–913. doi:10.1016/j.idairyj.2004.03.005.

Zagorska, J., and I. Ciprovica. 2013. Evaluation of Factors Affecting Freezing Point of Milk. World Acad. Sci. Eng. Technol. 7:393–398.

Zdunczyk, S., H. Zerbe, and M. Hoedemaker. 2003. Importance of estrogens and estrogenactive compounds for udder health in cattle. A review. Dtsch. Tierarztl. Wochenschr. 110:461–5.

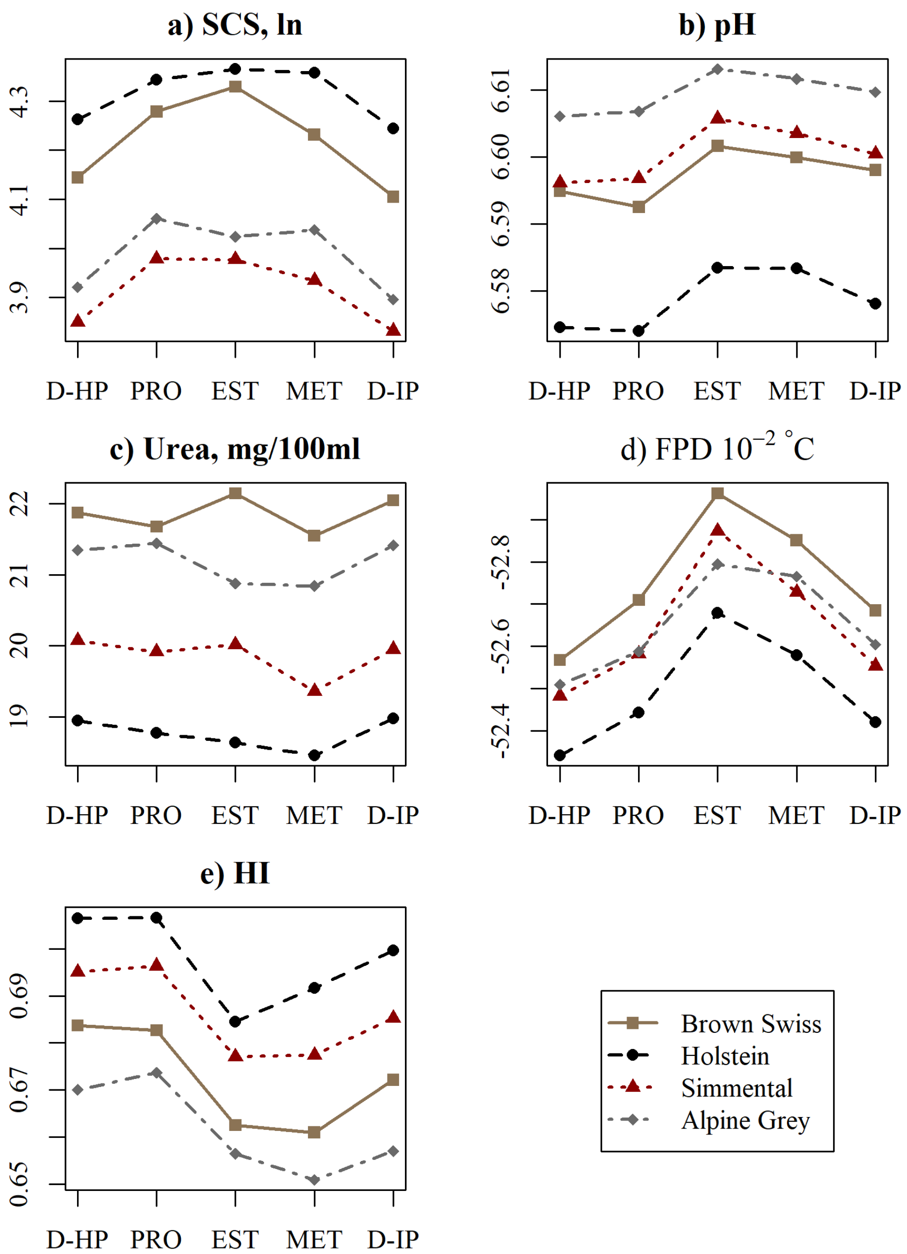
APPENDIX

Appendix 1. Least squares means of fat (Panel a), protein (Panel b), casein (Panel c), lactose (Panel d) and fat:protein ratio (Panel e) for the estrous cycle phases by breed.



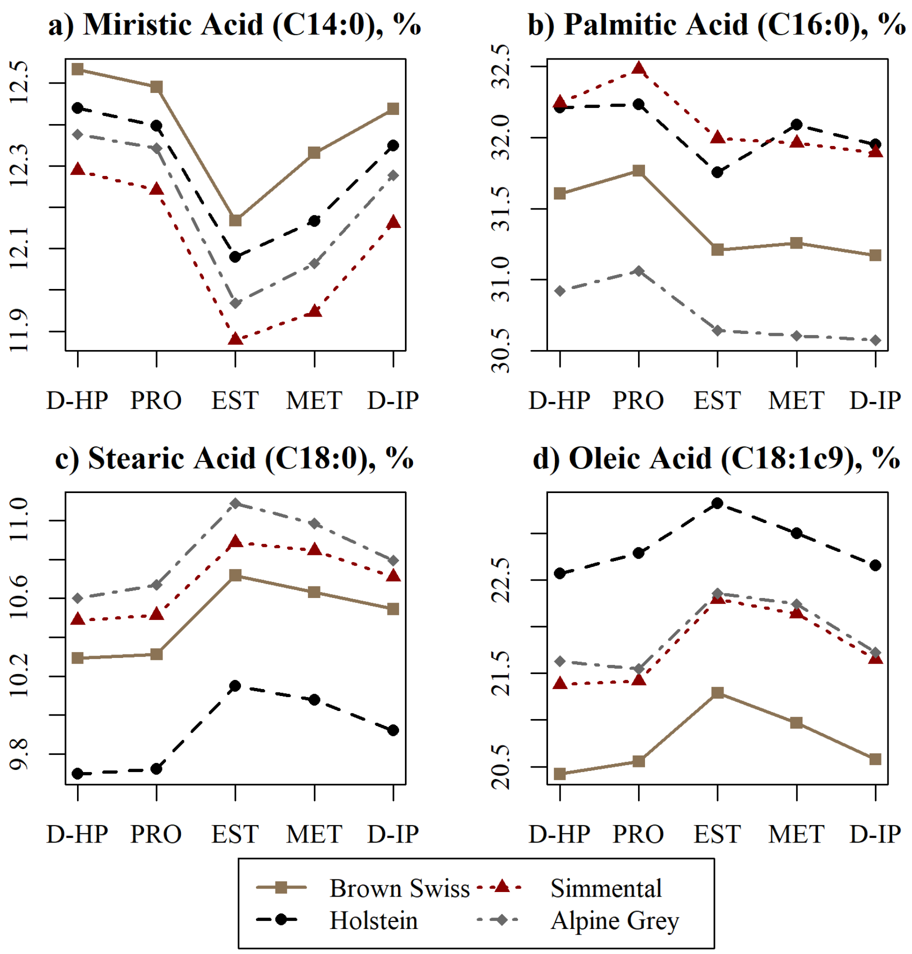
* D-HP = Diestrus High Progesterone; PRO = Proestrus; EST = Estrus; MET = Metestrus; D-IP = Diestrus Increasing Progesterone

Appendix 2. Least squares means of somatic cell score (SCS; Panel a), pH (Panel b), urea (Panel c), freezing point depression (FPD; Panel d), and homogenization index (HI; Panel e) for the estrous cycle phases by breed.



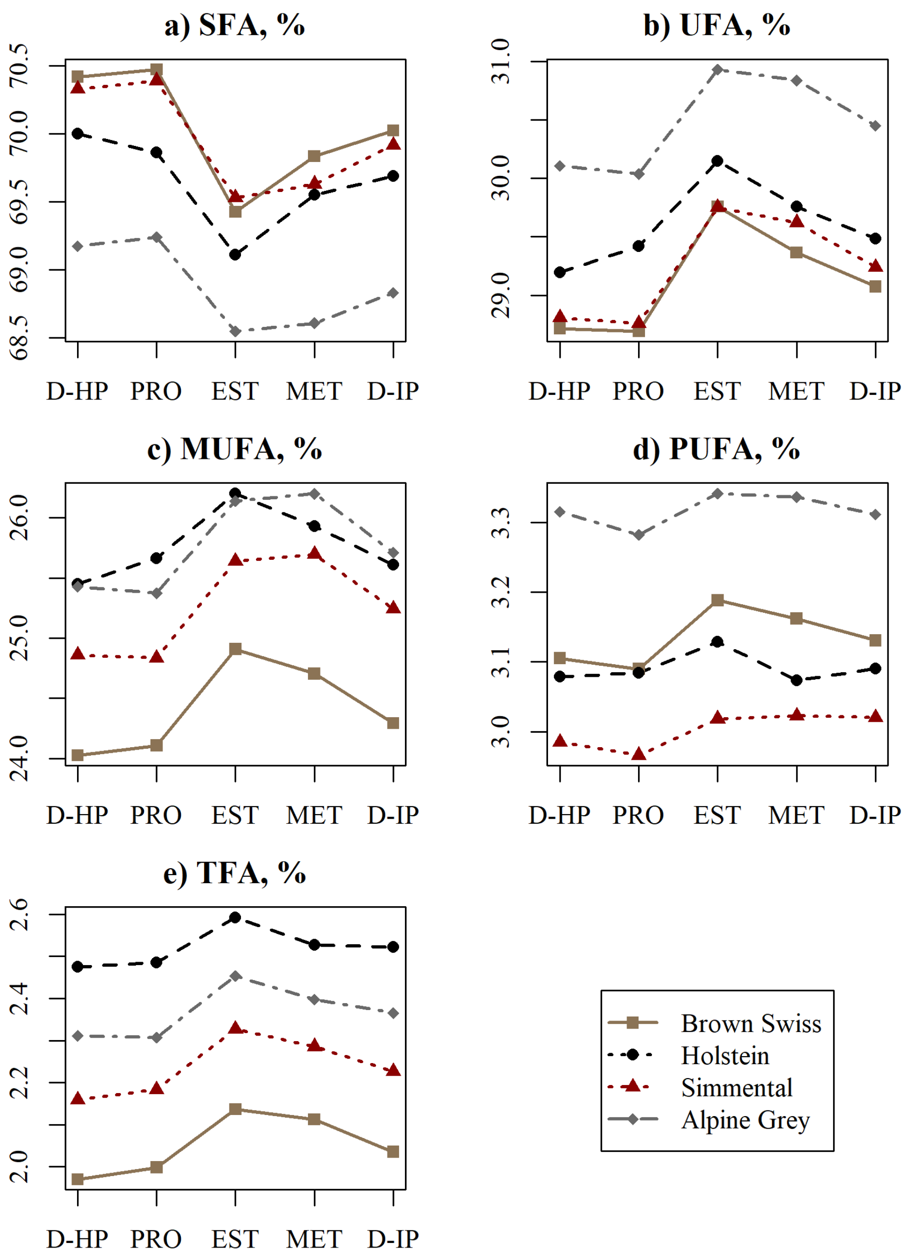
D-HP = Diestrus High Progesterone; PRO = Proestrus; EST = Estrus; MET = Metestrus; D-IP = Diestrus Increasing Progesterone

Appendix 3. Least squares means of miristic acid (C14:0; Panel a), palmitic acid (C16:0; Panel b), stearic acid (C18:0; Panel c) and oleic acid (C18:1*c*9; Panel d) for the estrous cycle phases by breed.



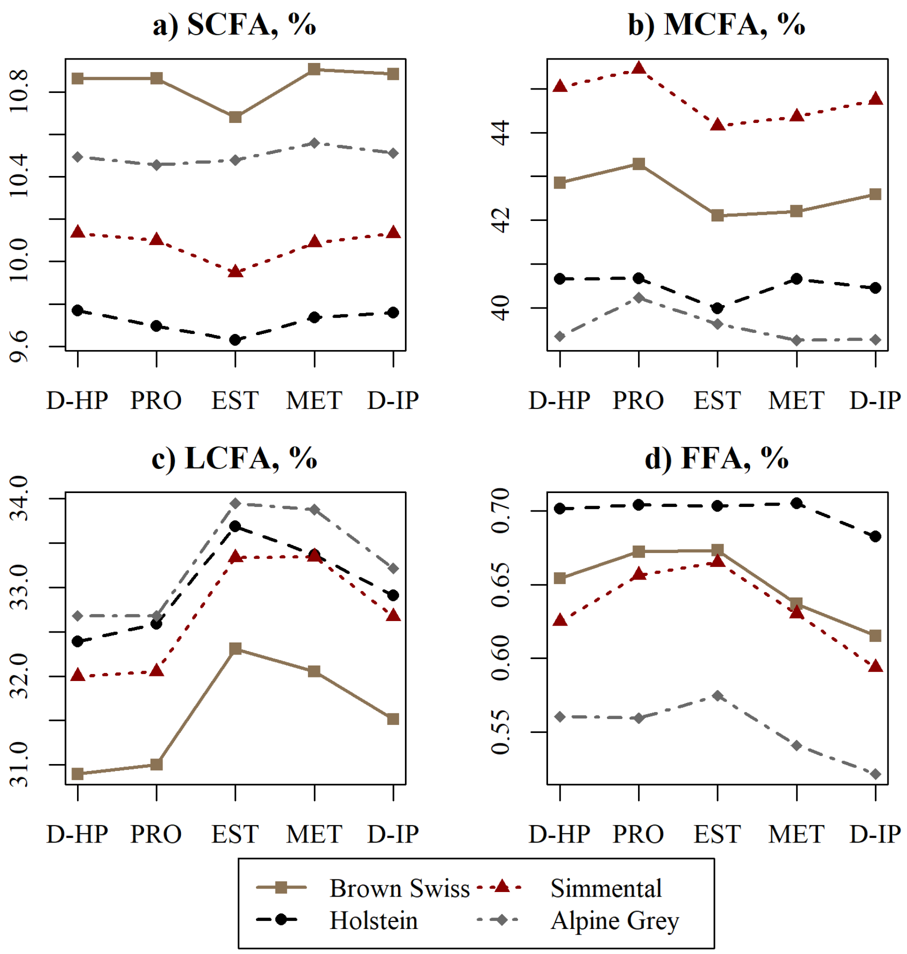
D-HP = Diestrus High Progesterone; PRO = Proestrus; EST = Estrus; MET = Metestrus; D-IP = Diestrus Increasing Progesterone

Appendix 4. Least squares means of saturated fatty acids (SFA; Panel a), unsaturated fatty acids (UFA; Panel b), monounsaturated fatty acids (MUFA, Panel c), polyunsaturated fatty acids (PUFA; Panel d) and trans-unsaturated fatty acids (TFA; Panel e) for the estrous cycle phases by breed.



* D-HP = Diestrus High Progesterone; PRO = Proestrus; EST = Estrus; MET = Metestrus; D-IP = Diestrus Increasing Progesterone

Appendix 5. Least squares means of short-chain fatty acids (SCFA; Panel a), medium-chain fatty acids (MCFA; Panel b), long-chain fatty acids (LCFA; Panel c) and free fatty acids (FFA; Panel d) for the estrous cycle phases by breed.



* D-HP = Diestrus High Progesterone; PRO = Proestrus; EST = Estrus; MET = Metestrus; D-IP = Diestrus Increasing Progesterone

CHAPTER IV

PREDICTING PREGNANCY STATUS WITH MILK

Diagnosing pregnancy status using infrared spectra and milk composition in dairy cows.

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INTERPRETIVE SUMMARY

Diagnosing pregnancy status using infrared spectra and milk composition in dairy cows.

By Toledo-Alvarado et al.

We assess the utility of individual wavelengths from milk infrared absorbance spectra, and complete milk infrared spectra and milk components for diagnosing pregnancy status of dairy cows. Overall, the most accurate predictions were obtained for the model that included the complete infrared spectra. A combination of milk components or different wavelengths were also useful for diagnosing pregnancy, with relative performance of various models being similar across breeds. This study provides new possibilities for pregnancy status screening of cows using milk infrared spectroscopy.

ABSTRACT

Data on Holstein (16,890), Brown Swiss (31,441), Simmental (25,845) and Alpine Grey (12,535) cows reared in north-eastern Italy were used to assess the ability of milk components (fat, protein, casein and lactose) and Fourier Transform Infrared **(**FTIR) spectral data to diagnose pregnancy. Pregnancy status (PS) was defined as to whether or not a pregnancy was confirmed by a subsequent calving and no other subsequent inseminations within 90 days of the breeding of specific interest. Milk samples were analyzed for components and FTIR full spectrum data using a MilkoScan FT+ 6000. The spectrum covered 1,060 wavenumbers (wn) from 5,010 to 925 cm^{-1}. The PS was predicted using generalized linear models (GLM) using fat, protein, lactose, casein and single FTIR spectral bands as predictors. We also fitted a GLM as a simultaneous function of all wavelengths (1,060 wn) with a Bayesian variable selection model using the BGLR R-package. Prediction accuracy was assessed using the area under a receiver operating characteristic curve assessed in a 10-fold cross validation (CV-AUC) based on sensitivities and specificities of phenotypic predictions. Overall, the best prediction accuracies were obtained for a model that included the complete FTIR spectral data. We also observed differences between breeds, the highest CV-AUC value being obtained for Alpine Grey cows (CV-AUC = 0.645), whereas Brown Swiss and Simmental had similar performance (CV-AUC = 0.630 and 0.628 respectively) followed by Holsteins (CV-AUC = 0.607). For single wavelength analyses, important peaks were detected at: from wn 2,973 to wn 2,872 cm^{-1} where Fat-B is usually filtered; wn 1,773 cm^{-1} where Fat-A is filtered; wn 1,546 cm^{-1} where protein is filtered; wn 1,468 cm^{-1} associated with urea and fat; wn 1,399 cm^{-1} and wn 1,245 cm^{-1} associated with acetone; from wn 1,025 cm^{-1} to wn 1,013 x cm^{-1} where lactose is filtered. In conclusion, this research provides new insights to alternative strategies for pregnancy screening on commercial herds.

Key words: FTIR-Spectroscopy, milk, milk components, pregnancy.

INTRODUCTION

Changing metabolic and energy requirements due to pregnancy in cows are likely to also change milk yield and milk composition. For example, a decline of milk yield during gestation in pregnant cows has been reported in several studies, becoming more evident after the third month of pregnancy (Olori et al., 1997; Loker et al., 2009) where the requirements of the fetus demand a significant amount of nutrients (Moe and Tyrrell, 1972). Pregnancy also affects milk composition with an increase of fat, protein, lactose and casein as pregnancy advances (Olori et al., 1997). Consequently, the effect of pregnancy has been suggested as an adjustment factor to increase the accuracy of genetic evaluations on production traits (Bohmanova et al., 2009). In fact, several countries have included pregnancy stage in their genetic evaluations for milk yield, fat and protein (Interbull, 2016).

A gradual decline in fertility, together with an increase of milk production, has motivated researchers to modify breeding programs (VanRaden et al., 2004) and management practices to reverse this trend. Early diagnosis tools that would help farmers to monitor the status for each animal are also needed. Fourier Transform Infrared (FTIR) spectroscopy is already globally used to routinely assess milk composition in milk recording programs (ICAR, 2016); for instance, FTIR data is routinely used to assess milk components (fat, protein, casein, lactose, total solids, urea, citric acid, free fatty acids, some individual fatty acids and groups), freezing point, pH and ketosis screening. In addition, FTIR spectroscopy has been used to predict many other detailed phenotypes such as fatty acid profiles, protein fraction compositions, free amino acids and milk coagulation properties (De Marchi et al., 2014). Other phenotypes having direct relationships with milk composition have been also studied, such as body energy status, feed and energy intake (McParland and Berry, 2016). In relation to health and fertility of cattle, Bastin et al., (2016) studied the use of FTIR spectroscopy in milk as an effective indicator of health and fertility, associating acetone and β-hydroxybutyrate with ketosis, and various fatty acids (e.g., C18:1 *cis*-9 and C10:0) to fertility. Finally, Lainé et al., (2017) reported a direct effect of pregnancy on milk composition of Holsteins, and on their milk FTIR spectrum: the absorbance of 212 waves were affected by pregnancy, especially in the infrared spectral region from wavenumber 1,577 to 968 × cm^{-1} (transition from mid- to long-infrared sections of the spectrum).

What has not yet been studied is the possibility to discriminate between pregnant versus open cows simply using whole-spectrum FTIR profiles. Therefore, the objective of this study was to assess and to compare the prediction accuracies of a reproductive outcome (pregnancy status) that can be achieved using milk components derived from spectra data (fat, protein, casein and lactose) as well as single-band and whole-spectrum FTIR data. Our study is based on data generated within the Italian milk recording systems of Holstein, Brown Swiss, Simmental and Alpine Grey cattle breeds.

MATERIALS AND METHODS

Data

Production and female fertility data was collected from farms in the northeastern Bolzano/Bozen province in Italy by the Breeders Federation of Alto Adige/Südtirol (Associazione Provinciale delle Organizzazioni Zootecniche Altotesine / Vereinigung der Südtiroler Tierzuchtverbände, Bolzano/Bozen, Italy). Management systems were rather heterogeneous ranging from the traditional small farms of the mountainous areas to more modern and larger operations elsewhere. A good description of the dairy farms in the region is provided by Sturaro et al., (2013) and by (Stocco et al., 2017a). Data included records generated from 2010 to 2016, on Holstein, Brown Swiss, Simmental and Alpine Grey cows.

Pregnancy Definition and Data Editing

A cow's pregnancy status (PS) was coded as a binary variable, based on whether a subsequent insemination was not recorded within 90 days after putative conception and confirmed by subsequent calving (PS = 1) versus an insemination being registered within the 90 days period (PS = 0). Otherwise PS was set to unknown. The interval between consecutive inseminations was required to be greater than 3 days in accordance with ICAR guidelines (ICAR, 2016). Only records made ≤ 91 days after each insemination were kept, since the percentage of open cows was very low by week 13 after insemination: (7%, 6%, 3%, and 3% for Holstein, Brown Swiss, Simmental and Alpine Grey respectively). The proportions of pregnant and open cows by weeks after the inseminations for each breed are available in Appendix 1. Gestation length was restricted to be within 30d from the average for each breed (Holstein = 281d; Brown Swiss = 290d; Simmental = 285d; Alpine Grey = 287d). The calving interval was restricted to be less than $(\mu + 3sd)$ for all breeds. Only records with DIM ≤ 305 d were considered whereas parity was classified as 1, 2, 3 and ≥ 4 parities. A detailed description of fertility traits as well as data editing is reported in Toledo-Alvarado et al., (2017).

FTIR Spectra

All milk samples were analyzed using a MilkoScan FT+ 6000 (Foss Electric, Hillerød, Denmark) in the laboratory of the Federazione Latterie Alto Adige / Sennereiverband Südtirol (Bolzano/Bozen Italy). The spectrum covers from the Short-Wavelength Infrared (SWIR) to the Long-Wavelength Infrared (LWIR) regions with 1,060 spectral points from wavenumber 5,010 to 925 × cm^{-1}, which correspond to wavelengths 1.99 to 10.81 μm and frequencies from 150.19 to 27.73 THz. The spectrum transmittances (T) were transformed to absorbances (A) with the equation $A = \log(1/T)$. A principal component analysis was performed on the FTIR Spectra with Mahalanobis distances calculated from the first 5 principal components scores. The probability level for the chi-squared distribution of a sample's Mahalanobis distance was calculated from the incomplete gamma function with 5 degrees of freedom. Samples having a probability level < 0.01 were considered to be outliers and removed from the data set (Shah and Gemperline, 1989). To explore spectra variation over time, the first 5 principal components were plotted over time and inspected. Major shifts were detected over different year periods, therefore to overcome the spectral variations the absorbance values for every wave were centered to a null mean and standardized to a unit sample variance within year periods.

Milk Yield and Composition

Milk yield and composition records were obtained from the official milk recording system of Bolzano/Bozen province from Associazione Provinciale delle Organizzazioni Zootecniche Altoatesine / Vereinigung der Südtiroler Tierzuchtverbände (Bolzano/Bozen Italy) and consisted of daily milk yield (kg/d), and of fat, protein, casein and lactose percentages analyzed from FTIR spectra according to internationally approved methods (ICAR, 2016). After editing, the number of records with milk production, composition, FTIR-Spectrum and fertility information per breed were 88,980 for Holstein, 176,698 Brown Swiss, 150,596 Simmental and 73,825 Alpine Grey (Table 1).

Calibration Models

Separate analyses for each breed including different effects in the model were used to predict the PS of cows. First, PS was predicted using milk components, one component at a time (fat, protein, casein, and lactose) with and without the inclusion of the effects of parity and days in milk (DIM). Then the PS was predicted using each of the 1,060 spectral points, one wavelength at a time. Finally, we included the complete FTIR-Spectra, and then the complete FTIR spectra along with parity and DIM effects to predict PS. The description of the covariates included in each model, labeled as Models 0 through 10, is in Table . All our analyses were based on a generalized linear model with a probit link. Specifically, at the liability level, we assumed a linear model of the form

$$l_i = \beta_0 + \sum_{j=1}^{p} \beta_j x_{ij} + \varepsilon_i,$$

where β_0 is an intercept, $[x_{i1}\, x_{i2} \ldots x_{ip}]$ represent the covariates (or dummy variables derived from it in the case of categorical predictors) listed in Table 2, $\beta_j = (\beta_1, \ldots, \beta_p)'$ are the effects of the covariates and ε_i is an error term assumed to be IID with mean zero and unit variance. The classification rule was set to $\{\, y_i = 1 \; if \; l_i > 0 \; ; 0 \; otherwise\}$. Therefore,

$$Pr(y_i = 1|x_i) = Pr(l_i > 0|x_i) = \Phi\left(\beta_0 + \sum_{j=1}^{p} \beta_j x_{ij}\right)$$

where $\Phi(.)$ represents the cumulative distribution function of the standard normal distribution.

Models 0-8 include a number of predictors that is small relative to sample size; therefore for these models effects were estimated by maximum likelihood using the GLM function of R. On the other hand models 9 and 10 include large numbers of predictors. In this case effects were estimated using a Bayesian model with effects of covariates (DIM and parity) treated as fixed and with the effects of the FTIR spectra treated as random; specifically following Ferragina et al., (2015) for the effects of FTIR spectra we used a mixture prior with a point of mass at zero and a t-slab; a modified version of model BayesB (Meuwissen et al., 2001) implemented in the BGLR R-package (Pérez and De Los Campos, 2014). For models 9 and 10 a total of 50,000 samples were drawn and the first 10,000 were discarded as burn-in.

Assessment of Prediction Accuracy

Sensitivity (TPr) defined as

$$TPr = true\ positives/(true\ positives + false\ negatives)$$

and specificity (FPr) defined as

$$FPr = true\ negatives/(false\ positives + true\ negatives)$$

are the most commonly used measures of classification accuracy. However, both measures depend upon the decision threshold such as the threshold increases, the number of TPr and FPr both monotonically increase. The projection of these pairs on a plane defines a curve, often referred as to the Receiver Operating Characteristic (**ROC**) Curve, which is typically used to assess the performance of a diagnostic tool (Fawcett, 2006). The most usual performance measure is the Area Under the ROC Curve (**AUC**), denoted as:

$$AUC = \frac{1}{mn}\sum_{i=1}^{m}\sum_{j=1}^{n} 1_{p_i > p_j},$$

where i runs over all m data points having a true positive classification and j runs over all data points having a true negative classification p_i and p_j denote the probability score assigned by classifier to data point i and j. For interpretation, the AUC is the probability that a classifier will rank a randomly chosen true positive instance higher than a randomly chosen true negative case. Since the AUC is a portion of the area of the unit square, its value will always be between 0 and 1, where an AUC of 0.5 is a random guess (Fawcett, 2006). Therefore, in order to assess the performance of milk components and FTIR-spectra as a diagnosis tool of pregnancy status of cows, the cross validation AUC (**CV-AUC**) was estimated as a summary metric between the predictions and the phenotypes in the testing sets of the ten-fold cross-validation with the R package pROC (Robin et al., 2011). All models were compared using Wilcoxon's test (paired tests corrected with false discovery rate method for multiple testing) applied to the CV-AUC achieved by each model. In addition each CV-AUC mean was tested (Wilcoxon's test) to be greater than 0.5 which is the CV-AUC of a random classifier. For the individual wavelength analysis, a Manhattan plot was created for each breed with the $-\log_{10}(P\text{-value})$ of the wavelength effect corrected with the false discovery rate method (Figure 1). All data editing and statistical analysis was done in the R environment (R Core Team, 2016)

RESULTS AND DISCUSSION

Fertility, Milk Yield and Composition

Table 1 shows the number of cows and insemination records for the four breeds analyzed. The number of records per cow was 5.6 for Brown Swiss cows (*n* = 176,698), 5.8 for Simmental (n = 150,596), 5.2 for Holstein (*n* = 88,980) and 5.8 for Alpine Grey (*n* = 73,825) across different parities. Additionally, the proportion of pregnant and open cows by weeks after the insemination is available for each breed in Appendix 1. The pregnancy rate was over 10 percentage points higher for the dual-purpose breeds Simmental (78.1%) and Alpine Grey (79.4%), compared to the specialized dairy breeds Holstein (67.9%) and Brown Swiss (68.9%). Dairy and dual-purpose breeds are different in terms of average fertility, as well as they react differently to the variations of milk productivity, both in terms of herd average and of individual cow production within herd (Toledo-Alvarado et al., 2017). Similar pregnancy rates, measured as non-return rate after 56 d, have been obtained by Tiezzi et al., 2011, 2015) on a different dataset relative to Brown Swiss cows in the same mountainous area.

Descriptive statistics for milk yield and components used for predicting PS are presented in Table 1. Holstein cows had the highest milk production but the lowest content of protein, casein and lactose. Relative to the Holsteins, the Brown Swiss cows had a lower daily milk yield (-16%), but higher percentages for fat (+5%), protein (+9%) and casein (+9%). The double purpose Simmental cows had a slightly greater milk production than the Brown Swiss cattle and lower milk contents. The Alpine Grey cows had the lowest mean milk yield (-36%) but similar milk composition relative to the Holsteins. These differences are due to both breed specific genetic and herd management conditions (Stocco et al., 2017a; b). For example, Holstein cows are generally reared within intensive dairy management systems (farms using free stall housing, milking parlors and total mixed rations), whereas the Alpine Grey cows typically are raised on traditional farms (tie stalls, hay feed supplemented with some concentrates, etc.). Conversely, Brown Swiss and Simmental cows can be readily found in both types of dairy management systems. More details on different farming systems in the Alps have been reported in Sturaro et al. (2013).

Diagnosis of Pregnancy Status according to Milk Composition

The CV-AUC for all the prediction models was estimated between predicted and observed PS for each fold created in the cross-validation procedure. Then the mean, minimum and maximum CV-AUC were estimated and the results for the four breeds are presented in the Table 3.

Using just the fat content of milk as a predictor showed a trivial increase of CV-AUC respect to the value (0.500) expected for a random classifier. Lactose content was only slightly more informative. On the contrary, the protein content was informative to predict PS, whereas casein content yielded slightly lower CV-AUC values than protein ($P < 0.05$). A model jointly fitting fat and protein had predictive ability similar than the protein or casein alone indicating further that the fat content did not give valuable information for predicting PS once differences in protein were accounted for. Similarly, the addition of casein and lactose only mildly increased CV-AUC. The cow's information of parity number and DIM had predictive ability higher than the milk components alone or combined with each other; however, it was only slightly improved when combined with milk components (fat, protein, casein and lactose) added to the model (Table 3).

We are not aware of any previous work on the prediction of PS from milk composition. Nevertheless, it is interesting to note that pregnancy affects milk composition (Olori et al., 1997; Loker et al., 2009); therefore milk composition should be useful for the diagnosis of PS. Nevertheless, prediction accuracy of PS is limited and only seems useful for preliminary screening. Prediction of PS directly from milk composition is more practical than the one also considering the information from the cow as parity and DIM; these data sources are often disconnected.

It is also interesting to note that the ranking of predictive ability of different models are about the same for all the 4 breeds tested (Table 3) despite their widely different production levels, genetic backgrounds, and management conditions. Also, CV-AUC values were similar for all models based on milk components, even though it should be noted that a slight increase in CV-AUC from Holstein and Brown Swiss cows to Simmental and Alpine Greys cows when the model also included milk composition and other cow-specific information.

Association of Pregnancy Status to Individual Wavelength's Absorbances of Milk FTIR Spectrum

Milk composition is generally estimated either based on the absorbance of milk at specific infrared wavelengths (Kaylegian et al., 2006) or based on the entire absorbance spectrum using specific chemometrics procedures (ICAR, 2016). In our study, we predicted PS directly from the absorbance of milk spectra, as well as using milk composition inferred by spectra data to, in turn, predict PS. Therefore, the wavenumber 1,546 cm^{-1}, which obtained the highest CV-AUC, was included in Table 3. In this region the protein is usually filtered from 1,546 x cm^{-1} (6.46 μm) to 1,492 x cm^{-1} (6.70 μm) (Kaylegian et al., 2006; Lynch et al., 2006). Therefore, it is not surprising that the CV-AUC of this wavenumber was similar to the values obtained with the models that included the protein or casein.

The milk FTIR absorbance measures of individual wavelengths or the whole spectra in the MIR range can be used to predict phenotype (Karoui et al., 2010). Several chemical bonds are responsible for the absorption of electromagnetic radiations at specific wavelengths in the area of short-wavelength infrared (SWIR [NIR]) and mid-wavelength infrared (MWIR [MIR]) (Bittante and Cecchinato, 2013). A preliminary analysis was carried out to associate each individual wavelength, based on the graphical representation of the $-\log_{10}(P\text{-value})$ obtained by the 1,060 models (one for each wavelength) to predict PS, the Manhattan plot is presented in Figure 1. One can easily identify the specific regions with important prediction capability on PS. The SWIR (or NIR) region from wavenumber 5,010 to 3,673 x cm^{-1} (wavelengths 2.00 to 2.72 μm) has no specific chemical bounds related to milk (Soyeurt et al., 2011; Bittante and Cecchinato, 2013); however a small significant peak was observed around the individual wavenumber 3,683 cm^{-1} (wavelength 2.72 μm) at the border between the SWIR and SWIR-MWIR regions of the spectrum. In this same area, Bittante and Cecchinato, (2013) founded a decrease of genetic variance and heritability coefficients of absorbance. Nevertheless, this wave is very close to those known for characterizing the bonds $C{=}CH_2$, and O-H (typical of alcohols, phenols and carboxylic acids). The region SWIR-MWIR (transition between NIR and MIR) extents from wavenumber 3,669 to 3,052 x cm^{-1} (wavelengths 2.73 to 3.28 μm), is known for being affected by a very high absorbance variance due to the effect of water content of milk, and it is often excluded from chemometrics for predicting milk components. In this area, estimated *P*-values were seemingly non-important so it could be also excluded for prediction of PS.

The MWIR-1 region (MIR) spans from 3,048 to 1,701 x cm^{-1} (corresponding to wavelengths 3.28 to 5.88 μm), this area is important for prediction of fat. The major absorbance peaks detected are bonds: C–H, C=O, C–N and N–H (Bittante and Cecchinato, 2013). In this region some peaks with medium prediction capability of PS were detected. The first important bandwidth is located between 2,973 to 2,872 x cm^{-1} (wavelengths 3.36 to 3.48 μm), in this region Fat-B (absorbance by the carbon-hydrogen stretch [C-H]) is usually filtered (2,873 to 2,777 x cm^{-1} [3.48 to 3.60 μm])(Kaylegian et al., 2006; Lynch et al., 2006), in this same bandwidth Lainé et al., (2017) found that the effect of pregnancy was higher than that of milk fat contents at an early stage of pregnancy. Then, after a gradual increase of significant values it is reached a flat region of prediction between 2,344 to 1,777 x cm^{-1} (wavelengths 4.27 to 5.63 μm). The second wavenumber notable in this region is 1,773 x cm^{-1} (5.64 μm) where usually the so-called Fat-A (absorbance by the ester carbonyl stretch [C=O]) is filtered from 1,785 to 1,747 x cm^{-1} (wavelengths 5.60 to 5.72 μm) when fat is to be predicted using a single small spectrum fraction (Kaylegian et al., 2006; Lynch et al., 2006). The small MWIR-2 region covers from 1,698 to 1,586 cm^{-1} (corresponding to wavelengths 5.89 to 6.31 μm) and it is also related with the absorbance of water and H-O-H bending, which increase the variability coefficients for the transmittance among different milks samples and decrease the capability of prediction for PS and no important peaks were detected here.

The last MWIR-LWIR region (from mid- to long-infrared) spans from wavenumber interval 1,582 to 925 x cm^{-1} (corresponding to wavelengths 6.32 to 10.81 μm), it is the so-called fingerprint area. This region had the highest *P*-values for the single wavelengths. This region harbors several peaks of absorbance relative to bonds: C-H, aromatic C=C, C–O and N–O (Bittante and Cecchinato, 2013). The most important signal for the prediction of PS is found at 1,546 cm^{-1} (6.46 μm), where the protein is usually filtered from 1,546 x cm^{-1} (6.46 μm) to 1,492 x cm^{-1} (6.70 μm) (Kaylegian et al., 2006; Lynch et al., 2006). Therefore, the wavenumber 1,546 cm^{-1}, which obtained the highest CV-AUC, was included in Table 3, and it is not surprising that the CV-AUC of this wavenumber was similar to the values obtained with the models that included the protein or casein content of milk. Lainé et al., (2017) described a relative effect of pregnancy on this same wavelength bigger than that of the protein content itself in the early stage of pregnancy.

The second important wavenumber is 1,468 x cm^{-1} (6.81 μm) that has been associated with urea at 1,469 x cm^{-1} (6.81 μm) and fat 1,460 x cm^{-1} (6.85 μm)(Hansen, 1998). The next two peaks 1,399 x cm^{-1} (7.15 μm) and 1,245 x cm^{-1} (8.03 μm), are related to acetone predictions (1,238 x cm^{-1}, [8.08 μm]) (Hansen, 1999). Finally, the last important waves are located from 1,025 x cm^{-1} (9.76 μm) to 1,013 x cm^{-1} (9.87 μm) close to the region where usually lactose is filtered (1,040 x cm^{-1}, [9.62 μm])(Kaylegian et al., 2006; Lynch et al., 2006). Also in this case the CV-AUC values obtained for the four breeds tested were very similar demonstrating that the relationship between spectrum and PS is based on basic physiological functions and are not breed specific, despite the differences in both milk yield and composition and also fertility among the different breeds.

Diagnosis of Pregnancy Status according to Whole Milk FTIR Spectrum

The second approach consisted in using all wavelengths of the FTIR spectrum (1,060 absorbance values for each milk sample) as predictors of PS in a Bayes B model (Pérez and De Los Campos, 2014). The absorbance at some waves is characterized by the effect of specific chemical bonds, thus a procedure based on variable selection and shrinkage seems to present some advantages respect to a statistical approach based on principal components analyses, like PLS frequently used, especially for complex traits like PS. Ferragina et al., (2015), compared the Bayesian Ridge Regression, Bayes A, Bayes B, and with PLS models, obtaining the best prediction accuracy using Bayes B model for the prediction of complex traits. The use of the entire spectrum lead to the greatest CV-AUC estimate in all the breeds (Table 3) than those achieved using individual wavelengths, the composition of milk (fat, protein, casein and lactose) and also composition of milk with the inclusion of cow's information (parity and DIM). Integrating information from the cow together with the spectral data exerted a negative effect slightly depressing the CV-AUC values in all breeds.

These results are in opposite direction than the ones for prediction accuracy of milk components. As the model including fat, protein, casein and lactose increased the CV-AUC when the parity and DIM were included, pointing out that these effects are more informative when using the milk components alone, but not as explicative as the all FTIR-Spectrum.

Here, the FTIR-Spectra for Alpine Grey cows with an CV-AUC of 0.645 was the best classifier of PS, followed by Brown Swiss and Simmental breeds (0.630 and 0.628, respectively) and the lowest value for FTIR-spectrum was observed in Holstein cows with 0.607.

These results show that, among the models tested, the entire FTIR spectrum without any supplemental information allows the most informative prediction. This means that this prediction could be directly implemented in the FTIR spectrometer simply installing a proper calibration and the results could be obtained on all milk sample routinely analyzed for their chemical composition (fat and protein) without additional costs and work-time required. The possibility of practical application for pregnancy screening requires further research for improving the basic knowledge of the relationships between milk infrared spectrum and the physiological change of the lactating cows following conceptions and for improving the predictive ability of spectral data.

CONCLUSIONS

Predicting PS using milk FTIR spectra is very difficult because of its complicated nature limiting the predictive ability and it is probably due to the fact that PS is a phenotype indirectly correlated with milk composition. However, it was demonstrated that milk composition, especially its contents of protein and casein, has low but positive predictive ability of PS and that the direct use of the entire FTIR spectrum allows an increase of accuracy of prediction, without the need of specific information on the cow. The results are still limited with low CV-AUC for practical use in the milk lab. But these results showed that some important regions in the milk spectrum are useful for predictions of PS and pose the basis for a deeper understanding of the complex relationships between the milk spectrum, milk composition and the physiological status of the cows. The signal seems to have similar effects and in the same regions in dairy cows and also in the dual-purpose breeds here evaluated. Further, parts of the spectrum where associated to or not associated at all to PS, revealing which type of milk composition could be more affected in early pregnancy. Potential of FTIR-spectrum as an extra tool for the farmer for surveillance of PS in the cows requires further research to improve accuracy of prediction and to implement the methodologies in farming conditions.

ACKNOWLEDGMENTS

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TABLES

Table 1. Number of cows and records, rate percentage of pregnant cows (PS) and descriptive statistics of days in milk (DIM) days open (DO) and milk yield and composition for each breed.

	Holstein	Brown Swiss	Simmental	Alpine Grey
Cows	16,890	31,441	25,845	12,535
Inseminations (N):				
Total	88,980	176,698	150,596	73,825
Successful	60,434	121,855	117,677	58,635
Pregnancy (%)	67.9	68.9	78.1	79.4
Interval traits (d):				
DIM	164.9±60.0	158.6±59.5	142.6±54.6	142.9±52.6
DO	156.5±76.2	148.9±76.5	119.1±63.3	118.0±60.4
Milk yield, kg/d	28.3±6.6	23.6±5.9	24.5±6.1	17.9±4.9
Milk composition,%:				
Fat	3.95±0.65	4.17±0.60	3.97±0.61	3.72±0.52
Protein	3.31±0.31	3.62±0.33	3.43±0.31	3.38±0.31
Casein	2.60±0.24	2.84±0.25	2.69±0.24	2.65±0.23
Lactose	4.76±0.17	4.76±0.18	4.76±0.17	4.81±0.19

Table 2. Effects included in each model for the prediction of pregnancy status[1]

Model	DIM	Parity	Fat	Protein	Casein	Lactose	wn_j	FTIR-Spectrum
0	✓	✓						
1			✓					
2				✓				
3					✓			
4						✓		
5			✓	✓				
6			✓	✓	✓	✓		
7	✓	✓	✓	✓	✓	✓		
8							✓	
9								✓
10	✓	✓						✓

[1]wn_j = individual wavenumbers (j = 1…1,060)

Table 3. Mean, minimum and maximum estimated for the CV-AUC estimated between predicted and observed pregnancy status (PS) for each breed after 10 fold cross-validation using different effects

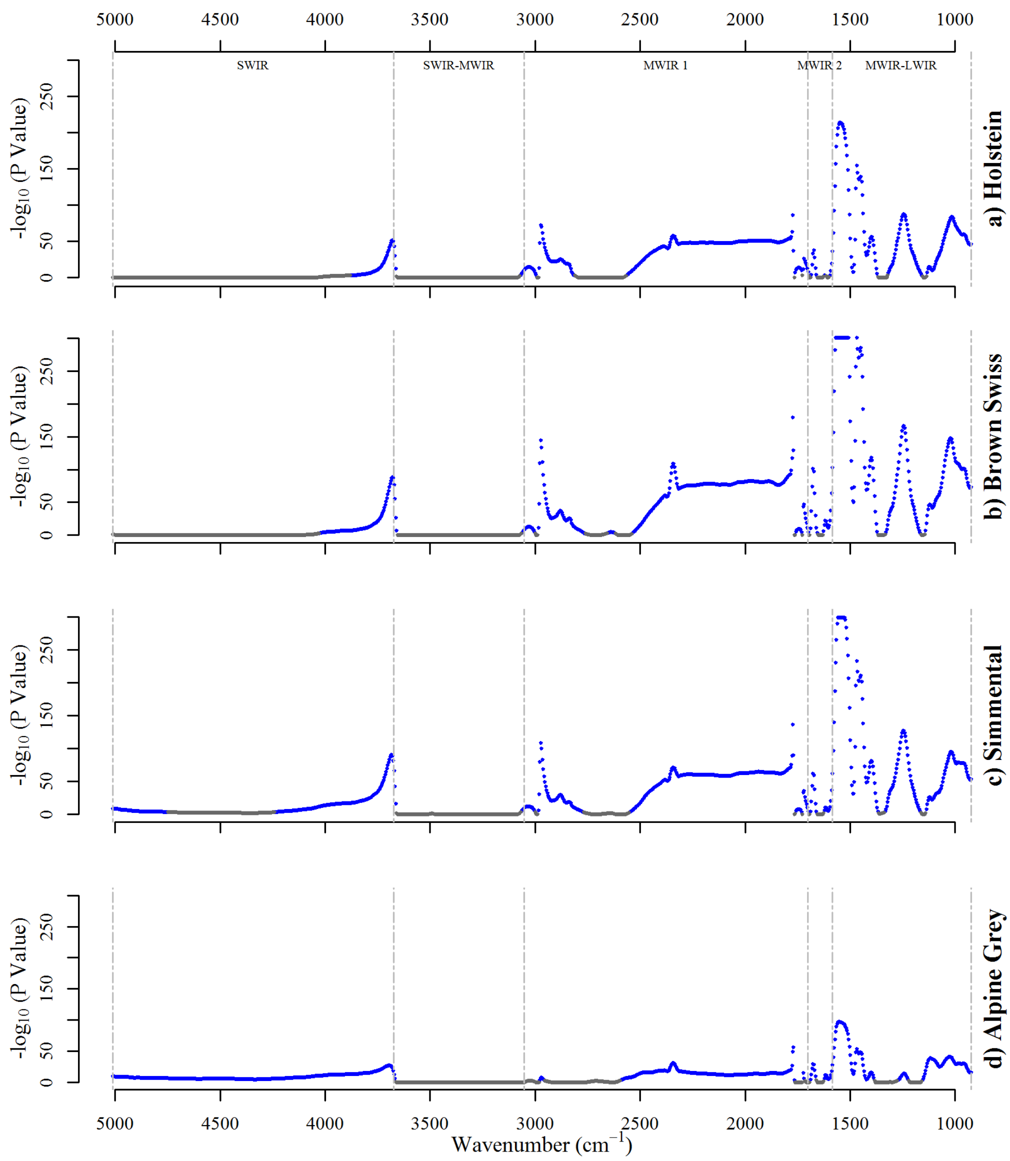
	Holstein			Brown Swiss			Simmental			Alpine Grey		
Effects in the model	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Parity, DIM	[f]0.598	0.587	0.604	[g]0.593	0.583	0.601	[h]0.612	0.603	0.620	[g]0.630	0.618	0.639
Fat	[a]0.522	0.512	0.530	[a]0.514	0.507	0.522	[a]0.515	0.506	0.529	[a]0.511	0.502	0.536
Protein	[d]0.566	0.557	0.572	[d]0.566	0.559	0.575	[de]0.571	0.566	0.578	[d]0.557	0.545	0.568
Casein	[c]0.563	0.556	0.570	[c]0.565	0.557	0.572	[c]0.569	0.565	0.578	[c]0.555	0.544	0.566
Lactose	[b]0.536	0.528	0.544	[b]0.540	0.536	0.545	[b]0.534	0.527	0.545	[b]0.536	0.523	0.551
Fat, Protein	[d]0.566	0.558	0.574	[e]0.567	0.561	0.576	[e]0.571	0.566	0.580	[cd]0.557	0.546	0.572
Fat, Protein, Casein, Lactose	[e]0.576	0.566	0.583	[f]0.577	0.568	0.585	[f]0.580	0.574	0.586	[e]0.570	0.557	0.587
Fat, Protein, Casein, Lactose, Parity, DIM	[g]0.603	0.590	0.611	[h]0.600	0.591	0.606	[i]0.615	0.605	0.622	[g]0.632	0.616	0.644
Wavenumber 1,546 (cm^{-1})	[cd]0.566	0.555	0.576	[cde]0.566	0.561	0.574	[cd]0.569	0.564	0.577	[cd]0.556	0.547	0.570
FTIR-Spectrum	**[g]0.607**	0.601	0.616	**[j]0.630**	0.626	0.639	**[j]0.628**	0.618	0.635	**[h]0.645**	0.626	0.656
FTIR-Spectrum, Parity, DIM	[e]0.580	0.573	0.594	[i]0.611	0.605	0.617	[g]0.597	0.588	0.601	[f]0.612	0.592	0.624

Wilcoxon-test [$H_1 = \mu > 0.5$]: all values reported are significantly different than the expected value of a random classifier ($P < 0.001$).

[a-j] Means within the same column with different superscript letters are significantly different ($P < 0.05$).

FIGURES

Figure 1. Manhattan plot of $-\log_{10}$(P-value) for the FTIR-spectrum wide association studies on pregnancy status (PS) of Holstein (Panel a), Brown Swiss (Panel b), Simmental (Panel c) and Alpine Grey (Panel d) cows. Blue dots indicate a significant effect of the single wavenumber on PS (P-value < 0.001); grey dots are non-significant wavenumbers (P-value ≥ 0.001). The P-values were corrected with the false discovery rate procedure.



*SWIR = short-wavelength infrared or near-infrared (1.40-3.0 μm); MWIR = mid-wavelength infrared (3.0-8.0 μm); LWIR = long-wavelength infrared (8.0-15 μm).

REFERENCES

Bastin, C., L. Théron, A. Lainé, and N. Gengler. 2016. On the role of mid-infrared predicted phenotypes in fertility and health dairy breeding programs. J. Dairy Sci. 99:4080–4094. doi:10.3168/jds.2015-10087.

Bittante, G., and A. Cecchinato. 2013. Genetic analysis of the Fourier-transform infrared spectra of bovine milk with emphasis on individual wavelengths related to specific chemical bonds.. J. Dairy Sci. 96:5991–6006. doi:10.3168/jds.2013-6583.

Bohmanova, J., J. Jamrozik, and F. Miglior. 2009. Effect of pregnancy on production traits of Canadian Holstein cows. J. Dairy Sci. 92:2947–2959. doi:http://dx.doi.org/10.3168/jds.2008-1782.

Fawcett, T. 2006. An introduction to ROC analysis. Pattern Recognit. Lett. 27:861–874. doi:10.1016/j.patrec.2005.10.010.

Ferragina, A., C. Cipolat-Gotet, A. Cecchinato, M. Pazzola, M.L. Dettori, G.M. Vacca, and G. Bittante. 2017. Prediction and repeatability of milk coagulation properties and curd-firming modeling parameters of ovine milk using Fourier-transform-infrared spectroscopy and Bayesian models. J. Dairy Sci. 1–13. doi:10.3168/jds.2016-12226.

Ferragina, A., G. de los Campos, A.I. Vazquez, A. Cecchinato, and G. Bittante. 2015. Bayesian regression models outperform partial least squares methods for predicting milk components and technological properties using infrared spectral data.. J. Dairy Sci. 98:8133–51. doi:10.3168/jds.2014-9143.

Hansen, P.W. 1998. Urea Determination in Milk Using Fourier Transform Infrared Spectroscopy and Multivariate Calibration. Milchwissenschaft 53:251–255.

Hansen, P.W. 1999. Screening of dairy cows for ketosis by use of infrared spectroscopy and multivariate calibration.. J. Dairy Sci. 82:2005–2010. doi:10.3168/jds.S0022-0302(99)75437-8.

ICAR (International Committee for Animal Recording). 2016. International Agreement of Recording Practices - Recording Guidelines.

Interbull. 2016. National Genetic Evaluation Forms Provided by Countries. Accessed February 7, 2017. http://www.interbull.org/ib/geforms.

Karoui, R., G. Downey, and C. Blecker. 2010. Mid-infrared spectroscopy coupled with chemometrics: A tool for the analysis of intact food systems and the exploration of their molecular structure-quality relationships-A review. Chem. Rev. 110:6144–6168. doi:10.1021/cr100090k.

Kaylegian, K.E., G.E. Houghton, J.M. Lynch, J.R. Fleming, and D.M. Barbano. 2006. Calibration of Infrared Milk Analyzers: Modified Milk Versus Producer Milk. J. Dairy Sci. 89:2817–2832.

Lainé, A., C. Bastin, C. Grelet, H. Hammami, F.G. Colinet, L.M. Dale, A. Gillon, J. Vandenplas, F. Dehareng, and N. Gengler. 2017. Assessing the effect of pregnancy stage on milk composition of dairy cows using mid-infrared spectra. J. Dairy Sci. 1–14. doi:10.3168/jds.2016-11736.

Loker, S., F. Miglior, J. Bohmanova, J. Jamrozik, and L.R. Schaeffer. 2009. Phenotypic analysis of pregnancy effect on milk, fat, and protein yields of Canadian Ayrshire, Jersey, Brown Swiss, and Guernsey breeds. J. Dairy Sci. 92:1300–1312. doi:http://dx.doi.org/10.3168/jds.2008-1425.

Lynch, J.M., D.M. Barbano, M. Schweisthal, and J.R. Fleming. 2006. Precalibration Evaluation Procedures for Mid-Infrared Milk Analyzers. J. Dairy Sci. 89:2761–2774.

De Marchi, M., V. Toffanin, M. Cassandro, and M. Penasa. 2014. Invited review: Mid-infrared spectroscopy as phenotyping tool for milk traits. J. Dairy Sci. 97:1171–1186. doi:10.3168/jds.2013-6799.

McParland, S., and D.P. Berry. 2016. The potential of Fourier transform infrared spectroscopy of milk samples to predict energy intake and efficiency in dairy cows. J. Dairy Sci. 99:4056–4070. doi:10.3168/jds.2015-10051.

Moe, P.W., and H.F. Tyrrell. 1972. Metabolizable Energy Requirements of Pregnant Dairy Cows. J. Dairy Sci. 55:480–483. doi:http://dx.doi.org/10.3168/jds.S0022-0302(72)85519-X.

Olori, V.E., S. Brotherstone, W.G. Hill, and B.J. McGuirk. 1997. Effect of gestation stage on milk yield and composition in Holstein Friesian dairy cattle. Livest. Prod. Sci. 52:167–176. doi:http://dx.doi.org/10.1016/S0301-6226(97)00126-7.

Penasa, M., M. De Marchi, and M. Cassandro. 2016. Short communication: Effects of pregnancy on milk yield, composition traits, and coagulation properties of Holstein cows. J. Dairy Sci. 99:4864–4869. doi:10.3168/jds.2015-10168.

Pérez, P., and G. De Los Campos. 2014. Genome-wide regression and prediction with the BGLR statistical package. Genetics 198:483–495. doi:10.1534/genetics.114.164442.

R Core Team, R. 2016. R: A Language and Environment for Statistical Computing. R Found. Stat. Comput. 1:409. doi:10.1007/978-3-540-74686-7.

Robin, X., N. Turck, A. Hainard, N. Tiberti, F. Lisacek, J.-C. Sanchez, and M. Müller. 2011. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 77. doi:10.1186/1471-2105-12-77.

Shah, N.K., and P.J. Gemperline. 1989. A program for calculating Mahalanobis distances using principal component analysis. Trends Anal. Chem. 8:357–361.

Soyeurt, H., F. Dehareng, N. Gengler, S. McParland, E. Wall, D.P. Berry, M. Coffey, and P. Dardenne. 2011. Mid-infrared prediction of bovine milk fatty acids across multiple breeds, production systems, and countries.. J. Dairy Sci. 94:1657–1667. doi:10.3168/jds.2010-3408.

Sturaro, E., E. Marchiori, G. Cocca, M. Penasa, M. Ramanzin, and G. Bittante. 2013. Dairy systems in mountainous areas: Farm animal biodiversity, milk production and destination, and land use. Livest. Sci. 158:157–168. doi:10.1016/j.livsci.2013.09.011.

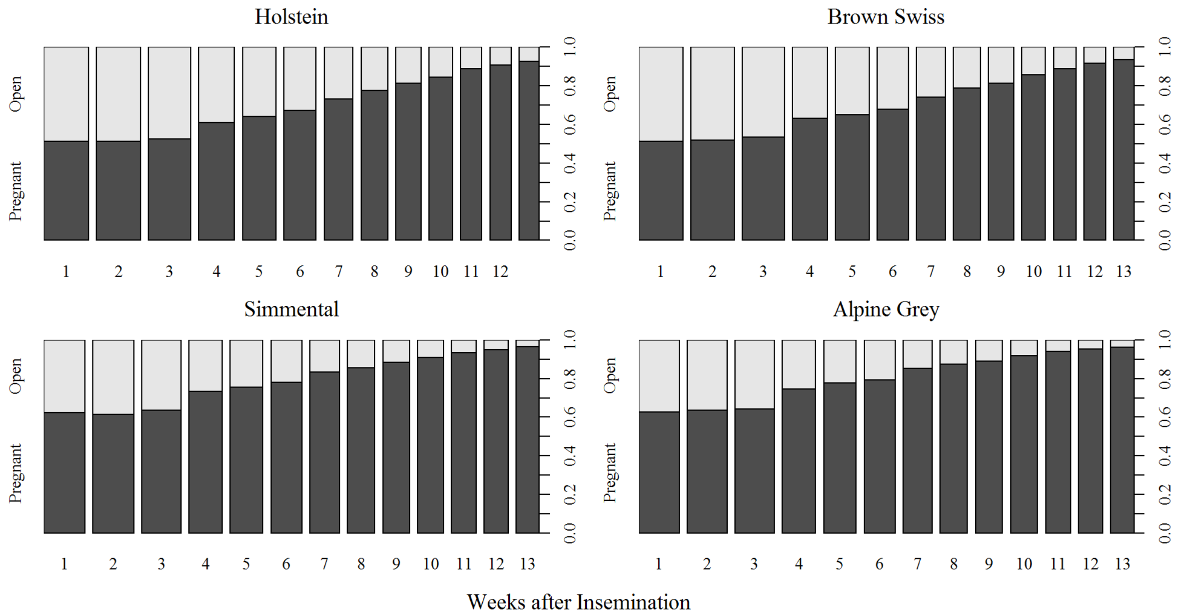
Tiezzi, F., C. Maltecca, A. Cecchinato, and G. Bittante. 2015. Comparision between different statistical models for the prediction of direct genetic component on embryo survival in Italian Brown Swiss dairy cattle. Elsevier.

Tiezzi, F., C. Maltecca, M. Penasa, A. Cecchinato, Y.M. Chang, and G. Bittante. 2011. Genetic analysis of fertility in the Italian Brown Swiss population using different models and trait definitions. J. Dairy Sci. 94:6162–72. doi:10.3168/jds.2011-4661.

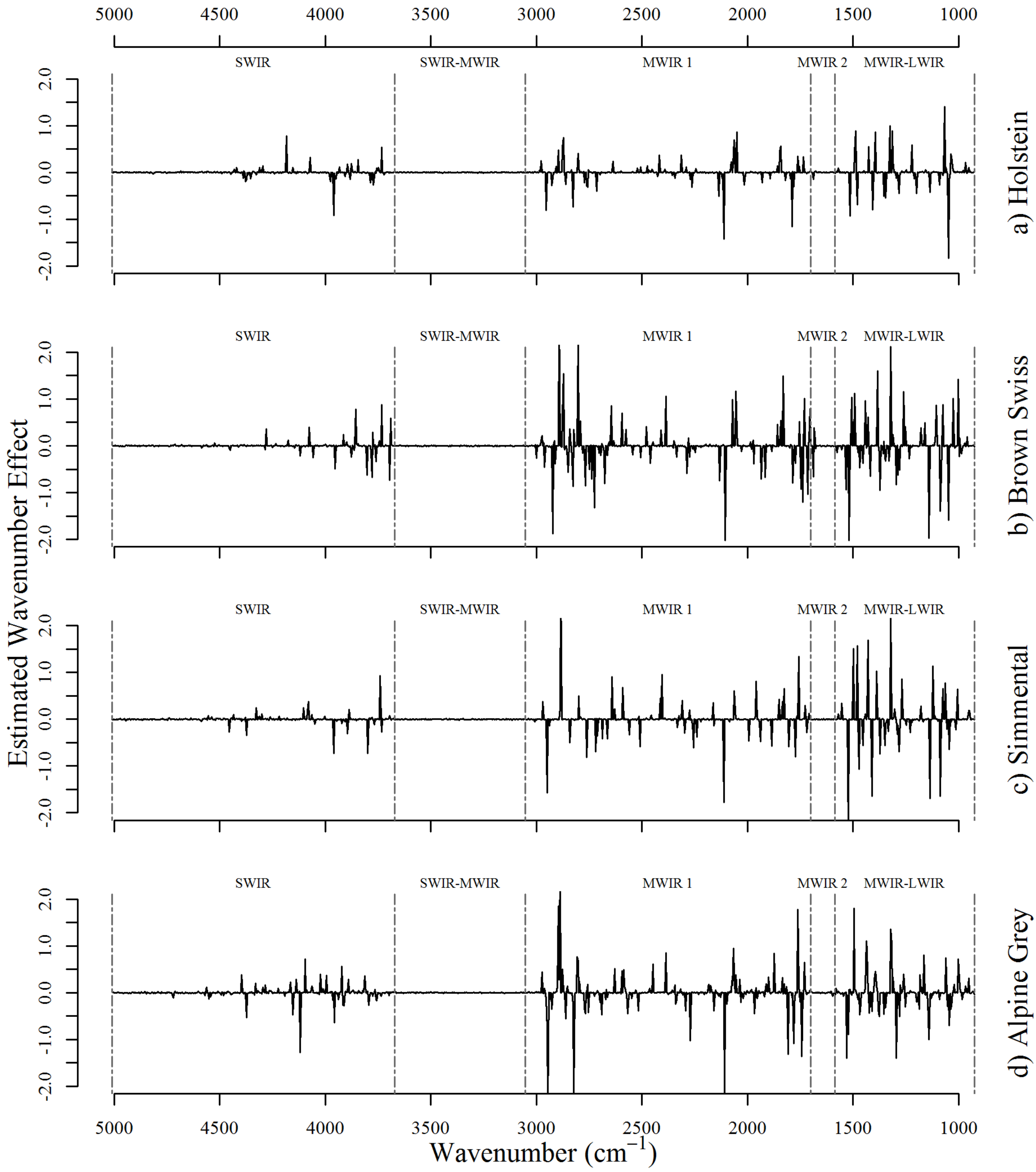
VanRaden, P.M., A.H. Sanders, M.E. Tooker, R.H. Miller, H.D. Norman, M.T. Kuhn, and G.R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285–92. doi:10.3168/jds.S0022-0302(04)70049-1.

APPENDIX

Appendix 1. Proportion of cows that are pregnant and open by weeks after insemination by breed.



Appendix 2. Estimated effects standardized for FTIR-Spectra using Bayes B to predict the Pregnancy Status (PS) in Holstein (a), Brown Swiss (b), Simmental (c), Alpine Grey (d).



*SWIR = short-wavelength infrared or near-infrared (1.40-3.0 μm); MWIR = mid-wavelength infrared (3.0-8.0 μm); LWIR = long-wavelength infrared (8.0-15 μm).

GENERAL CONCLUSIONS

Reproductive traits are highly affected my milk productivity and important differences were observed among the breeds. Whether comparing herds with different production levels or comparing cows with different milk yields in a similar production environment. The effects of herd and cow productions are clearly different each other and vary among breeds. The Alpine Grey and Simmental breeds have better reproductive ability than the specialized breeds Holstein and Brown Swiss, this difference is only partly due to different yield production. Days open can be considered an overall indicator of fertility, and it showed that herd productivity has the opposite effect on individual productivity. A better production environment could also lead to better overall fertility responses, while an increase in the milk yield of individual cows within the herd leads to worsening fertility. These associations are more evident moving from low to medium milk yields, and they, therefore, affect the dual-purpose more than the dairy breeds.

The milk composition showed high variability among the estrous phases: The fat, protein, casein and lactose and the milk's FA profile were highly affected by the reproduction cycle of the cow, probably induced by the hormonal and behavioral changes of the cows. Additionally, the estrous cycle also affected the urea, SCS, FPD, pH and HI. Assessments of the relation of milk composition with estrous phases could lead to new on-farm low-cost indicators of reproductive changes of the cow and potentially improve the design of breeding programs in dairy cattle. The milk profile could perhaps be useful in automated management-systems to identify cows in estrus or predict cows with incoming estrus and should be taken into account for breeding purposes.

It was observed that milk composition, especially the milk's contents of protein, has low but positive predictive ability to discriminate open versus pregnant cows. The direct use of the entire FTIR spectrum allows an increase of accuracy of prediction, without the need of specific information on the cow. It was possible to identify important regions that are useful for predictions of pregnancy status and pose the basis for a deeper understanding of the complex relationships between the milk spectrum, milk composition and the physiological status of the cows. The regions of the spectra related to pregnancy status have similar effects in specialized dairy cows and also in the dual-purpose breeds here evaluated. Specific regions of the spectrum where associated to or not associated at all with the pregnancy status of the cow, revealing the milk's components that could be more affected in early pregnancy.

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