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Thesis committee

Promotors
Prof. Dr AMC van Hooijdonk
Professor of Dairy Science and Technology
Wageningen University

Prof. Dr K Holtenius
Professor of Animal Nutrition and Management
Swedish University of Agricultural Sciences

Co-promotors
Dr KA Hettinga
Associate professor, Food Quality and Design
Wageningen University

Prof. Dr HLM Lindmark-Månsson
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Prof. Dr K Persson-Waller, SVA, Sweden

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Chapter One

General introduction

1.1 Historical Perspectives on Dry Period Management

During the last century, the dairy chain has developed rapidly, from farm level to the dairy plant (von Keyserlingk et al., 2013). The average milk yield of dairy cows in Western countries has increased over 3-fold since the first half of the last century (Capper et al., 2009, Kristensen et al., 2015). At the same time, industrial milk processing has been optimised, and a constant milk composition is required for obtaining the desired product yield and quality in the dairy plant. The increased demand for long shelf life products such as ultra-high temperature milk, and globalisation of the dairy product market, have also increased the quality requirements of raw milk (ZuivelNL, 2014). Ongoing developments in dairy farming, such as increasing milk production of cows and increasing attention for sustainability and animal welfare, require optimisation of dairy cow management (von Keyserlingk et al., 2013). Optimising the dry period length of dairy cows could be a way to improve cow health, whilst maintaining high quality milk.

A dry period in between lactations has been commonly applied since the early 1900's (Woodward et al., 1926, Arnold and Becker, 1936). Although the different experiences all described the need of a dry period for high milk production in the successive lactation, there was no consensus about the optimal length of the dry period (Woodward et al., 1926). The first scientific research indicating that a dry period of 6-8 weeks maximizes milk yield in the next lactation was reported by Arnold and Becker (1936). Applying a dry period was also reported to be important for restoring the body condition of the cow (Woodward et al., 1926), and for regeneration of the mammary epithelium (Swanson, 1965, Smith et al., 1966). In the last 2 decades, researchers started re-evaluating the optimal length of the dry period, taking metabolic health of cows into consideration. Although no data are available on the number of farmers that apply a different dry period length to their cows, applying a dry period of 6-8 weeks is still common practice among dairy farmers (Steeneveld et al., 2013).

1.2 Energy Balance, Milk Production and Composition

One of the main reasons for shortening the dry period is the improvement of the energy balance of the cow. At the start of lactation, cows switch from a relatively low feed intake and no milk production, to a high milk production. Therefore, in early lactation, cows are generally in a negative energy balance (NEB) (de Vries and Veerkamp, 2000). The severity of the NEB is affected by the milk yield, with an improved energy balance at a lower milk yield (van Knegsel et al., 2005). In the last decades, several controlled trials have been performed in which the performance of dairy cows with different dry period lengths was assessed. The influence of dry period length on milk production traits was assessed in a meta-analysis by van Knegsel et al. (2013). The authors reported a loss in milk yield in the successive lactation

of 5.9 kg/d when no dry period was applied, compared with a conventional dry period of 56 - 63 days. Shortening the dry period to 28 - 35 days also resulted in a statistically significant milk yield loss (-1.4 kg/d). Milk yield loss as a result of dry period reduction was reported to be stronger for primiparous cows than for multiparous cows (Annen et al., 2004, Pezeshki et al., 2007). Cows without a dry period were reported to produce approximately 800 - 1200 kg milk during the last 56 - 60 days of lactation (Annen et al., 2004, Schlamberger et al., 2010, van Knegsel et al., 2014). The total energy corrected milk yield over a whole lactation of continuously milked cows was 600 kg lower compared with cows with a 60 d dry period (Schlamberger et al., 2010). A study that evaluated milk production on practical dairy farms reported a milk yield loss of 1000 kg when the dry period was omitted (Steeneveld et al., 2013), which however was partly compensated by an increased fat and protein yield.

Milk protein content was higher in milk of cows with no (+0.25 percent point) or a short dry period (+0.06 percent point), compared with a conventional dry period (van Knegsel et al., 2013). Schlamberger et al., (2010) reported that cows without a dry period had a higher milk protein content than cows with a 60 d dry period until 20 weeks in lactation. Due to the increased protein content of milk from cows without a dry period, 305 d protein yield was similar for cows with a 0 or a 60 d dry period (Schlamberger et al., 2010). According to a meta-analysis by van Knegsel et al. (2013), milk fat content was not affected by dry period length. Consequently, cows with a 0 d dry period had a lower 305 d fat yield (417 kg) compared with cows with a 60 d dry period (541 kg) (Schlamberger et al., 2010). Steeneveld et al. (2013) reported a somewhat smaller loss in 305 d fat yield of 82 kg. The effect of dry period omission on lactose content in milk is not fully clear. Andersen et al. (2005) reported that cows with a 0 d dry period had a higher lactose content than cows with a 50 d dry period during the first 5 weeks in lactation, whereas van Knegsel et al. (2014) reported that cows without a dry period had a lower lactose content (4.52%) than cows with a 60 d dry period (4.59%) during the first 14 weeks in lactation. Differences in lactose content are relatively small compared with milk protein content, as is commonly the case for healthy cows due to the osmoregulatory function of lactose in milk (Linzell and Peaker, 1971). In the last 8 weeks before parturition, milk protein content of cows without a dry period increased, to an average protein content of 5.1% (van Knegsel et al., 2014). Milk protein content increased up to 7.5% in the last week before calving (Schlamberger et al., 2010), possibly due to increased immunoglobulin transfer into milk during the last weeks before calving (Guy et al., 1994b). Milk fat content of cows without a dry period was 5.2% during the last 8 weeks before parturition (van Knegsel et al., 2014). Little reports are available on detailed compositional analyses of milk from the last weeks of gestation from cows without a dry period, and how this composition relates to the status of the mammary gland during this period.

The reduced milk production of cows without a dry period results in an improved energy balance in early lactation. The NEB of cows with a reduced dry period is less deep, and lasts for a shorter period of time (van Knegsel et al., 2014). Cows with a 60 d dry period were in a NEB during the first 14 weeks of their lactation. Cows with a 30 d dry period were in a NEB for 8 to 12 weeks, and cows with a 0 days dry period were in a NEB for 4 to 5 weeks (van Knegsel et al., 2014, Van Hoeij, Submitted). Besides, the peak NEB can be reduced 1.5 times by applying a dry period of 30 days and 3 times by applying a dry period of 0 days instead of 60 days (van Knegsel et al., 2014).

1.3 Metabolic Status of the Cow in Relation to Dry Period Length

The NEB is related to various health problems of the cow, such as weight loss, ketosis, and reduced fertility (Butler, 2003, Grummer et al., 2004). In controlled trials there has been little evidence for the relation between dry period length and energy balance-related disease incidence. This is probably due to the insufficient power of the trials performed, with relatively low numbers of animals in controlled trials compared to the number that would be needed for examining disease incidence. Only ketosis incidence tended to reduce when no dry period was applied (van Knegsel et al., 2013). For fertility traits, no uniform relation with dry period length was found (van Knegsel et al., 2013). The metabolic status of cows was improved by dry period omission. Cows without a dry period had increased glucose concentrations and lower non-esterified fatty acid concentrations in blood than cows with a conventional dry period. This indicates that cows with an improved energy balance have lower mobilization of body fat reserves, which is also reflected in the fatty acid composition of milk. Cows with a conventional dry period had higher concentrations of long chain fatty acids in early lactation milk than cows without a dry period (van Knegsel et al., 2014). High concentrations of long chain fatty acids of cows in severe NEB originate from body fat mobilisation (Jorjong et al., 2014). Cows in severe NEB had lower ATP production in mammary epithelial cells due to reduced activity of the citric acid cycle. Lower mitochondrial activity during NEB can result in less *de novo* synthesis of fatty acids in mammary epithelial cells (Lu et al., 2013). Therefore, cows with a 0 d dry period had higher concentrations of *de novo* synthesised short-chain fatty acids in early lactation milk than cows with a 60 d dry period (van Knegsel et al., 2014).

Milk proteomics and metabolomics were found to reflect the energy balance of cows in early lactation. Compounds such as stomatin and galactose-1-phosphate were found to be higher in milk of cows with a positive energy balance (Lu et al., 2013). Milk proteomics has provided further understanding of mammary health of dairy cows. The milk proteome can give a detailed insight in the response towards infections of the mammary gland (Danielsen et al., 2010, Alonso-Fauste et al., 2012, Smolenski et al., 2014). Proteomics was also used to

show the changes occurring during transition from colostrum to milk in the immune protein profile after 2-3 days in lactation (Zhang et al., 2015). The metabolome of milk consists of low molecular weight molecules that are formed by rumen fermentation and in the energy metabolism pathways (Antunes-Fernandes et al., 2016). Another metabolomics study showed that the glycerophosphocholine and choline concentrations in milk could be used as markers for ketosis (Klein et al., 2012). All in all, milk proteomics and metabolomics were proven to be useful tools to improve understanding of the health of dairy cows in general, and mammary gland health in particular.

1.4 Mammary Epithelial Health Status

A conventional dry period allows the mammary gland of the cow to turn into a high-productive state before the successive lactation. During the dry period, accelerated mammary epithelial cell (MEC) renewal takes place compared with a lactating mammary gland in the same stage prior to parturition (Capuco et al., 1997, Collier et al., 2012). Since extensive regeneration of MECs was observed during a 60 d dry period, less viable MECs seem to be present after a reduced dry period (Capuco et al., 1997, Collier et al., 2012). Epithelial cell proliferation and apoptosis in the first 20 days of lactation did not differ between mammary glands of cows with either a conventional or no dry period (Annen et al., 2007). Only at 2 and 4 days postpartum, cows without a dry period had a higher MEC proliferation index than cows with a 60 d dry period (Annen et al., 2008).

Cows without a dry period have a lower colostrum production at first milking (5.1 kg) than cows with a 60 d dry period (7.7 kg) (Mayasari et al., 2015). Continuous milking until parturition results in the absence of an accumulation of milk prepartum. Immunoglobulin G (IgG), an essential immune protein in colostrum for the calf, is secreted into milk starting several weeks before parturition (Guy et al., 1994a). Milking until parturition reduces accumulation of IgG in the last weeks of lactation, resulting in a 2.5 fold lower IgG concentration in colostrum of cows with a 0 d dry period compared with cows with a 30 d or 60 d dry period (Mayasari et al., 2015). Hence, applying no dry period negatively affects both the volume and IgG concentration in colostrum. However, dry period length of the cow did not affect growth of calves and immunoglobulin concentrations in blood plasma of calves after immunisation (Mayasari et al., 2015).

Milk stasis does not only cause accumulation of IgG before parturition, but also residual milk accumulates after drying off. Residual milk in the udder serves as a nutrient source for bacteria, making the dry period a risk period for udder infections (Bradley and Green, 2004). Therefore, it was hypothesized that no accumulation of milk due to dry period omission would reduce the incidence of mammary infections (Collier et al., 2012). However, no relation was found between the incidence of mastitis in early lactation and dry period length

(Church et al., 2008, Watters et al., 2008, Santschi et al., 2011). One of the main indicators in milk for mammary health in general, and infection status in particular, is somatic cell count (SCC). Milk that contains pathogens usually has SCC > 600,000 cells/ml (Dohoo and Meek, 1982), whereas subclinical mastitis is indicated by SCC > 250,000 cells/ml (Runciman et al., 2010). SCC was reported to be higher in early lactation milk of cows that had no dry period compared with a dry period of either 30 or 60 days (van Knegsel et al., 2014). In other work, however, no differences in SCC in early lactation milk were found between cows with different dry period lengths (Andersen et al., 2005, Rastani et al., 2005, Watters et al., 2008, Schlamberger et al., 2010).

Studies that indicated no effect of dry period length on mastitis incidence all used intramammary antibiotic treatment at drying off. In the Netherlands, it used to be a common practice to treat the cow with an intramammary antibiotic at drying off. A stricter antibiotics policy nowadays does, however, not allow the use preventive antibiotics at drying off of healthy cows. In Sweden, application of medical products, such as antibiotics, without motivation has been prohibited since 1979 by the Swedish board of Agriculture. Therefore, cow management practices are being explored in Sweden to reduce the risk of microbial infections after drying off (Ekman, 2003). Between 2005 and 2012, 44% of the antibiotics in dairy farming in the Netherlands were used for dry-off therapy of cows (Kuipers et al., 2016). Without considering potential benefits of lower veterinary costs when no dry period is applied, the lower management costs and higher milk protein contents seem to result in sufficient cost reduction and income to compensate for the reduction in milk yield in the Dutch situation. Hence, omitting the dry period may be economically beneficial for Dutch dairy farmers (Heeren et al., 2014). No economic analysis has been reported for Swedish dairy farmers.

1.5 Milk Protein Composition

In contrast to milk fatty acid composition, milk protein composition has not been studied extensively in relation to dry period length. Bovine milk has a protein content of about 3.4%, of which 20% are whey proteins and 80% are caseins. The whey protein fraction predominantly consists of α-lactalbumin, which plays an essential role in lactose synthesis, and β-lactoglobulin. The casein fraction consists of 4 major proteins, α_{s1}-, α_{s2}-, β- and κ-casein, in a ratio of 11 : 3 : 10 : 4 (Walstra et al., 2006). These caseins form casein micelles due to hydrophobic interactions and calcium phosphate nanoclusters of phosphorylated serine residues and colloidal calcium and inorganic phosphate. The α_{s1}-, α_{s2}- and β-caseins are predominantly located in the core of the casein micelle, whereas κ-casein is located more on the outer part, forming a 'hairy layer' at the surface of the micelle, stabilizing the micelle. Caseins undergo posttranslational modifications in the Golgi apparatus of mammary

epithelial cells. Phosphorylation is a posttranslational modification that occurs in different degrees for all casein fractions, whereas glycosylation is unique for κ-casein (Walstra et al., 2006). During a lactation with a conventional dry period, the glycosylated κ-casein fraction is increasing towards the end of lactation (Bonfatti et al., 2014).

The casein composition of milk can be affected by proteolytic activity. The main proteolytic enzyme in milk is plasmin, which is the active form of its zymogen plasminogen (Kelly et al., 2006). Plasminogen is transported transcellularly from blood to milk by Golgisomes, in the presence of casein micelles (Silanikove, 2016). Plasmin can hydrolyse α_{s1}- α_{s2}- and β-casein fractions (Grufferty and Fox, 1988), with the β-casein fraction being the most susceptible in milk (Politis et al., 1989). Plasminogen can be activated by either tissue-type or urokinase-type activators, the latter being related to somatic cells (Ismail and Nielsen, 2010). Plasmin activity is related to mammary epithelial tissue remodelling (Nørgaard et al., 2008), which increases in the last 20 days prior to calving when the dry period is omitted (Collier et al., 2012). Plasmin activity in milk was increasing in the last two weeks before parturition of cows that did not have a dry period, with maximum activity around parturition (Dupont et al., 1998). The effect of dry period length on plasmin activity in early lactation milk has not been studied yet, neither the consequence this would have for casein composition.

1.6 Processing Quality of Milk

Few studies have been done on the relation between dry period length of cows and milk composition and quality for processing. The current work includes analyses of protein composition, plasmin activity and mineral composition of milk. Two products that are sensitive for changes in milk protein composition and plasmin activity are cheese and ultra-high temperature (UHT) milk.

A high casein content in milk has been related to an increased cheese yield per kg milk (Walstra et al., 2006, Wedholm et al., 2006). Cheese making properties, such as rennet coagulation time and curd firmness also improved with increasing milk casein content (Walstra et al., 2006, Joudu et al., 2008). Milk with a large κ-casein fraction generally showed good coagulation during renneting (Wedholm et al., 2006). A high degree of glycosylation of κ-casein has been related to higher curd firmness after renneting of milk (Robitaille et al., 1993), possibly as a result of small casein micelles (Bijl et al., 2014). Very late lactation milk, which was collected > 46 weeks in lactation, also showed good coagulation properties (Wedholm et al., 2006). The authors did not explicitly report the dry period length that was applied, so the weeks until calving were not indicated.

The physical stability and sensory properties of ultra-high temperature (UHT) milk can be affected by proteolytic action of plasmin (Rauh et al., 2014a, Rauh et al., 2014b). Although most plasmin is inactivated during UHT treatment, residual plasmin activity can hydrolyse

caseins into peptides during prolonged storage at room temperature (Rauh et al., 2014b). Casein hydrolysis can cause age gelation of UHT milk. Besides, the peptides that are formed during casein hydrolysis may have bitter off flavours in UHT milk (Rauh et al., 2014a). Hence control of plasmin activity is an important quality attribute of milk for UHT processing.

1.7 Aim of the Study and Thesis Outline

The aim of this work is to evaluate the effect of shortening or omitting the dry period on milk composition and quality. The biological background of dry period-induced differences in milk composition is addressed, and differences in milk composition are used for predicting the processing quality of milk. Most dry period research thus far has focused on major production traits of cows, whereas this more detailed study provides an indication for the applicability of a reduced dry period from a milk quality perspective.

In chapter 2, **Influence of shortening or omitting the dry period of Holstein Friesian cows on casein composition of milk**, casein composition in early and late lactation milk is analysed of cows with either a 0, 30 or 60 days dry period. The possible biological origin of differences between casein compositions is discussed, which is further evaluated in chapters 3, 4 and 6.

In chapter 3, **Influence of shortening the dry period of Swedish dairy cows on plasmin activity in milk**, plasmin and plasminogen activity and casein composition of milk from cows with a dry period of either 4 or 8 weeks is evaluated. The influence of the factors parity and somatic cell count on plasmin activity of cows with a shortened dry period are addressed. In addition, the relation between plasmin activity and casein composition is evaluated.

In chapter 4, **Influence of dry period length of dairy cows on casein micelle composition in early and mid-lactation milk**, outcomes of more detailed compositional analyses are presented to predict processing quality of milk from cows with a dry period of either 0 or 30 days. Plasmin activity and mineral composition are related to casein composition of casein micelles and milk serum.

In chapter 5, **Influence of dry period length of Swedish dairy cows on the proteome of colostrum**, the proteomes of colostrum serum (first secretion after calving) and transition milk serum (fifth milking after calving) of cows with a dry period of either 4 or 8 weeks are compared. This comparison was done for 2 cow breeds, Swedish Holsteins and Swedish Reds. Differences in proteome are related to the status of the mammary epithelium.

In chapter 6, **Blood glucose concentration relates to milk composition of dairy cows in early lactation**, variation in glucose concentration of blood in cows is related to milk protein composition and lactose production in early lactation. The milk metabolome is presented to indicate the involvement of metabolic pathways that use glucose as a substrate in milk component synthesis in the mammary gland.

In chapter 7, **General discussion**, the relation between milk composition of cows with different dry period lengths and mammary gland physiology is evaluated. Also effects of dry period length on processing quality of milk are discussed. Finally, the applicability of different dry periods is evaluated.

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Chapter Two

Influence of shortening or omitting the dry period of Holstein Friesian cows on casein composition of milk

Ruben de Vries, Ariëtte van Knegsel, Monika Johansson,
Helena Lindmark-Månsson, Toon van Hooijdonk,
Kjell Holtenius, Kasper Hettinga

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Abstract

The aim of this study was to evaluate the effect of shortening or omitting the dry period of dairy cows on milk casein composition. For this study, milk samples were analysed of 90 cows with either a dry period of 0, 30 or 60 d and either a glucogenic or lipogenic ration in early lactation. Milk was sampled 6 and 2 wk prepartum, and 2, 6 and 12 wk postpartum. Milk was analysed for casein (CN) composition by capillary zone electrophoresis, and isoforms of κ-CN were measured by reversed phase - high performance liquid chromatography. Shortening the dry period from 60 d to 30 d reduced the α_{s1}-CN fraction by 3.8%, and increased the α_{s2}-CN fraction by 5.5%. In milk from cows with a 0 d dry period, the glycosylated κ-CN fraction in late lactation increased from 8% to 12% between 6 and 2 wk prepartum. After calving, the glycosylated κ-CN fraction in milk was higher for cows with a 0 d dry period (6.7%) compared with cows with a 60 d dry period (5.2%). The glycosylated κ-CN fraction 2 wk postpartum was negatively correlated with milk yield, suggesting that glycosylation was related to reduced productivity of mammary epithelial cells. In early lactation, the β-CN fraction was reduced in milk of cows with a 0 d dry period. A lowered β-CN fraction was associated with high SCC and greater parity, indicating that it was a result of proteolytic activity. In conclusion, casein composition changes as a result of shortening the dry period from 60 d to 30 d are not expected to affect processing characteristics of milk. Applying a 0 d dry period may affect processability of milk because of a higher glycosylated κ-CN fraction, and possibly because of higher proteolytic activity compared with a 60 d dry period.

Introduction

A dry period of 6 to 8 weeks is known to maximize milk yield in the subsequent lactation (Kuhn et al., 2005). Shortening the dry period reduces milk yield in the subsequent lactation, but improves the energy balance and metabolic health of dairy cows in early lactation (Rastani et al., 2005, Van Knegsel et al., 2014). A meta-analysis showed that milk production after calving decreases with 1.4 kg/d as a result of shortening (28 - 35 d), and 5.9 kg/d as a result of omitting the dry period. At the same time, milk protein content increased when the dry period was shortened (+0.06%) or omitted (+0.25%) (Van Knegsel et al., 2013). By applying a 30 d or 0 d dry period, late lactation milk is obtained very close to calving. This late lactation milk (50 - 0 d prepartum) was related to short renneting time and high gel strength after renneting (Remond et al., 1997), but casein composition of this milk was not determined.

Caseins comprise approximately 80% of all proteins in milk and are present in four major forms: α_{s1}-, α_{s2}-, β- and κ-casein (CN) (Walstra et al., 2006). Before secretion into milk, these caseins can undergo various post-translational modifications (PTMs). Phosphorylation is a PTM that can occur in all caseins, whereas glycosylation only occurs in κ-CN (Walstra et al., 2006). On average, approximately 60% of all κ-CN is glycosylated. Up to 6 glycans can be attached to the protein (Holland et al., 2006). Glycans attached to κ-CN exist as mono-, di-, tri- and tetra-saccharides. These glycans can consist of N-acetylneuraminic acid, galactose and N-acetylgalactosamine (Saito and Itoh, 1992). The degree of glycosylation of κ-CN is highly variable (Holland et al., 2006).

Variation in casein composition and PTM profiles results in variation in functional properties of milk. The proportion of κ-CN of total casein was positively related to renneting properties of milk (Wedholm et al., 2006). Influence of glycosylation degree of κ-CN on chymosin hydrolysis rate of κ-CN has been shown in simplified model systems but not in milk (Dziuba and Minkiewicz, 1996). Curd firmness after chymosin hydrolysis of κ-CN in milk was positively correlated with N-acetylneuraminic acid content of κ-CN (Robitaille et al., 1993). Small casein micelles resulting from a high degree of glycosylation could explain this increased curd firmness (Bijl et al., 2014, Robitaille et al., 1993). Several potential functionalities for the new-born calf have been ascribed to glycosylation of κ-CN, such as immune regulating, probiotic and anti-microbial properties (Brody, 2000). However, the main reason for naturally occurring variation in glycosylation degree of κ-CN in milk is not clear.

Milk casein composition varies between different stages of lactation, and is rather constant during mid-lactation (de Kruif and Huppertz, 2012, Ostersen et al., 1997). Progressing lactation decreased α_s-CN and κ-CN fractions in milk, whereas it increased the β-CN fraction (Ostersen et al., 1997). Progressing lactation increased glycosylation of κ-CN (Bonfatti et al., 2014, Robitaille et al., 1991), though colostrum κ-CN has the highest glycosylation degree

(Fiat et al., 1988, Guerin et al., 1974). Another factor influencing κ-CN glycosylation is the genetic variant of κ-CN. In general, the B variant of κ-CN is more glycosylated than the A variant (Bijl et al., 2014, Robitaille et al., 1991). The influence of parity on glycosylation of κ-CN is unclear; Robitaille et al. (1991b) observed a decrease in glycosylation with increasing parity, whereas Bonfatti et al. (2014) reported highest levels in second lactation. Plasmin, the main proteolytic enzyme in milk, is another cause of variation in casein composition. Of the caseins, β-CN is most prone to degradation by plasmin in milk (Politis et al., 1989). Plasmin activity increases with increasing parity and stage of lactation (Bastian et al., 1991, Politis et al., 1989).

The aim of this study was to evaluate the effect of shortening or omitting the dry period on casein composition, both in pre- and postpartum milk. Outcomes can be used for better understanding of the impact of dry period length of the cow on processability of milk.

Materials and Methods

Experimental design, animals and sampling

The Institutional Animal Care and Use Committee of Wageningen University and Research centres approved the experimental protocol. Milk samples were obtained from an experiment that has been described before (Van Knegsel et al., 2014). In short, Holstein-Friesian dairy cows (N = 168) were selected from the Dairy Campus Research dairy herd (WUR Livestock Research, Lelystad, The Netherlands). Cows were blocked for parity (primiparous or multiparous), expected calving date, milk production in the previous lactation and body condition score and randomly assigned to length of dry period (0, 30 or 60 d dry) and early lactation ration (glucogenic or lipogenic) resulting in a 3 x 2 factorial design. Cows were housed in a free-stall with slatted floor and cubicles. During lactation, cows were milked twice daily (05.00h and 16.30h). The drying off protocol for cows with the 30 d and 60 d dry period consisted of a transition to the far-off ration at day 7 before drying-off, and milking once daily at day 4 before drying-off cows. At drying-off cows were treated with an intramammary antibiotic (Supermastidol, Virbac Animal Health, Barneveld, the Netherlands). Milk yield was recorded daily. Milk samples for fat, protein, and lactose contents and somatic cell count analysis (ISO 9622, Qlip, Zutphen, the Netherlands) were collected four times per week (Tuesday afternoon, Wednesday morning, Wednesday afternoon and Thursday morning).

For the current study, 90 cows within the main experiment were selected, based on dry period length and lactation ration, resulting in 6 groups of 15 cows. Milk samples were taken on Friday morning 6 and 2 wk prepartum, and 2, 6 and 12 wk postpartum. Samples were stored at -20°C immediately after collecting. All milk samples of the 90 cows were included in capillary zone electrophoresis (CZE) analysis to determine casein composition. The results of the CZE analysis show that dietary energy source did not influence casein composition of milk. For sample availability reasons, only milk samples from cows with a lipogenic lactation ration (45 cows) were used for quantification of glycosylated and non-glycosylated κ-CN fractions by reversed phase - high performance liquid chromatography (RP-HPLC).

Analysis of protein composition

Milk protein composition was measured by capillary zone electrophoresis (CZE), which is an appropriate method for all caseins, apart from the glycosylated forms of κ-CN (Heck et al., 2008). CZE was chosen because of its ability to separate different forms of α-CN, and genetic variants of β-CN. Sample preparation, buffer composition, equipment and run conditions for CZE were described before (Åkerstedt et al., 2012). D,L-dithiothreitol (DTT) was added to the sample buffer at the day of sample preparation. Milk samples of 300 µL were mixed with

700 µL sample buffer, and subsequently defatted after centrifugation (10 min, 10,000 x *g*). All chemicals originated from Sigma-Aldrich (Steinheim, Germany). Samples were stored at -20°C prior to analysis. Fractions of individual proteins were calculated by dividing their peak area by the cumulative peak area of all caseins, α-lactalbumin (α-LA) and β-lactoglobulin (β-LG). Because of limited detection of glycosylated isoforms of κ-CN, the total κ-CN fraction was calculated according to the method of Gustavsson et al. (2014). The β-CN fraction was corrected for co-eluting minor forms of κ-CN, as described by Gustavsson et al. (2014).

Analysis of κ-CN isoforms

It is known that glycosylated and non-glycosylated isoforms of κ-CN can be quantified using RP-HPLC (Bobe et al., 1998, Bonfatti et al., 2008, Visser et al., 1991). Buffer composition and sample preparation for reversed phase-high performance liquid chromatography (RP-HPLC) were according to Bobe et al. (1998). The only exception was solvent A, used for sample dilution, which consisted of 0.1% trifluoroacetic acid (TFA) in water (Bonfatti et al., 2008). Separations were carried out by an Ultimate 3000 LC module equipped with an Aeris WIDEPORE 3.6 µm XB-C18 column, 250x4.6 mm (Phenomenex, Utrecht, the Netherlands). A security guard cartridge system was used as a pre-column (AJ0-8769, Phenomenex). Temperature in the auto-sampler was 4°C. An injection loop of 100 µL was used, injection volume was 5 µL. UV detection wavelength was 214 nm. Two solvents (A and B), consisting of 0.1% TFA in milliQ water and 0.1% TFA in acetonitrile respectively, were used for protein elution. An increasing gradient of solvent B was applied, starting at 33% (v/v). Increases of B were 0.2% per min for 5 min, 0.5% per min for 4 min, 0.33% per min for 9 min, 0.25% per min for 2 min, 5% per min for 30 s, 0% per min for 4.5 min, 0.33% per min for 3 min, 0.67% per min for 4.5 min, 0% per min for 5 min, followed by a linear return to the starting conditions in 30s. A flow rate of 0.25 mL/min was applied for 24 minutes, followed by an increase to 0.4 mL/min in 3 min. This flow was applied until the end of the run at 41 min. All chemicals originated from Sigma-Aldrich (Steinheim, Germany), apart from trifluoroacetic acid, tri-sodium citrate dihydrate (both from Merck, Darmstadt, Germany) and acetonitrile (HPLC-ultra gradient, Biosolve chemicals, Valkenswaard, the Netherlands). Dionex Chromeleon 7.1.2 (Thermo Scientific) was used for data processing. After every milk sample, a blank sample was ran to avoid carry-over effects. Elution times of milk proteins were validated using protein standards (α_s-CN, α-LA, β-CN, β-LG, κ-CN; purities 70-85%, all from Sigma-Aldrich, St Louis, MO, USA).

Statistical analysis

Prepartum and postpartum data were analysed separately. For prepartum comparison of the dry period groups, only data of sample week -6 were included in a mixed model (Proc mixed, SAS 9.3, SAS Institute Inc., Cary, NC, USA). Fixed factors in the model were dry period (0 or 30 d dry), parity (prepartum: 1, 2 or > 2), and the interaction dry period x parity (model 1). Other variables were analysed with a mixed model accounting for repeated measures (Proc mixed, SAS 9.3, SAS Institute Inc., Cary, NC, USA). Cow was the repeated subject. For comparison of the prepartum test week, only data from cows in the 0 d dry period group were included. Fixed factors in the model were test week (-2 or -6 wk), parity (prepartum: 1, 2 or > 2) and the interaction test week x parity (model 2). Prepartum parity results are based on the model for comparison of the dry period groups. The postpartum model included the fixed factors dry period (0, 30 or 60 d dry), test week (2, 6 or 12 wk), parity (postpartum: 2, 3 or > 3) ration (glucogenic or lipogenic), and the interactions dry period x test week, dry period x parity and test week x parity (model 3). For all variables apart from milk yield and SCC, an autoregressive covariance-structure was the best fit and was used to account for within-cow variation. Compound Symmetry (CS) was the best fit for milk yield and SCC and was used to account for within-cow variation. *P*-values for dry period length were corrected by Bonferroni adjustment. Differences are considered significant when the corrected *P*-value < 0.05. Correlation coefficients were determined using Proc corr in SAS.

Results

Milk yield and composition

Cows in the 30 d dry period group had higher milk fat (5.7%) and protein (4.3%) content than cows in the 0 d dry period group (5.1% fat and 4.1% protein) at 6 wk prepartum (Table 2.1). First parity cows had higher lactose (4.6%) than second (4.4%) or greater (4.3%) parity cows. A parity x week interaction was found for lactose content prepartum (P = 0.02). Milk of cows in third or greater parity at 2 weeks prepartum had lower lactose content in milk than younger cows or cows in week 6 prepartum. Cows in third or greater parity had higher SCC (321,000 cells/mL) than first parity cows (124,000 cells/mL). Between 6 and 2 wk prepartum, milk yield decreased from 16.9 to 9.1 kg/d and lactose content decreased from 4.4% to 4.2%. Milk protein (4.1% to 6.1%) and SCC (193,000 cells/mL to 551,000 cells/mL) increased between 6 and 2 wk prepartum.

Postpartum (2, 6 and 12 wk) milk yield was lower for cows with a 30 d dry period (4.9 kg/d) and cows with a 0 d dry period (8.6 kg/d) compared with cows with a 60 d dry period (Table 2.1). Second parity cows had lower milk yield (35.9 kg/d) than third (41.8 kg/d) or greater parity (41.4 kg/d) cows. The interaction dry period x test week indicated that milk yield increased with increasing dry period length in the second and sixth postpartum test week, but milk yield did not differ between cows with a 0 d or 30 d dry period at 12 weeks postpartum (P = 0.02). Cows with a 0 d or 30 d dry period had higher milk protein content than cows with a 60 d dry period. Also, cows with 0 d dry period had greater milk protein content compared with cows with 30 d dry period (P = 0.03). Second parity cows with a dry period of 0 d, had higher milk protein content (4.2%) than older cows or cows with a longer dry period. Ration did not affect milk yield and macronutrient composition apart from fat content. Cows fed the lipogenic ration had greater fat content than cows fed the glucogenic ration (lipogenic: 4.7%, glucogenic: 4.4%, P = 0.03). Cows of greater than third parity had lower lactose content (4.5%) than cows of second (4.7%) or third (4.7%) parity at 6 weeks postpartum. In the second and twelfth postpartum sampling week, no difference in lactose content was found between parities. Cows that had a 0 d dry period had higher SCC than cows that had a dry period of 30 d or 60 d. At 2 wk in lactation, SCC was higher than at 6 wk in lactation. Second and third parity cows had lower SCC than fourth or greater parity cows.

TABLE 2.1. Milk production, macronutrient composition and SCC of cows with a dry period of 0, 30 or 60 d (Mean ± SEM). All dry period groups consist of 30 cows.

	Dry period (days)				*P*-values[1]		
	0	30	60	SEM	Dry period	Week[2]	Parity
Prepartum[3]							
Milk yield (kg/d)	16.9	14.7		1.5	0.10	<0.01	0.15
Fat (%)	5.1	5.7		0.2	<0.01	0.18	0.58
Protein (%)	4.1	4.3		0.1	0.04	<0.01	0.13
Lactose (%)	4.4	4.4		0.07	0.15	<0.01	<0.01
SCC[4] (x10^3 cells/mL)	193	223		49	0.48	<0.01	<0.01
Postpartum[5]							
Milk yield (kg/d)	35.5^c	39.2^b	44.1^a	0.4	<0.01	<0.01	<0.01
Fat (%)	4.7	4.6	4.4	0.1	0.07	<0.01	0.47
Protein (%)	3.8^a	3.7^a	3.4^b	0.02	<0.01	<0.01	<0.01
Lactose (%)	4.5	4.6	4.6	0.01	0.04	<0.01	<0.01
SCC[4] (x10^3 cells/mL)	238^a	163^b	110^b	24	<0.01	<0.01	<0.01

$^{a-c}$ Values with different superscript within a row differ ($P < 0.05$) between different dry periods.

[1] Also included in the model: lactation ration and the interactions dry period x parity, dry period x test week and parity x test week.

[2] Prepartum week effects are based on 2 and 6 wk prepartum samples of 0 d dry cows.

[3] Prepartum means, SEM, and dry period and parity effects are based on 6 wk prepartum samples of 0d and 30d dry cows.

[4] *P*-values are based on the natural logarithm of SCC.

[5] Postpartum test weeks are 2, 6 and 12 wk after calving.

Casein composition

At 6 wk prepartum, cows in the 0 d dry period group had a higher α_{s2}-CN fraction (7.7%) in milk than cows in the 30 d dry period group (6.9%) (Table 2.2). Cows in the 0 d dry period group had a lower β-CN fraction (32.2%) than cows in the 30 d dry period group (34.5%). First parity cows had a higher α_{s1}-CN fraction (30.8%) than second parity cows (29.3%) 6 wk prepartum. First parity cows had a lower α_{s2}-CN fraction (6.4%) than second (7.8%) or greater (7.8%) parity cows. First parity cows had a higher β-CN fraction (35.1%) than third or greater parity cows (31.6%), and lower total κ-CN fraction (11.0%) than cows of third or greater parity (12.2%).

Postpartum fractions of α_{s1}-, α_{s2}- and β-CN in milk were different for cows with different dry period lengths. Cows with a 60 d dry period had 0.8% higher α_{s1}-CN fraction than cows with a 0 d dry period, and 1.2% higher α_{s1}-CN fraction than cows with a 30 d dry period. Cows with a 30 d dry period had the highest β-CN fraction. Cows with a 0 d dry period had the lowest β-CN fraction. The β-CN fraction was inversely related with parity: 32.9%, 32.4% and 31.5% (parities 2, 3 and >3 respectively). The interaction dry period x test week influenced the β-CN fraction ($P < 0.01$): milk samples 2 wk postpartum of cows with a 0 d dry period had a lower β-CN fraction (29.0%) than samples from any other postpartum dry period x test week combination. At 12 wk in lactation, the β-CN fraction of cows with a 0 d dry period (31.3%) did not differ from the

β-CN fraction of 30 d dry cows (32.5%). The β-CN fraction was negatively correlated with SCC (R = 0.37, $P < 0.01$). The α_{s2}-CN fraction was higher at 2 wk after calving (7.9%) than in later postpartum samples. Casein composition was not influenced by feeding either a glucogenic or a lipogenic ration.

TABLE 2.2. Milk casein fractions (% of the sum of total casein, α-LA and β-LG) from cows with a dry period of 0, 30 or 60 d (30 cows per group), measured with CZE (Mean ± SEM).

	Dry period (days)				*P*-values[2]		
	0	30	60	SEM	Dry period	Week[2]	Parity
Prepartum[3]							
α_{s1}-CN	29.8	30.2		0.5	0.58	0.81	0.02
α_{s2}-CN	7.7	6.9		0.3	0.02	0.62	<0.01
β-CN	32.2	34.5		0.9	0.01	0.78	0.01
κ-CN	11.4	11.3		0.4	0.93	0.03	0.02
Postpartum[4]							
α_{s1}-CN	30.8^{ab}	30.4^{b}	31.6^{a}	0.1	0.02	0.24	0.63
α_{s2}-CN	8.1^{a}	7.7^{a}	7.3^{b}	0.1	<0.01	<0.01	0.27
β-CN	30.7^{b}	33.8^{a}	32.4^{a}	0.2	<0.01	<0.01	0.04
κ-CN	12.1	11.9	11.8	0.1	0.57	0.04	<0.01

[a-c] Values with different superscript within a row differ ($P < 0.05$) between different dry periods.

[1] Also included in the model: lactation ration and the interactions dry period x parity, dry period x test week and parity x test week.

[2] Prepartum week effects are based on 2 and 6 wk prepartum samples of 0 d dry cows.

[3] Prepartum means, SEM, and dry period and parity effects are based on 6 wk prepartum samples of 0 and 30 d dry cows.

[4] Postpartum test weeks are 2, 6 and 12 wk after calving.

Glycosylation of κ-CN

Separation of milk proteins with RP-HPLC obtained 5 different κ-CN fractions for milk of cows heterozygous for κ-CN (Figure 2.1). The non-glycosylated, mono-phosphorylated A, B and E genetic variants could be distinguished, as well as 3 regions of isoforms with different PTM patterns (Bobe et al., 1998, Bonfatti et al., 2008). The glycosylated κ-CN fraction is described as the sum of the 3 glycosylated κ-CN regions (peaks 1, 2, 4; Figure 2.1). Of cows with a 0 d dry period that were used for detailed κ-CN analysis, 8 had κ-CN genotype AA, 6 had AB and 1 had BB.

Between 6 and 2 wk prepartum, the glycosylated κ-CN faction increased from 7.8% to 12.0% and the non-glycosylated κ-CN fraction decreased from 6.2% to 3.7% (Figure 2.2). Differences were stronger for cows that have the B genetic variant of κ-CN (6.2% increase) than for cows that are homozygous AA for κ-CN (2.5% increase). In postpartum milk, no differences in non-glycosylated κ-CN were found between the different dry period lengths (Figure 2.3A). Postpartum milk of cows with a 0d dry period contained a higher glycosylated

κ-CN fraction (6.7%) than milk of cows with a 60d dry period (5.2%) ($P < 0.01$, Figure 2.3B). Differences were still present ($P < 0.05$) in 6 and 12 wk postpartum. The glycosylated κ-CN fraction was not correlated with milk yield and SCC when all postpartum sampling moments were combined. At 2 wk postpartum, the glycosylated κ-CN fraction correlated negatively (R = -0.69, $P < 0.01$) with milk yield (Figure 2.4).

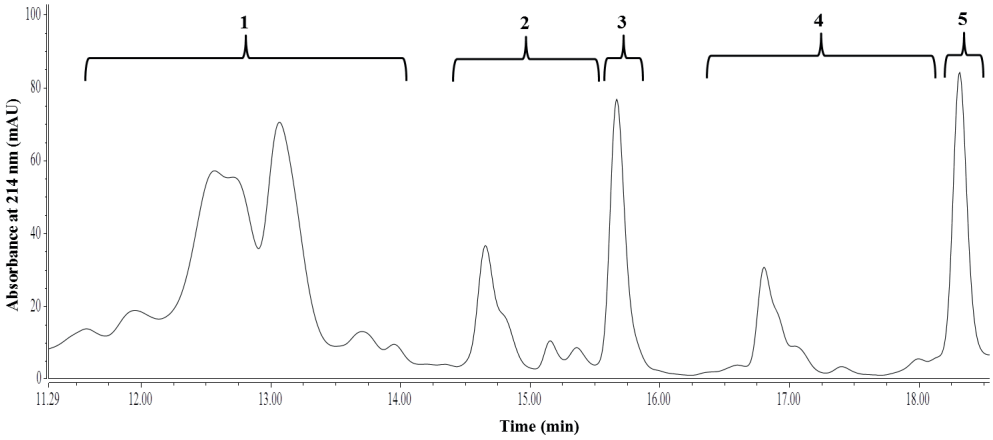

FIGURE 2.1. Example of a RP-HPLC chromatogram of raw bovine milk, zoomed-in on the κ-CN fractions. Peak 1, 2 and 4: glycosylated κ-CN isoforms, 3: non-glycosylated κ-CN A, 5: non-glycosylated κ-CN B.

Discussion

The current results show that dry period length influenced postpartum casein composition of milk. Cows with a 30 d dry period had a lower α_{s1}-CN fraction in milk than cows with a 60 d dry period. Applying a 0 d dry period did not affect the α_{s1}-CN fraction. Both cows with a 0 d and a 30 d dry period had increased α_{s2}-CN fractions in milk compared with cows with a 60 d dry period. Differences in α_{s1}-CN and α_{s2}-CN between cows with a 60 d dry period and cows with a 0 or 30 d dry period cannot be compared with prepartum casein composition since no prepartum samples were available before 6 wk prepartum. Postpartum differences may thus be a result of dry period length. The nature of the differences in α-CNs cannot be explained by the current results. Cows with a 0 d dry period had a 5.2% lower postpartum β-CN fraction than cows with a 60 d dry period, and 9.2% point lower than cows with a 30 d dry period. In 6 wk prepartum samples, a 6.7% lower β-CN fraction was found in milk of cows in the 0 d dry period group compared with milk of cows in the 30 d dry period group. This unexpected prepartum difference in β-CN fractions may partly explain the postpartum difference. All in all, it cannot be excluded that postpartum differences in casein composition between dry period groups originate from prepartum differences.

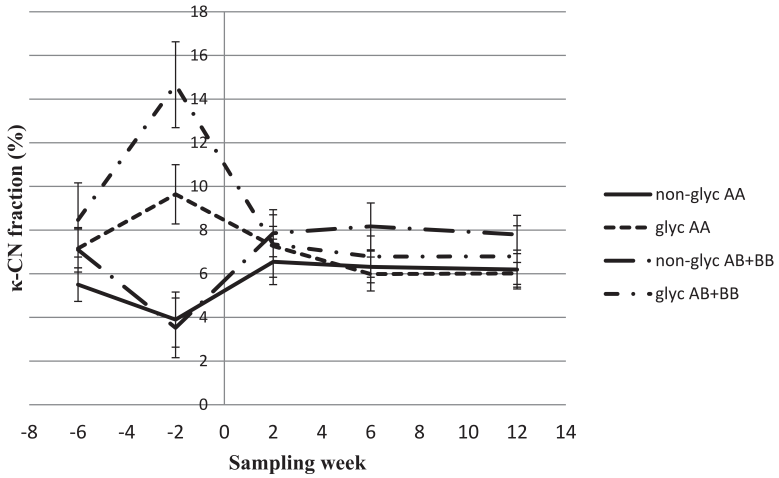

FIGURE 2.2. Changes in glycosylated (glyc) and non-glycosylated (non-glyc) κ-CN fractions in the peripartum period of continuously milked cows with κ-CN genotype AA (8 cows) or AB (+BB) (7 cows), measured with RP-HPLC. Fractions are represented as percentage of the sum of total casein, α-LA and β-LG.

In the current study, the α_{s2}-CN fraction was found to be maximal at 2 wk postpartum, whereas the α_{s1}-CN fraction was not influenced by the postpartum sampling week. Previously α_s-CN was reported to decrease as lactation progresses, without distinguishing between α_{s1}- and α_{s2}-CN (Ostersen et al., 1997). An increase in β-CN fraction as a result of lactation week in early lactation has been observed before (Ostersen et al., 1997).

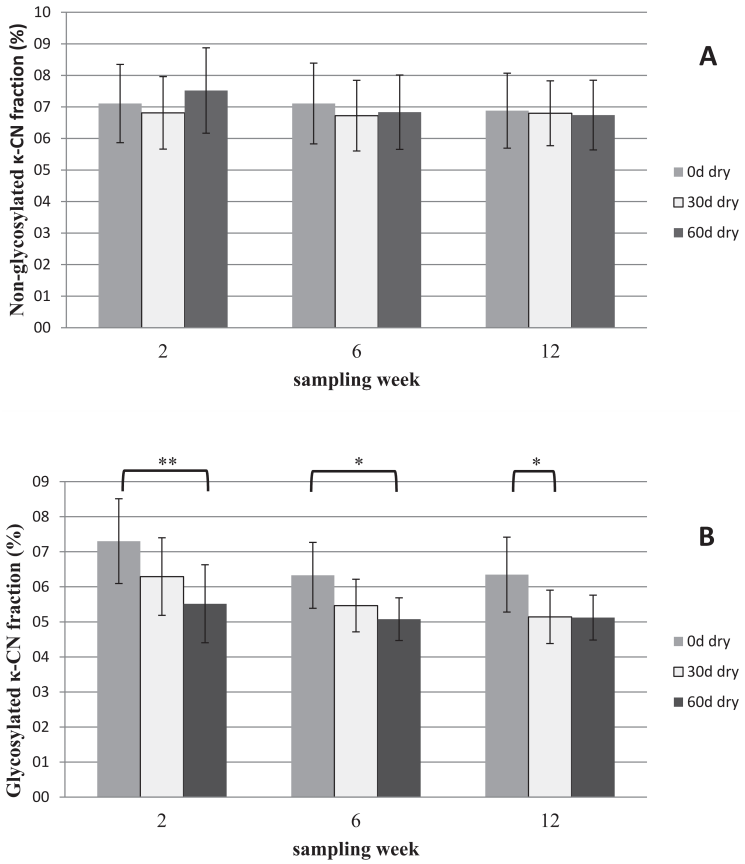

FIGURE 2.3. Postpartum fractions of non-glycosylated (A) and glycosylated (B) κ-CN in milk of cows with a 0, 30 or 60 d dry period (15 cows per dry period group), measured with RP-HPLC. Fractions are represented as percentage of the sum of total casein, α-LA and β-LG.

* $P < 0.05$

** $P < 0.01$

Besides the separate effects of dry period length and lactation stage on postpartum β-CN fractions, an interaction between dry period and lactation stage was found. At 2 wk in lactation, the β-CN fraction of cows with a 0 d dry period was lower than the β-CN fraction of cows with a 30 d or 60 d dry period. At 12 wk in lactation, the β-CN fraction was not related to dry period length anymore. The β-CN fraction was corrected for co-eluting minor forms of κ-CN using a fixed value. This correction factor may be underestimated for cows with a 0 d dry period due to an increased glycosylated κ-CN fraction. Therefore, the β-CN fraction of cows with a 0 d dry period may be slightly overestimated. The reduced β-CN fraction of cows with a 0 d dry period at 2 wk postpartum may be the effect of proteolytic breakdown by plasmin. Previous work has shown that plasmin activity increases with increasing lactation stage (Bastian et al., 1991, Politis et al., 1989), so a reduction in plasmin

activity in milk can be expected between late lactation and the successive early lactation. These studies did not emphasise on dry period length of the cows. For cows with a 0 d dry period, plasmin activity was found to be similar pre- and postpartum (Dupont et al., 1998). This may indicate increased plasmin activity in early lactation milk of cows with a 0 d dry period compared with cows with a 60 d dry period. In the study of Dupont et al. (1998), no cows with a dry period were included to study the effect of dry period length on plasmin activity postpartum. In the current study, the β-CN fraction was lowered for cows in fourth or higher parity. A negative correlation was found between β-CN fraction and SCC. Plasmin activity is known to increase with increasing parity and elevated SCC (Bastian et al., 1991, Politis et al., 1989). Increasing plasmin activity at increasing SCC may be the result of increased activation of plasminogen due to decreased epithelial barrier integrity, like is the case for mastitic cows (Politis et al., 1989). The current results indicate that the reduced β-CN fraction in 2 wk postpartum milk samples of cows with a 0d dry period may result from an increased plasmin activity.

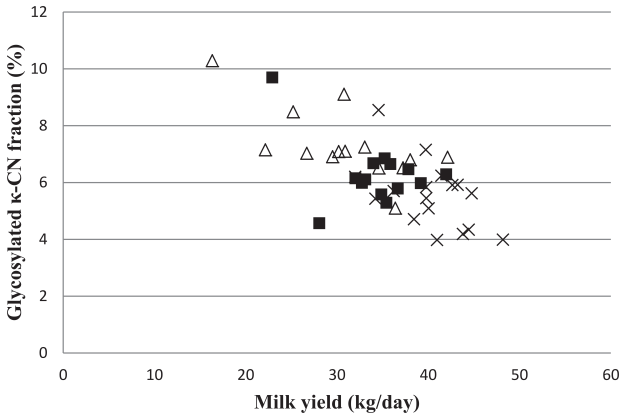

FIGURE 2.4. Glycosylated κ-CN fraction as a percentage of total protein related to milk yield at 2 wk in lactation, measured with RP-HPLC. A fraction is represented as percentage of the sum of total casein, α-LA and β-LG. Different markers indicate different dry period lengths (Δ 0 d dry, ■ 30 d dry, x 60 d dry, 15 cows per dry period group).

In the current study, the glycosylated κ-CN fraction increased approximately one and a half fold between 6 and 2 wk prepartum. The increase in glycosylation was stronger when the B variant of κ-CN was present than in milk of cows homozygous for κ-CN A. Only in this late stage of lactation a genotype effect on κ-CN glycosylation was observed, whereas the B variant of κ-CN is known to be associated with higher levels of glycosylation in mid-lactation as well (Bijl et al., 2014, Robitaille et al., 1991). Increasing glycosylated and decreasing non-glycosylated κ-CN fractions have been observed before with progressing lactation (Bonfatti et al., 2014). The N-acetylneuraminic acid content of κ-CN, an indication for the amount of glycosylated κ-CN, has also been shown to increase when lactation progresses (Robitaille

et al., 1991). From the study of Bonfatti et al. (2014) an approximately one and a half fold increase in glycosylated κ-CN can be calculated between early (5-30 d) and late (350 d) lactation. The strong increase in glycosylation of κ-CN in the last weeks before calving may be related to the onset of colostrum production, since colostrum κ-CN is known to be highly glycosylated (Fiat et al., 1988, Guerin et al., 1974). Secretion of colostrum components such as IgG_1 has also been shown to increase in the last 21 d before calving (Guy et al., 1994). The one and a half fold increase in glycosylated κ-CN between 6 and 2 wk prepartum that was observed in the current study indicates that not the number of days in lactation, but the number of days until calving is most important in determining the level of κ-CN glycosylation.

The current study shows that the glycosylated κ-CN fraction was higher in postpartum milk samples of cows with a 0 d dry period compared with cows with a 30 d or 60 d dry period. Glycosylation was higher at 2 wk in lactation than in later postpartum samples. At this early moment in lactation, the glycosylated κ-CN fraction of milk correlated negatively with milk yield. In previous work, a higher N-acetylneuraminic acid content was found in the casein fraction of mastitic milk (Robitaille et al., 1991). This is in agreement with increased activation of the O-glycan biosynthesis pathway found in bovine mammary cells with *E. coli* mastitis (Yang et al., 2014). In other cell types, inhibition of O-glycosylation was shown to result in increasing apoptosis (Chatham et al., 2008). In all 3 aforementioned studies, protein glycosylation was related to dysfunctional cells. Applying a 0 d dry period to dairy cows was shown to result in reduced repair or replacement of damaged mammary epithelial cells prepartum (Capuco et al., 1997), possibly resulting in increased numbers of dysfunctional cells postpartum. Reduced milk yield and increased κ-CN glycosylation may be markers for the presence of an increased proportion of senescent mammary epithelial cells. In the current study, the glycosylated κ-CN fraction was not correlated with SCC. Since no mastitic cows were included, this does not disagree with the work of Robitaille et al. (1991b) and Yang et al. (2014). Both high κ-CN glycosylation and β-CN degradation in milk were related to a 0 d dry period. In contradiction to a high glycosylated κ-CN fraction, a low β-CN fraction was related to high parity cows and elevated SCC. Therefore, increased κ-CN glycosylation and β-CN degradation in postpartum milk of cows with a 0 d dry period seem to have a different origin. Increased κ-CN glycosylation in milk of cows with a 0 d dry period at 2 wk in lactation seems to be related to not optimally functioning mammary epithelial cells.

Cows that are used in the current study are a sub-group of the population described by Van Knegsel et al. (2014). Similar changes in milk yields and macronutrient composition were found when the dry period was shortened or omitted as in the previous study. Differences in milk protein percentages when the dry period was shortened from 60 d to 0 or 30 d were larger in the current study (0 d: 0.4% higher, 30 d: 0.3% higher) than in a previously reported meta-analysis (0 d: 0.25% higher, 30 d: 0.06% higher) (Van Knegsel et al., 2013).

The difference between the current study and the previous meta-analysis may be a result of the early stage of lactation in which the samples were taken in the current study. Including samples from a later stage of lactation may reduce differences between groups of cows with different dry period lengths.

Differences in milk composition as a result of different dry period length may affect processing properties of the milk. Total protein content increased both in early and late lactation when a 0 d or 30 d dry period was applied. It is known that high protein content is related to high cheese yield (Wedholm et al., 2006). In previous work, a variation in α_{s1}-CN and β-CN fractions larger than differences found in the current study did not influence milk coagulation properties or cheese yield (Wedholm et al., 2006). Therefore the observed α_{s1}-CN and β-CN reductions in the current study as a result of dry period reduction may not influence processability of milk. It is known that plasmin activity negatively affects cheese milk quality and UHT milk stability (Kelly and McSweeney, 2003). The suggested increase in plasmin activity in relation to dry period length reduction should be studied in more detail in future work. We found increased κ-CN glycosylation in both pre- and postpartum milk of cows with a 0 d dry period. If the increase in κ-CN glycosylation would be a separate effect, it may result in increased curd firmness (Robitaille et al., 1993). Though, besides protein content and κ-CN glycosylation, increases in calcium concentration (Ostersen et al., 1997, White and Davies, 1958) and milk pH (White and Davies, 1958) in late lactation may be expected. All these milk compositional properties are known to affect cheese making properties (Walstra et al., 2006), and may thus influence cheese making properties of milk from cows with a 0 d dry period. Protein content, composition and proteolytic activity are all factors that may influence milk processing. Future work is required to investigate the influence of these factors on rennetability, gel strength and storage stability of early lactation milk in relation to dry period length.

Conclusions

Shortening the dry period of dairy cows from 60 to 30 d resulted in lower α_{s1}-CN and higher α_{s2}-CN fractions in milk protein. Applying a 0 d dry period resulted in an increase of glycosylated κ-CN, in both late and early lactation. A lower β-CN fraction in early lactation milk of cows with a 0 d dry period is likely a result of increased plasmin activity. Changes in α_{s1}-CN and α_{s2}-CN fractions as a result of shortening the dry period from 60 to 30 d are unlikely to influence processability of milk. The contribution of changes in protein percentages, the glycosylated κ-CN fraction and possible increase in plasmin activity of milk from cows with a 0 d dry period to milk processing properties should be subject of future work in this field.

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Chapter Three

Influence of shortening the dry period of Swedish dairy cows on plasmin activity in milk

Ruben de Vries, Melanie Brandt, Åse Lundh, Kjell Holtenius, Kasper Hettinga, Monika Johansson

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Abstract

The aim of this study was to evaluate the influence of shortening the dry period of Swedish dairy cows on plasmin activity and casein composition in milk. Swedish Holstein and Swedish Red cows, 45 in total, were assigned to a dry period of either 4 or 8 weeks. Milk samples were taken 10 and 5 weeks prepartum, and 6 and 12 weeks postpartum. Plasmin activity and plasminogen activity were measured with a spectrophotometric assay. Casein composition was measured by capillary zone electrophoresis. Prepartum plasminogen activity increased by 22% between 10 and 5 weeks prepartum, whereas no change in plasmin activity was observed during the same period. Cows with a 4 weeks dry period had 61% higher plasmin activity in postpartum milk than cows with an 8 weeks dry period. Cows of third or greater parity tended to have a stronger increase in plasmin activity as a result of applying a short dry period than cows of second parity. Although the α_{s1}- and β-casein fractions declined with increasing plasmin activity, no dry period effects were found on casein composition. Based on postpartum differences in plasmin activity, it was concluded that particularly multiparous cows require more than 4 weeks between lactations for recovery of the mammary epithelium. Changes in casein composition as an effect of plasmin activity are not expected to have a great impact on processing quality of milk, although future work is needed to verify this.

Introduction

A conventional dry period of 6-8 weeks prior to parturition is known to maximise milk yield of dairy cows in their successive lactation (Kuhn et al., 2005, Watters et al., 2008, van Knegsel et al., 2014). High peak milk production of cows subjected to a conventional dry period may result in a deep negative energy balance in early lactation, which is related to increased risk of metabolic problems (Rastani et al., 2005, van Knegsel et al., 2013) and reduced fertility (Butler, 2003, Gumen et al., 2005). Shortening the dry period is a way to improve the energy balance in early lactation (Rastani et al., 2005, van Knegsel et al., 2014).

When applying such a shorter (28-35 d) instead of a conventional 56-64 d dry period, postpartum milk yield was reported to be 1.4 kg/d lower. Protein content was 0.06% higher in milk of cows with a short dry period compared with a conventional dry period, whereas milk fat content was not affected by shortening the dry period (van Knegsel et al., 2013). The postpartum α_{s1}-casein (CN) fraction was 3.8% lower in milk of cows with a 30 d dry period compared with a 60 d dry period, whereas the α_{s2}-CN fraction was 5.5% lower when a 30 d dry period was applied (de Vries et al., 2015). Omitting the dry period resulted in a reduced β-CN fraction, which was suggested to be a result of increased proteolytic activity (de Vries et al., 2015). These recent findings have provided an overview of compositional changes of milk as a result of shortening the dry period of the cow. The cause of compositional changes are, however, not well understood.

Plasmin is the main endogenous protease in bovine milk. It is converted from its inactive zymogen plasminogen by the action of plasminogen activators. Plasmin and plasminogen originate from blood and are transported passively to milk through tight junctions in the mammary epithelium (Kelly and McSweeney, 2003). In the mammary gland, plasmin facilitates tissue remodelling by protein degradation and activation of other enzymes (Politis, 1996). The function of plasmin in milk is not clear yet. Plasmin activity in milk was shown to increase with advancing stage of lactation (Politis et al., 1989a, Bastian et al., 1991), fourth or greater parity (Politis et al., 1989a, Bastian et al., 1991) and somatic cell count (SCC) in milk higher than 300,000 cells/mL (Politis et al., 1989a). Plasmin can hydrolyse α_{s1}-CN, α_{s2}-CN and β-CN in milk. Reduced casein fractions in milk may lead to defects in processing such as reduced cheese yield and curd firmness (Mara et al., 1998, Srinivasan and Lucey, 2002). However, no relation was found between naturally present plasmin activity and clotting parameters of milk (Bastian et al., 1991). Plasmin is a relatively heat stable enzyme (Prado et al., 2007) that may affect protein stability during storage of UHT milk (Rauh et al., 2014) or milk protein ingredients (Gazi et al., 2014). The aim of this study was to evaluate the influence of shortening the dry period of dairy cows on plasmin and plasminogen activity, and the consequence of plasmin activity for casein composition.

Materials and Methods

Animals and milk samples

Forty-five clinically healthy cows with proper udder health of either the Swedish Holstein (SH, N = 21) or Swedish Red (SR, N = 24) breed were included in this study. A dry period of either 4 weeks (N = 26, of which 13 SH, 13 SR) or 8 weeks (N = 19, of which 8 SH, 11 SR) was applied to the cows. The dry period groups consisted of both primiparous and multiparous cows (4 wk dry, primiparous N = 15, multiparous N = 11; 8 wk dry primiparous N = 13, multiparous N = 6). Cows were randomly assigned to either a 4 wk or an 8 wk dry period. Cows that, one week prior to the experiment, yielded less than 15 kg milk/day, or had signs of reduced udder health, were excluded from the study. The cows were housed at the Swedish Livestock Research Centre, Lövsta in an indoors loose house system with slatted floor and cubicles with rubber mats and chopped straw as bedding. Lactating cows were batch-milked twice daily at 06.00 and 16.00 in an automatic milking rotary (DeLaval AMR™, Tumba, Sweden). Before drying-off, the cows were fed silage *ad libitum* and concentrate according to milk production. The week prior to drying-off, concentrate was withdrawn and during the dry off procedure the cows were fed 4 kg DM of silage and straw *ad libitum* and no concentrate. No intramammary antibiotics were used at drying off. During the dry period, cows were fed a blend of silage and straw *ad libitum* and concentrate was stepwise increased to 3 kg at parturition. After calving, silage was provided *ad libitum* while the supply of concentrate was increased stepwise to 13.5 kg/d. Water was always available *ad libitum*.

Milk samples from the morning milking were taken 10 and 5 weeks prepartum, and 6 and 12 weeks postpartum. Bronopol (0.3%) was added as a preservative to milk samples. Milk samples were stored at -20°C directly after collection. Milk yields were automatically recorded in the robot, whereas milk fat and protein percentage and somatic cell counts (SCC) were analysed at the Department of Animal Nutrition and Management, SLU Uppsala, using Fourier Transform Infra-Red (FTIR) spectroscopy and flow cytometry with fluorescence cell staining (Foss Electric, Hillerød, Denmark). Prepartum plasmin and plasminogen activity were only analysed in milk of cows with a 4 wk dry period, allowing the comparison of week 5 and 10 prepartum. No prepartum comparison between dry period groups was made for plasmin and plasminogen activity. The Uppsala Local Ethics Committee approved the experimental protocol (C178/12).

Plasmin and plasminogen activities in milk serum

Milk samples were analysed for both plasmin activity and plasminogen activity by a method modified from Korycha-Dahl et al. (1983). Chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., SE-135 70, Stockholm, Sweden), except where stated differently. The reaction mixture in which plasmin and plasminogen activity were measured consisted of 0.5 mL milk and 7.5 mL plasmin buffer (50 mM Trizma-HCL pH 7.4, 117 mM NaCl, 25 mM EACA, pH 7.4). After 1 h incubation at room temperature, the milk serum containing the plasmin and plasminogen was separated from casein micelles by ultracentrifugation (LKB Ultrospin, Sweden) with an RP55T angle rotor, 12 mL x 12 at 100,000 x g for 1 h at 4°C. Plasmin activity was measured in the serum with the pyro-GLU-Phe-Lys-p-nitroanilide hydroxychloride chromogenic substrate (2.5 mg/mL) (Aniara, Biophen CS-41(03), USA) in a Sarstedt 96 wells plate (Sarstedt, Helsingborg, Sweden). Plasminogen activity was determined after addition of urokinase (49.5 plough units) for activation of plasminogen into plasmin. Plasmin and total activity (i.e. combined plasmin and plasminogen activity) were measured continuously every third minute during 120 minutes by a multi-mode microplate reader (FLUOstar Omega, BMG LABTECH, Germany) at 37°C. The change in absorbance ($\Delta A405/\Delta t$) was used for the measurement of plasmin activity, where the formation of p-nitroanilide (pNA) was calculated from the linear part of the absorbance versus time curve. Plasminogen activity was calculated as the difference between the total activity and plasmin activity. Plasmin and plasminogen activities were expressed in the same units, with one unit being defined as the amount of enzyme that produces a $\Delta A405$ of 0.001 per minute at pH 7.4 and 37°C due to pNA released from CS-41(03) substrate in the defined reaction mixture. The intra-assay coefficient of variation was 5% for both plasmin and plasminogen activity.

Casein composition

Casein composition of milk samples was determined by capillary zone electrophoresis as described by de Vries et al. (2015).

Statistical analysis

A mixed model accounting for repeated measures (SAS 9.3, SAS Institute Inc., Cary, NC, USA) was used for comparison between treatments, in which cows were the repeated subject. Prepartum and postpartum results were analysed separately. *P*-values were corrected by Bonferroni adjustment. Differences were considered to be significant if $P < 0.05$. Prepartum comparison between dry period lengths was done with samples of week -10. Fixed factors in the mixed model were dry period (4 wk or 8 wk), breed (Swedish Holstein or Swedish Red) and parity (first, second or greater). The interactions dry period x breed, dry period x parity and breed x parity were also included in the model. Prepartum comparison between sampling weeks was done with milk samples from cows in the 4 wk dry period group. Fixed

factors in the model were sampling week (-10 or -5 wk), breed (Swedish Holstein or Swedish Red) and parity (first, second or greater). The interactions sampling week x breed, sampling week x parity and breed x parity were also included in the model. One model was used for postpartum samples, including the fixed factors dry period (4 wk or 8 wk), sampling week (6 or 12 wk), breed (Swedish Holstein or Swedish Red) and parity (second, third or greater). The interactions dry period x sampling week, dry period x breed, dry period x parity, sampling week x breed, sampling week x parity and breed x parity were also included in the postpartum model. An autoregressive covariance structure had the best fit for all variables, both pre- and postpartum, and was used to account for within-cow variation.

Results and Discussion

Milk yield

Cows in the 4 wk dry period group had a higher milk yield (24.4 kg/d) than cows in the 8 wk dry period group (19.9 kg/d) at 10 wk prepartum ($P < 0.01$). Swedish Reds (SR) had lower milk yield (18.5 kg/d) than Swedish Holsteins (SH) (24.3 kg/d) at 10 wk prepartum ($P < 0.01$). Milk yield was significantly influenced by the interaction dry period x breed ($P = 0.04$). SH with a 4 wk dry period (28.2 kg/d) had higher milk yield than SH with an 8 wk dry period (20.4 kg/d), whereas SR with a 4 wk or an 8 wk dry period had similar milk yield. Milk yield of cows with a 4 wk dry period was higher ($P < 0.01$) at 10 wk prepartum (24.4 kg/d) than at 5 wk prepartum (19.5 kg/d) (Table 3.1). Postpartum milk yield of cows that had an 8 wk dry period (42.9 kg/d) was higher compared with cows that had a 4 wk dry period (38.2 kg/d) (Table 3.2), which is was larger than was reported before (-1.5 kg/d) in a meta-analysis (van Knegsel et al., 2013). The unexpected prepartum difference seems not to have influenced postpartum results, but rather lead to underestimation of the effect size. Postpartum milk yield was higher at 6 weeks (41.6 kg/d) than at 12 weeks of lactation (38.6 kg/d) (Table 3.2).

TABLE 2.1. Means for milk yield and composition of cows with a 4 wk dry period. *P*-values for week are based on milk samples of cows with a 4 wk dry period (N = 25) at 5 or 10 wk prepartum.

	Week relative to calving			*P*-value
	-10	-5	SEM	Week[1]
Milk yield (kg/day)	24.4	19.5	1.7	<0.01
Fat (%)	4.82	4.76	0.20	0.36
Protein (%)	3.91	4.10	0.12	0.06
SCC (x10^3 cells/mL)[2]	94	100	28	0.15
Plasmin activity (units/mL)	7.3	8.0	1.9	0.14
Plasminogen activity (units/mL)	74.3	90.9	6.7	<0.01
α$_{s1}$-CN (%)[3]	28.4	28.2	0.8	0.35
α$_{s2}$-CN (%)[3]	7.8	8.3	0.5	0.04
β-CN (%)[3]	34.0	34.0	1.2	0.60
κ-CN (%)[3]	10.1	9.6	0.5	0.57
α-LA (%)[3]	3.0	2.9	0.1	0.64
β-LG (%)[3]	10.7	10.7	0.4	0.86

[1] Also included in the model are the interactions week x breed, week x parity and breed x parity.

[2] Statistical analysis based on log-values.

[3] Milk protein fractions are expressed as percentage of the sum of all proteins in the CZE electropherogram.

Milk composition prepartum

Milk macronutrient composition prepartum did not differ between cows with either a 4 wk or an 8 wk dry period, neither did milk protein composition. SH had lower milk protein content (3.93%) than SR (4.12%) at 10 wk prepartum ($P = 0.04$). Milk protein content was particularly lower for SH with a 4 wk dry period (3.73%), compared with SH with an 8 wk dry period (4.14%) or SR ($P < 0.01$). At 10 wk prepartum, 3 milk samples had SCC between 250,000 and 500,000 cells/mL. None of the prepartum milk samples had SCC > 500,000 cells/mL. Plasminogen activity was higher ($P < 0.01$) at 5 wk prepartum (90.9 units/mL) than at 10 wk prepartum (74.3 units/mL). Such an increase in plasminogen activity with advancing lactation has been reported before (Bastian et al., 1991, Nicholas et al., 2002). Plasminogen activity correlated negatively with milk yield (R = -0.45, $P < 0.01$) and positively with milk protein conent (R = 0.67, $P < 0.01$), whereas plasmin activity did not. These results indicate a concentration effect of plasminogen during late lactation due to the correlations with milk yield, as was reported before (Schaar and Funke, 1986). Plasmin activity did not differ between milk samples taken either 5 or 10 wk prepartum, which is in accordance with Dupont et al. (1998). Other work, however, indicated an increase in plasminogen activation towards the end of lactation (Politis et al., 1989b), which was not confirmed by the current results. Primiparous cows had lower plasmin activity in milk at either 5 or 10 wk prepartum (5.6 units/mL) than multiparous cows (11.9 units/mL). Proteolytic activity towards caseins did not seem to increase between 10 and 5 weeks prepartum, since none of the casein fractions reduced between 10 and 5 wk prepartum. The α_{s2}-CN fraction was higher ($P = 0.04$) at 5 wk prepartum (8.3%) than at 10 wk prepartum (7.8%). Milk of SH had a lower α-LA fraction (2.7%) than milk of SR (3.1%) at 10 wk prepartum. Negative correlations were found between plasmin activity and α_{s1}- and β-CN fractions, but these were strongly determined by 1 milk sample with high plasmin activity. All in all, the current work shows that milking cows until 4 wk prepartum does not result in increased plasmin activity or casein breakdown in late lactation milk.

TABLE 3.2. Milk yield and composition of cows after a dry period of 4 wk (N = 25) or 8 wk (N = 18) at 6 and 12 wk postpartum (Mean ± SEM).

	Dry period length		Week			*P*-values[1]			
	4 wk	8 wk	6	12	SEM	Dry period	Breed	Parity	Week
Milk yield (kg/day)	38.2	42.9	41.6	38.6	1.8	<0.01	0.05	0.83	<0.01
Fat (%)	3.83	3.65	3.65	3.87	0.18	0.11	<0.01	0.61	0.09
Protein (%)	3.39	3.30	3.33	3.39	0.05	0.24	0.54	0.70	0.04
SCC (x10^3 cells/mL)[2]	280	356	268	333	155	0.31	0.01	0.20	0.92
Plasmin activity (units/mL)	5.0	3.1	4.5	3.9	0.5	<0.01	0.63	0.02	0.49
Plasminogen activity (units/mL)	56.2	56.6	56.3	56.5	3.7	0.90	0.91	0.03	0.57
α_{s1}-CN (%)[3]	29.5	30.2	29.9	29.6	0.3	0.06	0.10	0.09	0.81
α_{s2}-CN (%)[3]	8.6	8.3	8.8	8.1	0.3	0.24	0.19	0.27	<0.01
β-CN (%)[3]	33.8	33.4	33.5	33.8	0.6	0.83	0.46	0.93	0.12
κ-CN (%)[3]	10.3	10.9	10.5	10.6	0.4	0.53	0.20	0.31	0.90
α-LA (%)[3]	3.6	3.6	3.7	3.5	0.1	0.92	0.04	0.77	<0.01
β-LG (%)[3]	10.1	9.4	9.6	10.0	0.3	0.07	0.76	0.74	0.29

[1] Also included in the model are the interactions dry period x breed, dry period x parity, dry period x week, breed x parity, breed x week and parity x week.

[2] Statistical analysis based on log-values (SCC).

[3] Milk protein fractions are expressed as percentage of the sum of all proteins in the CZE electropherogram.

Milk composition postpartum

In samples taken either 6 or 12 wk postpartum, milk fat content of SR (3.96%) was higher than of SH (3.55%). Milk protein content was higher at 12 wk (3.39%) than at 6 wk postpartum (3.33%). Cows with an 8 wk dry period had a lower milk protein content at 6 weeks postpartum (3.21%) than at 12 weeks postpartum, whereas cows with a 4 wk dry period had similar protein content at either 6 or 12 weeks postpartum. This resulted in a significant dry period x week interaction ($P = 0.03$). SCC was higher in milk of SR (393,000 cells/mL) than SH (208,000 cells/mL). SH with an 8 wk dry period had lower SCC (51,000 cells/mL) than SH with a 4 wk dry period (270,000 cells/mL), or SR with any dry period, resulting in a dry period x breed interaction ($P = 0.03$). In total, 14 cows had SCC in milk > 250,000 cells/mL at either 6 wk postpartum (N = 3), 12 wk postpartum (N = 6) or both (N = 5). The α_{s2}-CN fraction was higher at 6 weeks postpartum (8.8%) than at 12 weeks postpartum (8.1%) ($P < 0.01$). The α-LA fraction was higher in the sixt (3.7%) than in the twelfth (3.5%) week postpartum ($P < 0.01$). Cows of parity greater than second had higher α-LA fraction at 6 weeks (3.9%) than at 12 weeks postpartum (3.3%), whereas no difference between weeks was observed for cows of second parity, which resulted in a parity x week interaction ($P < 0.01$). Like prepartum, the α-LA fraction was higher in milk of SR (3.8%) than SH (3.4%) ($P = 0.04$).

Plasmin and plasminogen activity

In previous work, it was suggested that complete omission of the dry period resulted in higher proteolytic activity in milk based on lower β-CN fractions. Applying a 30 d dry period did not affect the β-CN fraction in the same study (de Vries et al., 2015). The plasmin activity assay used in the current study gives a more sensitive measure for plasmin activity than the β-CN fraction that was used previously to indicate proteolytic breakdown. The current results show that plasmin activity in milk samples 6 or 12 wk postpartum was higher of cows with a 4 wk dry period (5.0 units/mL) than of cows with an 8 wk dry period (3.1 units/mL) ($P < 0.01$, Table 3.2). Cows of third or greater parity had higher plasmin activity (5.8 units/mL) than cows of second parity (3.5 units/mL, $P = 0.02$). Dry period length and parity tended to interact with each other ($P = 0.06$), and plasmin activity was particularly high in milk of cows of third or greater parity that had a 4 wk dry period (7.4 units/mL, Figure 3.1A). Cows with a 4 wk dry period had a 28% reduction in plasmin activity between 10 wk prepartum and 6 wk postpartum. Similar reductions in plasmin activity between late and early lactation have been reported in previous work, taking into consideration that all cows have a higher parity number after calving (Bastian et al., 1991). Plasmin activity at 6 wk postpartum did not correlate with milk yield 10 wk prepartum, and thus the postpartum difference in plasmin activity does not seem to be a result of the prepartum difference in milk yield between cows with a 4 wk or an 8 wk dry period. In contrast to plasmin activity, plasminogen activity was higher in milk of second parity cows (66.3 units/mL) than of cows with greater parity (54.3 units/mL) ($P = 0.03$). Plasminogen activity was not affected by dry period length, or by an interaction between dry period length and parity (Figure 3.1B). Plasminogen is transported transcellularly (Silanikove, 2016) and converted into plasmin by the influence of plasmin activators and plasminogen activator inhibitors. The current work shows an increase in plasmin activity in postpartum milk as a result of dry period reduction, though no change in plasminogen activity was found. If transcellular transport would be the main cause of increased plasmin activity in milk of cows with a 4 wk dry period, a proportional increase in plasminogen activity would be expected.

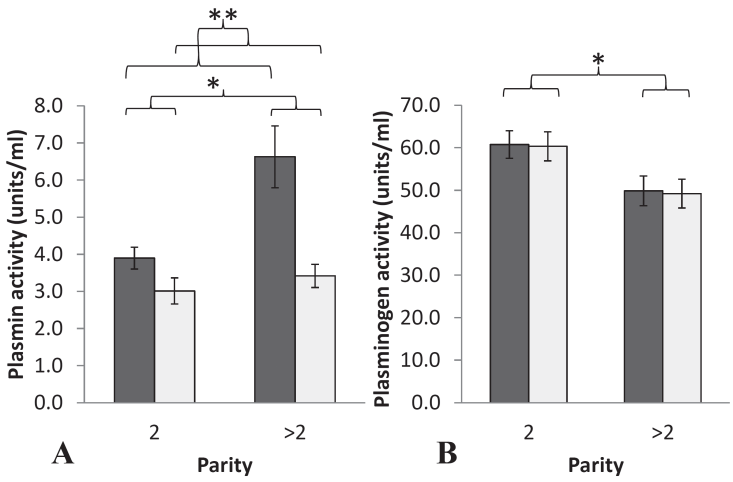

FIGURE 3.1. Plasmin activity (**A**) and plasminogen activity (**B**) in milk samples (6 and 12 wk postpartum) of cows with a dry period of either 4 wk (■) or 8 wk ( ) , and of second, or greater than second parity.
* $P < 0.05$
** $P < 0.01$

Role of plasmin in the mammary epithelium

Plasmin activity in milk can reflect several processes in the mammary epithelium, such as mammary involution and epithelial cell proliferation (Politis, 1996). Plasmin was suggested to enhance epithelial cell proliferation by matrix degradation between cells during cell division (Saskela and Rifkin, 1988). The epithelium of the bovine mammary gland undergoes significant proliferation both prepartum (Capuco et al., 1997), as well as postpartum (Capuco et al., 2001). Mammary epithelial cell proliferation was found to occur at a lower rate for multiparous cows than for primiparous cows (Miller et al., 2006). A dry period was suggested to be important for regeneration of the mammary epithelium in order to prepare for a successive lactation, which was based on a higher cell regeneration rate of non-lactating mammary tissue compared with lactating tissue (Capuco et al., 1997). A 4 wk dry period in combination with a low proliferation rate may therefore result in limited epithelial cell proliferation prepartum. A consequence may be increased proliferation postpartum compared with cows that had an 8 wk dry period. Ongoing cell proliferation in early lactation may be reflected by higher plasmin activity in milk, due to the involvement of plasmin in mammary epithelial cell proliferation. The group of cows with high plasmin activity in milk consisted of both SH and SR, and consequently, the breed did not influence plasmin activity in the current study, although one could speculate that a dual purpose breed (SR) may have a different mammary epithelial cell proliferation rate than a milk breed (SH). If there would be a breed effect on plasmin activity, this effect was less strong than the effect of parity, and therefore it did not appear in the current results. The role of plasmin activity

in tissue remodelling is generally believed to be mediated by urokinase-type plasminogen activators originating from somatic cells (Politis, 1996). Plasmin activity in postpartum milk of cows with a 4 wk dry period correlated exponentially with SCC (R = 0.55, $P < 0.01$). This indicates that SCC is may be related to plasmin activity in postpartum milk of cows with a 4 wk dry period, possibly due to tissue remodelling. No relation was found between plasmin activity and SCC in postpartum milk of cows with an 8 wk dry period. It can be concluded that high-parity cows with a high milk yield prepartum that had a short dry period, had high plasmin activity in milk postpartum, which seems to reflect increased mammary epithelial cell proliferation.

Influence of plasmin activity on casein composition

In contrast to plasmin activity, none of the casein fractions was affected by dry period length, although the α_{s1}-CN fraction tended to be lower in milk of cows with a 4 wk dry period (29.5%) than in milk of cows with an 8 wk dry period (30.1%) (Table 3.2). Plasmin activity correlated negatively with the β-CN fraction (R = -0.46, $P < 0.01$) and the α_{s1}-CN fraction (R = -0.45, $P < 0.01$) when including all postpartum milk samples. Plasmin activity correlated positively with the α_{s2}-CN fraction in postpartum milk samples (R = 0.41, $P < 0.01$) (Figure 3.2). However, all these correlations were strongly determined by samples (N = 2) with high plasmin activity (>10 units/mL). Like α_{s1}-CN and β-CN, α_{s2}-CN can be degraded by plasmin (Kelly and McSweeney, 2003, Rauh et al., 2014) and therefore the positive correlation was not expected. This correlation may be caused by the use of casein fractions, which represent individual casein concentrations relative to the total protein concentration in the capillary zone electrophoresis profile. Hence, reduction of one fraction may indirectly result in the increase of another fraction. Previous work by us suggested increased proteolytic activity in early lactation when no dry period was applied, based on a low β-CN fraction. In the same study, no effect was found for cows with a short (30 d) dry period compared with a 60 d dry period (de Vries et al., 2015). The current work indicates that shortening the dry period to 4 wk can affect plasmin activity in milk, although the effect on casein composition is limited.

Implications of plasmin activity in relation to milk processing

Increased plasmin activity may result in increased protein breakdown into peptides during ripening of (semi) hard types of cheese. Peptide formation resulting from a 1.5 to 3-fold increase in plasmin activity, a similar increase as in the current work (Table 3.2), may increase cheese flavour development (Farkye and Landkammer, 1992), but may also result in undesired bitterness (Bastian et al., 1997). The current work indicates that dry period reduction may induce a comparable 1.5-fold increase in plasmin activity in early lactation, but despite this increase it is still lower than in late lactation. No overall plasmin-mediated effect of dry period length on casein composition was found, and thus this is not expected

to influence cheese making properties. Since plasmin is a relatively heat resistant enzyme, it can still be active in milk protein ingredients (Gazi et al., 2014) and UHT milk (Ismail and Nielsen, 2010). In UHT milk, plasmin has been shown to increase age gelation (Kohlmann et al., 1988, Rauh et al., 2014). Quantitative data about the influence of endogenous plasmin activity on storage stability of UHT milk are difficult to compare due to variations between studies in analytical and processing conditions. Pre-treatment conditions of milk prior to UHT processing have a major influence on the impact of proteolysis on product properties (Rauh et al., 2014), and may therefore need to be considered. Future work is needed to evaluate UHT milk stability of early lactation milk from cows with a 4 wk dry period. It is recommended to compare cows of different parities and include cows with a 0 d dry period to obtain a complete overview of the effect of dry period induced changes in plasmin activity on processing characteristics of milk.

Conclusion

In conclusion, applying a 4 wk dry period to multiparous cows resulted in high plasmin activity in postpartum milk, compared with an 8 wk dry period. This result indicated that the epithelium of these cows regenerated to a lesser extent than that of other cows. Increased plasmin activity, but unaffected plasminogen activity, indicated that applying a short dry period resulted in increased activation of plasminogen. Shortening the dry period to 4 wk did not affect casein composition in milk. Milk with high plasmin activity was found to contain reduced α_{s1}-CN and β-CN fractions. However, the effect of shortening the dry period on casein composition is not expected to give rise to concern for cheese making industry.

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Chapter Four

Influence of dry period length of dairy cows on casein micelle composition in early and mid-lactation milk

Ruben de Vries, Toon van Hooijdonk, Kasper Hettinga

Manuscript in preparation

Abstract

The aim of this work was to evaluate the influence of applying a dry period of either 0 or 30 days to dairy cows on micellar casein and mineral composition, and plasmin activity of early and mid-lactation milk. Milk samples of 18 cows with a dry period of either 0 or 30 days were collected during early lactation (5-8 wk postpartum) and mid-lactation (20-28 wk postpartum). Milk protein composition was analyzed by reversed phase - high performance liquid chromatography, plasmin activity was analyzed spectrophotometrically, and mineral composition was analyzed by ion chromatography and inductive coupled plasma - atomic emission spectroscopy. Cows with a dry period of 0 days had a higher protein content in early and mid-lactation milk than cows with a dry period of 30 days. Milk of cows with a dry period of 0 days had a lower β-casein fraction in casein micelles. The β-casein fraction correlated with plasmin activity in milk. However, the lower β-casein fraction in milk of cows without a dry period did not seem to be a result of proteolytic activity. Micellar magnesium and phosphorus concentrations were higher in milk of cows without a dry period, but proportional to the casein content. Cows without a dry period had lower calcium concentration in milk serum, which was related to a higher casein content, and a lower citrate concentration in milk. It was concluded that dry period omission resulted in an increase in milk protein content, and consequently micellar mineral concentrations and plasmin activity in milk. Therefore, dry period omission did not affect casein micelle composition, apart from a small reduction of the β-casein fraction. It is therefore expected that the influence of dry period omission on processing quality of milk is very limited.

Introduction

Dairy cows commonly receive a dry period prior to parturition in order to maximize milk production in the successive lactation, and to treat infections of the mammary gland. However, a common dry period of approximately 6-8 weeks is associated with a severe negative energy balance in early lactation (van Knegsel et al., 2014), and consequently metabolic problems (Grummer, 1993). Omitting or shortening the dry period is a strategy to improve the energy balance and metabolic health of dairy cows in early lactation (van Knegsel et al., 2013).

Omitting the dry period influences the composition of milk in early and mid-lactation. Cows managed without a dry period have a higher milk protein content than cows with a dry period (van Knegsel et al., 2013, van Knegsel et al., 2014). The production of lactose, the driving force for milk volume, of cows without a dry period is lower, resulting in a lower milk yield (van Knegsel et al., 2014). Some studies have indicated that cows without a dry period have higher somatic cell count (SCC) in milk than cows with a dry period (Mantovani et al., 2010, van Knegsel et al., 2014), whereas no effect was found in other studies (Andersen et al., 2005). Somatic cells can be involved in the activation of the plasminogen into plasmin (Zachos et al., 1992), which was also found to be the case in early lactation milk of cows with a dry period of 4 weeks (de Vries et al., 2016). The effect of dry period omission on plasmin activity in early and mid-lactation milk has not been reported so far. The majority of plasmin and plasminogen in milk is attached to casein micelles (Ismail and Nielsen, 2010), and thus plasmin activity may affect the casein composition of milk. Of the individual casein fractions, particularly the β-casein fraction was reported to reduce when the dry period was omitted (de Vries et al., 2015). Shortening the dry period to 30 days did not affect the β-casein fraction (de Vries et al., 2015, de Vries et al., 2016).

Changes in plasmin activity may influence the amount of intact casein in micelles (Crudden et al., 2005). Casein micelles consist of different casein fractions, all containing serine residues that can be phosphorylated. The resulting phosphoserine residues form calcium phosphate nanoclusters together with calcium and inorganic phosphate (Walstra et al., 2006). Although proteins and minerals in casein micelles are in dynamic equilibrium with the milk serum phase, the calcium phosphate nanoclusters were reported to have a fixed composition (Holt, 1982). Differences in casein content are reflected in proportional differences in micellar calcium and micellar phosphorus concentrations (Bijl et al., 2013). Dry period omission is known to result in an increased protein content in early lactation milk (van Knegsel et al., 2013), and may thus influence the concentration of calcium and phosphate in casein micelles, and their distribution between casein micelles and the milk serum phase.

Although previous work has indicated effects of dry period omission on casein composition of milk (de Vries et al., 2015), the influence of dry period omission on plasmin activity and

mineral composition has not been reported so far. These parameters are of importance for casein micelle properties, and consequently for milk quality. Therefore, the aim of this work is to evaluate the influence of applying a dry period of either 0 days or 30 days on casein micelle composition, by analyzing casein composition, mineral composition and plasmin activity in milk.

Materials and Methods

Experimental design

Cows that were used are a subgroup of the experimental population described before (Van Hoeij, Submitted). The protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University (Protocol number 2014125). In the current study, 18 clinically healthy Holstein Friesian cows that had a dry period of either 0 d (N = 9) or 30 d (N = 9) were included. During the sampling period, cows were in second parity or greater than second parity, evenly distributed over the groups. During the experiment, all cows received a lactation ration that met the energy requirement of the cows based on their expected milk production. Of these 18 cows, milk samples were collected in early lactation (5 - 8 wk postpartum) and mid-lactation (20 - 28 wk postpartum). Cows were milked twice daily. Milk samples were taken at morning milking. Milk fat, protein and lactose content and somatic cell count were analyzed at milk control station Qlip (Zutphen, the Netherlands) according to ISO 9622. Milk samples for further analyses were frozen at -20°C immediately after collecting.

Milk and milk serum preparation

Milk samples were thawed at 37°C for 30 min, and cooled back to room temperature prior to compositional analyses or further preparations. Sodium azide (0.02%) (Sigma-Aldrich) was added as a preservative, and aprotonin (0.02 mg/kg) was added for protease inhibition to milk samples that were used for protein and mineral analysis. Protein and mineral composition were measured in both milk and milk serum. Milk serum was prepared after allowing the milk to restore their mineral balance for 3 days at room temperature. Milk was ultracentrifuged using a Beckman L-60 ultracentrifuge equipped with a 70 Ti rotor (117,500 x *g*, 60 min, 20°C). The supernatant was collected and stored at -20°C prior to further preparation.

Milk protein composition

Casein fractions and fractions of the whey proteins α-lactalbumin and β-lactoglobulin in milk and milk serum were analyzed with reversed phase-high performance liquid chromatography (RP-HPLC). The RP-HPLC method was described in this thesis (Chapter 6).

Plasmin and plasminogen activity

Plasmin and plasminogen activity were measured spectrophotometrically, using a method adjusted from Rollema et al. (1983). Chemicals were purchased at Sigma-Aldrich (Steinheim, Germany), unless mentioned differently. Milk (0.5 mL) was mixed with a clearing buffer (3.5 mL), that consisted of 40 mM tris-hydroxy(methyl)aminomethane, 40 mM potassium chloride (Merck, Darmstadt, Germany), 50 mM 6-aminocaproic acid, and 100 mM EDTA in MilliQ water (Millipore, Billerica, MA) (pH 7.4). The mixture was incubated at room temperature for 10 min. Samples were centrifuged in an Eppendorf 5430 R centrifuge (20817 x *g*, 60 min, 4°C) and consequently defatted and filtered over 0.2 µm using a RC syringe filter. Samples (0.2 mL) were mixed in a 96 wells microplate (PS, clear, F-bottom, Greiner Bio-one) with substrate solution (0.05 mL), that consisted of 3 mM Biophen CS-41(03) chromogenic substrate (Nodia BV, Amsterdam, the Netherlands) in MilliQ water. For every sample, a blank containing 0.05 mL MilliQ water instead of substrate solution was included. For total plasmin and plasminogen measurement, 10 µl urokinase solution (5,000 Plough units / mL) was added. After incubation (15 min, 37°C), contents of the microtiter plate were shaken gently in the plate reader (Spectramax M2, Molecular Devices). Absorbance was measured as a function of time at a wavelength of 405 nm. Measurement points were taken every 30 s during 30 min at 37°C. Every plate contained a substrate blank, in which the milk sample was replaced by MilliQ water. The analysis was performed in duplicate for all samples and blanks. The within-measurement coefficient of variation was 3% for both plasmin activity and plasminogen activity. Plasmin activity was calculated according to equation 1.

$$Plasmin\ activity = \left[\left(Slope_{sample+sub} - Slope_{sample+w} \right) - \left(Slope_{SB+sub} - Slope_{SB+w} \right) \right] * 10^4 \qquad (1)$$

In which sub = substrate solution, W = MilliQ water and SB = Substrate blank

The total plasmin + plasminogen activity was calculated similarly, using the samples to which urokinase solution was added. Plasminogen activity was calculated by subtracting the plasmin activity from total plasmin + plasminogen activity. One activity unit is expressed as ΔA405nm * 10^{-4} per minute at 37°C (pH 7.4) in the reaction mixture.

Mineral composition

Concentrations of calcium, magnesium and phosphorus in milk and milk serum were measured by inductive coupled plasma - atomic emission spectroscopy (ICP-AES; ISO 15151, 2010). Caloric compounds in milk (2.5 g) were removed by dry ashing according to ISO 8070 (IDF 119, 2007). Milk ash was dissolved in 0.14 M HNO_3 solution, in order to obtain a 200 x diluted milk sample. Milk serum was diluted 200 x in 0.14 M HNO_3 as well. Samples were filtered using an RC syringe filter (0.45 µm) prior to analysis. Citrate concentrations were measured by anion exchange chromatography using an IonPac AS19 column (4 x 250 mm, Dionex, Thermo Scientific, Sunnyvale CA) (Gaucheron et al., 1996). Milk was defatted after

centrifugation using an Eppendorf 5430 R centrifuge (20,817 x *g*, 5 min, 20°C). Defatted milk and milk serum samples were diluted 500 x in ultrapure water prior to analysis. Mineral concentrations are expressed in mmol/kg defatted milk. Citrate concentrations ranged from 6.5 - 15.4 mmol/kg defatted milk, apart from one milk sample. This data point of this sample was removed because it was 60-fold lower than the average citrate concentration in milk of the other cows, which is physiologically impossible.

Statistical analyses

A mixed model, accounting for repeated measures, was used for comparison of dry period length, lactation stage and parity (Proc Mixed, SAS 9.3, SAS Institute Inc., Cary, NC). Fixed factors in the model were dry period (0 or 30 d), lactation stage (early or mid) and parity (second or greater than second). Cows were the random factors in the model. An autoregressive covariance structure (AR1) was used to account for within-cow variation. *P*-values were corrected by Bonferroni adjustment. Differences were considered significant if $P < 0.05$. If not mentioned differently in the results section, average values of a dry period group included values of both early and mid-lactation. Average values of a lactation stage includes both cows with a 0 d or a 30 d dry period. Correlations between variables were analyzed using Proc corr in SAS 9.3.

Results

Milk protein composition

Cows with a 0 d dry period tended to have a lower milk yield than cows with a 30 d dry period ($P = 0.09$, Table 4.1). Milk yield was higher in early lactation (36.6 kg/d) than in mid-lactation (27.8 kg/d) ($P < 0.01$). Dry period length and lactation stage did not interact for milk yield, indicating that the milk yield reduction between early and mid-lactation was similar for cows with either a 0 d or a 30 d dry period. Cows with a 0 d dry period had higher milk protein content (3.89%) than cows with a 30 d dry period (3.42%) ($P < 0.01$).

TABLE 4.1. Milk yield, macronutrient composition and plasmin(ogen) activity in early and mid-lactation milk of cows with either a 0 d or a 30 d dry period (mean ± SEM).

	Early		Mid			*P*-value		
	0 d	30 d	0 d	30 d	SEM	Dry period	Lactation stage	Parity
Milk yield (kg/d)	35.04	39.14	25.04	30.28	3.27	0.09	<0.01	0.53
Fat (%)	4.51	4.33	4.99	4.68	0.34	0.28	0.02	0.88
Protein (%)	3.68	3.15	4.14	3.59	0.26	<0.01	0.01	0.18
Lactose (%)	4.64	4.67	4.52	4.56	0.10	0.79	0.02	0.05
SCC (x10^3 cells/mL)[1]	118	88	110	85	77	0.39	0.03	0.07
Plasmin activity (units/mL)	11.0	7.0	14.1	10.4	2.7	0.07	<0.01	0.67
Plasminogen activity (units/mL)	133.8	101.7	161.8	122.3	31.9	0.15	0.17	0.01

[1] Statistical analysis based on log-values (SCC).

Cows with a 0 d dry period tended to have higher plasmin activity in milk (12.4 units/mL) than cows with a 30 d dry period (9.1 units/mL) ($P = 0.07$). Plasmin activity was higher in mid-lactation (12.1 units/mL) than in early lactation (9.4 units/mL) ($P < 0.01$). Plasmin activity and milk yield correlated negatively in mid-lactation ($R = -0.75$, $P < 0.01$), but did not correlate in early lactation. Second parity cows had higher plasminogen activity (150.8 units/mL) than cows of third or greater parity (91.8 units/mL) ($P = 0.01$). Cows with a 0 d dry period had a lower β-casein fraction in milk (32.6%) than cows with a 30 d dry period (33.7%) ($P = 0.02$). The distribution of β-casein between casein micelles and milk serum was not influenced by dry period length (Table 4.2). Consequently, the β-casein fraction that was present in casein micelles was also lower in milk of cows with a 0 d dry period (31.3%) compared with a 30 d dry period (32.7%). Three protein fractions were found in milk serum that were not α-lactalbumin or β-lactoglobulin, of which 1 co-eluted with α_{s2}-casein and 1 co-eluted with β-casein. The sum of these fractions correlated positively with plasmin activity ($R = 0.69$, $P < 0.01$), and negatively with the micellar β-casein fraction ($R = -0.75$, $P < 0.01$). Therefore these 3 fractions may be breakdown products of β-casein. However, the sum of these potential breakdown products did not differ between cows with either a 0 or a 30 d dry period ($P = 0.11$).

TABLE 4.2. Casein composition of milk in early and mid-lactation milk of cows with either a 0 d or a 30 d dry period, expressed as fractions of all proteins. Of the caseins that were found in both milk and milk serum, the percentage that was present in the micelle is presented (mean ± SEM).

	Early		Mid			*P*-value		
	0 d	30 d	0 d	30 d	SEM	Dry period	Lactation stage	Parity
α_{s1}-CN (%)[1]	25.6	26.1	25.7	26.2	1.1	0.53	0.04	0.08
α_{s2}-CN (%)[1]	18.0	16.8	16.6	16.1	0.9	0.10	0.01	0.42
Of which micellar (%)	93.1	93.6	91.1	93.4	2.4	0.35	0.13	0.09
β-CN (%)[1]	32.5	34.1	32.7	33.5	0.6	0.02	0.86	0.36
Of which micellar (%)	96.2	96.8	95.5	96.8	1.2	0.19	0.27	0.13
κ-CN (%)[1]	12.2	11.7	12.1	13.1	1.1	0.60	0.30	0.15
Of which glycosylated (%)	42.5	39.4	43.9	41.6	3.1	0.13	0.05	0.43
α-LA (%)[1]	3.5	4.1	3.5	3.3	0.3	0.52	0.02	0.25
β-LG (%)[1]	8.3	7.3	9.4	7.8	0.9	0.09	<0.01	0.99

[1] Protein fractions are expressed as percentage of total protein measured with RP-HPLC.

The casein number, which was calculated as the sum of the micellar α_{s1}-, α_{s2}-, β- and κ-casein fraction, tended to be higher in milk of cows with a 30 d dry period (86.7%) compared with cows with a 0 d dry period (85.0%) (*P* = 0.07). Cows with a 0 d dry period had a higher casein number in early lactation (85.8%) than in mid-lactation (84.1%), whereas cows with a 30 d dry period had a similar casein number in early and mid-lactation. This resulted in an dry period x lactation stage interaction for casein number (*P* = 0.03). Early lactation milk contained lower fractions of α_{s1}-casein, non-glycosylated κ-casein and β-lactoglobulin than mid-lactation milk. The α-lactalbumin fraction in milk was higher in early lactation (3.7%) compared with mid-lactation (3.4%) (*P* = 0.02). The α-lactalbumin fraction correlated positively with the lactose yield (R = 0.48, *P* < 0.01).

Mineral composition of milk

Mineral composition in early or mid-lactation milk of cows with either a 0 d or a 30 d dry period, and its distribution over casein micelles and milk serum, is presented in Table 4.3. Cows with a 0 d dry period had higher magnesium concentration in casein micelles (2.0 mmol/kg defatted milk) than cows with a 30 d dry period (1.6 mmol/kg defatted milk, *P* = 0.02). Cows with a 0 d dry period had higher micellar phosphorus concentration (21.1 mmol/kg defatted milk) than cows with a 30 d dry period (18.5 mmol/kg defatted milk, *P* = 0.04). Dry period length did not affect the ratios between micellar casein and micellar phosphorus, calcium and magnesium.

TABLE 4.3. Mineral composition in milk, milk serum and the casein micelle fraction of cows with either a 0 d or a 30 d dry period in early or mid-lactation. Values are expressed as mmol/kg defatted milk (mean ± SEM).

	Early		Mid			*P*-value		
	0 d	30 d	0 d	30 d	SEM	Dry period	Lactation stage	Parity
Calcium	33.3	34.4	36.3	34.0	2.2	0.60	0.32	0.71
Soluble	8.9	11.7	9.4	10.2	0.9	<0.01	0.33	0.78
Micellar	24.4	22.7	26.9	23.8	2.4	0.14	0.22	0.61
Magnesium	4.8	4.9	5.5	4.9	0.3	0.17	0.04	0.27
Soluble	3.0	3.4	3.4	3.2	0.2	0.46	0.56	0.38
Micellar	1.9	1.5	2.2	1.7	0.2	0.02	0.03	0.45
Phosphorus	33.9	30.3	36.2	31.9	2.8	0.05	0.24	0.61
Soluble	13.8	12.8	14.0	12.7	1.2	0.33	0.36	0.78
Micellar	20.1	17.5	22.3	19.2	2.0	0.04	0.07	0.39
Total citrate	8.5	11.4	8.5	8.7	1.1	0.03	<0.01	0.02
Sodium[1]	14.0	15.4	16.5	16.3	2.1	0.99	0.14	0.12
Potassium[1]	38.9	40.6	39.5	39.5	1.2	0.04	0.09	<0.01
Chloride[1]	31.8	29.5	29.5	30.7	3.7	0.72	0.83	0.79

[1]Concentrations measured in milk serum

Early lactation milk had a lower magnesium concentration (4.9 mmol/kg defatted milk) than mid-lactation milk (5.2 mmol/kg defatted milk). A similar difference between lactation stages was found for the micellar magnesium fraction ($P = 0.03$), whereas the magnesium fraction in milk serum was similar for both lactation stages. Cows with a 0 d dry period had a lower calcium concentration in milk serum (9.1 mmol/kg defatted milk) than cows with a 30 d dry period (10.8 mmol/kg defatted milk) ($P < 0.01$). The calcium concentration in milk serum correlated negatively with milk protein content (R = -0.52, $P < 0.01$), and positively with the citrate concentration in milk (R = 0.51, $P < 0.01$). Cows with a 0 d dry period had a lower citrate concentration (8.5 mmol/kg defatted milk) in milk than cows with a 30 d dry period (9.7 mmol/kg defatted milk) ($P = 0.03$). The citrate concentration in milk correlated negatively with the milk protein content (R = -0.49, $P = 0.01$). Citrate concentrations in milk were higher in early lactation than in mid-lactation ($P < 0.01$). Cows with a 30 d dry period had a higher citrate concentration in milk in early lactation than in mid-lactation, whereas cows with a 0 d dry period had a similar citrate concentration in early and mid-lactation milk. This resulted in a dry period x lactation stage interaction ($P = 0.02$). Milk from cows with a 0 d dry period had a lower potassium concentration (39.2 mmol/kg defatted milk) than milk from cows with a 30 d dry period (39.9 mmol/kg defatted milk) ($P = 0.04$). Second parity cows had lower potassium concentration in milk (38.0 mmol/kg defatted milk) than cows of third or greater parity (40.2 mmol/kg defatted milk) (P < 0.01).

Discussion

The objective of this work was to evaluate the effect of applying a 0 d or 30 d dry period to dairy cows on the composition of casein micelles in milk. The current work showed that cows with a 0 d dry period had a higher milk protein content than cows with a 30 d dry period, and the casein number tended to be lower for cows with a 0 d dry period. Cows with a 0 d dry period had a lower micellar β-casein fraction and a higher plasmin activity in milk than cows with a 30 d dry period. Cows with a 0 d dry period had a lower calcium concentration in milk serum. The ratios of casein and micellar phosphorus, calcium and magnesium however were not affected by different dry period lengths. In this section, the nature and possible implications of these outcomes is discussed.

Milk synthesis

The milk yield loss of 3.3 kg resulting from dry period reduction from 30 to 0 d was not significant, but numerically similar to previous findings (van Knegsel et al., 2013). Milk yield was not affected by an interaction between dry period and lactation stage, indicating a similar lactation persistency of cows with either a 0 d or a 30 d dry period persistency until mid-lactation. The milk yield is strongly determined by the amount of lactose that is produced in the mammary epithelial cells. Lactose synthesis in the Golgi apparatus of mammary epithelial cells is facilitated by α-lactalbumin. The current results showed that the α-lactalbumin fraction of cows with a 30 d dry period in early lactation was higher than in mid-lactation or of cows with a 0 d dry period. This was reflected in a high lactose yield, and thus a high milk yield of cows with a 30 d dry period during early lactation. The lactose content in milk did not correlate with the α-lactalbumin fraction, likely due to the osmotic effect of lactose, resulting in a constant lactose content. Although the ratio between caseins and whey proteins was not significantly affected by dry period length, dry period length was found to affect the proportion between individual casein fractions.

Micellar casein composition

Cows with a 0 d dry period had a lower β-casein fraction than cows with a 30 d dry period, whereas other protein fractions were not affected by dry period length (Table 4.2). A reduced β-casein fraction in milk of cows with a 0 d dry period was reported before (de Vries et al., 2015). It was suggested that increased plasmin activity was the underlying cause of the β-casein reduction. The current results show that the micellar β-casein fraction correlated negatively with plasmin activity in mid-lactation ($R = -0.65$, $P < 0.01$), although the micellar β-casein fraction and plasmin activity did not correlate in early lactation. Plasmin activity correlated positively with the proportions of the α_{S2}- and β-casein fractions that were measured in milk serum (Figure 4.1), also when early and mid-lactation samples were

analyzed separately. Previous work has indicated that proteolysis of β-casein by plasmin results in γ-caseins which co-elute with β-casein on RP-HPLC (Bonfatti et al., 2008). Besides, the same study reported that the background noise for the α_{S2}-casein fraction increased with increasing plasmin activity, as is the case in the current work. These outcomes show that RP-HPLC can be used for analyzing intact micellar casein fractions, although correcting for co-eluting breakdown products in milk serum is needed. Despite a lower β-casein fraction in milk of cows with a 0 d dry period, there was no difference in fractions of potential breakdown products. Although the β-casein fraction was lower, and plasmin activity tended to be higher in milk of cows with a 0 d dry period compared with a 30 d dry period, there were several indications that plasmin activity did not result in a difference in intact casein fractions in milk of cows with different dry period lengths. Firstly, cows with a 0 d dry period did not only tend to have increased plasmin activity, but also had increased protein content; hence, the plasmin activity per amount of casein was not affected by dry period length. Secondly, no difference in breakdown products was found in milk of cows with either a dry period of 0 d or 30 d. Thirdly, plasmin activity only correlated with the β-casein fraction in mid-lactation, and not in early lactation, whereas the difference in β-casein fractions between cows with a 0 or a 30 d dry period was largest in early lactation (Table 4.2). It can be concluded that the effect of plasmin activity was reflected in the casein composition of milk, but that it is not the explanatory factor for the difference in β-casein fractions between cows with either a 0 d or 30 d dry period. In chapter 6 of this thesis, other regulatory factors that may influence protein fractions in early lactation milk are discussed.

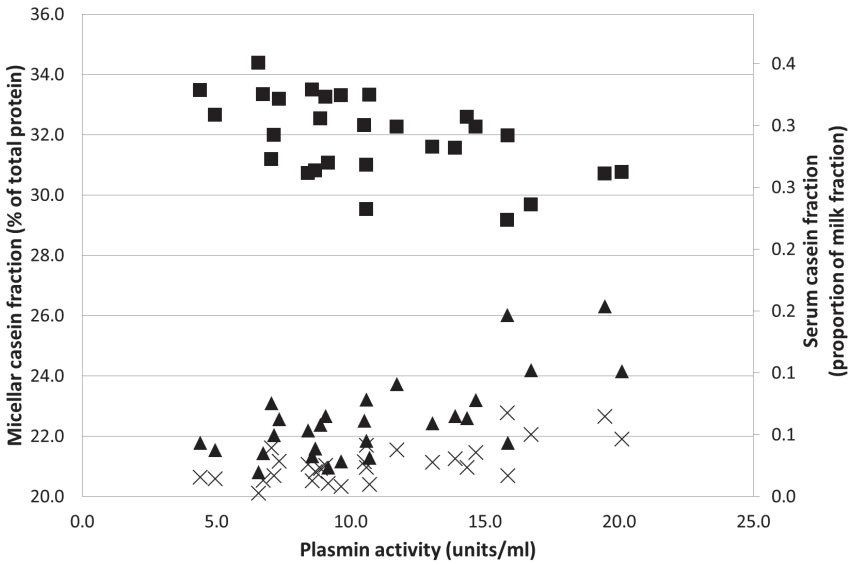

FIGURE 4.1. Correlations between plasmin activity in milk and (■) the β-casein fraction in casein micelles (R = -0.53, $P < 0.01$), and between plasmin activity and percentages in milk serum of the total fraction in milk of (▲) α_{S2}-casein (R = 0.70, $P < 0.01$) and (×) β-casein (R = 0.69, $P < 0.01$).

Previous work indicated higher plasmin activity in milk of cows with a 4 wk dry period, compared with a conventional 8 wk dry period (de Vries et al., 2016). This effect was ascribed to activation of plasminogen by activators originating from somatic cells. In the current work, cows with a 0 d dry period tended to have higher plasmin activity in milk than cows with a 30 d dry period (Table 4.1). Plasmin activity was previously shown to correlate with somatic cell count (SCC) at SCC > 300,000 cells/mL (Politis et al., 1989). In the current work, milk samples had an average SCC of 100,000 cells/mL, which was similar for cows with a 0 d and a 30 d dry period. The similar ratio between plasminogen and plasmin of cows with a 0 d or a 30 d indicate that plasminogen activation indeed did not differ between dry period groups. The negative correlation between plasmin activity and milk yield (R = -0.58, $P < 0.01$) indicated that numerical differences in plasmin activity are rather a concentration than an activation effect. Plasmin activity did not correlate with monovalent ions (Na, K, Cl). This is in line with the finding that plasmin is being transported transcellularly rather than paracellularly (Silanikove, 2016), and that no detectable increased activation takes place at an SCC of 100,000 cells/mL (Politis et al., 1989). All in all, this work indicates that dry period omission tends to result in increased plasmin activity in milk, which in cows with low SCC is an effect of concentration rather than activation of plasminogen.

Mineral composition of casein micelles

The higher milk protein content resulting from dry period omission may not only affect the casein fractions of casein micelles. Due to incorporation of calcium phosphate nanoclusters in casein micelles, the micellar mineral fractions may be influenced by omitting the dry period. Dry period omission only resulted in higher magnesium and phosphorus fractions in casein micelles (Table 4.3). The micellar calcium, magnesium and phosphorus fractions correlated positively with milk protein content (Figure 4.2). Hence, the ratio of casein and micellar phosphorus ($P = 0.45$), calcium ($P = 0.71$) and magnesium ($P = 0.12$) were similar for cows with either a 0 d or a 30 d dry period. Calcium, magnesium and phosphorus are all incorporated in calcium phosphate nanoclusters in casein micelles. These nanoclusters were reported to have a constant composition (Holt, 1982). The micellar calcium fraction was not affected by dry period length, but micellar calcium increased proportionally with increasing protein content, similar to micellar magnesium and phosphorus (Figure 4.2). In conclusion, variation in concentrations of the major micellar minerals seems to be a consequence of variation in milk casein content. This indicates that casein micelle composition remained constant during early and mid-lactation, and that the casein micelle composition was not influenced by dry period omission.

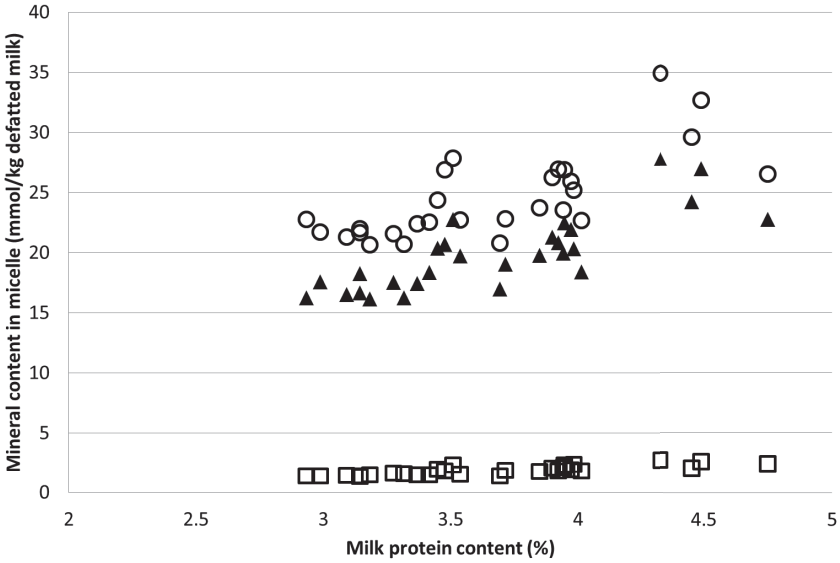

FIGURE 4.2. Correlations between milk protein content and micellar fractions of (o) calcium (R = 0.72, $P < 0.01$), (□) magnesium (R = 0.81, $P < 0.01$), and (▲) phosphorus (R = 0.80, $P < 0.01$), including data from both early and mid-lactation.

Average concentrations of calcium, phosphorus and magnesium, and their distribution between casein micelles and milk serum (Table 4.3) were similar as reported before in Dutch dairy herds (Bijl et al., 2013). Whereas the micellar mineral fractions were in proportion with protein content, most mineral fractions in serum were not related to protein content, apart from the calcium fraction. It is known that the total calcium concentration in milk depends on the amount of casein and citrate in milk (Neville, 2005). Whether calcium is in the soluble or in the micellar fraction is determined by the concentration of counter-ions in the serum phase, of which citrate is the most abundant (Gaucheron, 2005). At lower citrate concentration in milk serum, less calcium is able to remain in the serum, and more calcium sediments in calcium phosphate nanoclusters. Hence, the calcium citrate concentration in milk serum is in equilibrium with the micellar calcium phosphate concentration (Gaucheron, 2005). The calcium concentration in milk serum correlated negatively with the milk protein content (R = -0.52, $P < 0.01$), whereas the micellar calcium concentration correlated positively with protein content (Figure 4.2). These 2 correlations indicated a change in calcium distribution between milk serum and casein micelles with increasing milk protein content. The calcium concentration in milk serum correlated positively with the citrate concentration in milk (R = 0.51, $P < 0.01$), of which > 90% is present in the serum phase (Bijl et al., 2013). This was according to expectations, because minerals in milk serum are in equilibrium with each other, and the majority of calcium is associated with citrate in milk serum (Gaucheron, 2005). The total calcium concentration in milk did not correlate with the citrate concentration. Hence, the current results are in line previous findings that

the calcium concentration in milk is related to the secreted amounts of both casein and citrate (Neville, 2005), but in particular the distribution of calcium over the micellar and the serum phase. Mineral equilibria in milk are dependent on pH (Gaucheron, 2005). Cows with a 0 d or a 30 d dry period had similar milk pH (Personal communication Lex Oosterveld). Therefore it is assumed that variation in pH does not play a major role in explaining the difference between milk serum mineral composition of cows with either a 0d or 30 d dry period. Moreover, since the calcium concentration in serum and the citrate concentration in milk shift proportionally, the ionic equilibrium in milk remains similar, and no effect of dry period length on pH would be expected. From the current results it can be hypothesized that the casein content in milk is inversely related to citrate secretion, in order to maintain a constant micellar composition. Further work is needed to confirm this hypothesis. Following this hypothesis, milk of cows with a 0 d dry period in the current work had a low citrate concentration in milk, which allowed a relatively large amount of calcium to sediment in casein micelles.

Milk composition for processing

The current work showed that micellar protein fractions and mineral fractions in early and mid-lactation milk of cows that were managed without a dry period were concentrated proportionally. Plasmin activity also changed proportionally with the milk protein content, which resulted in similar fractions of casein breakdown products in milk of cows with either a 0 d or a 30 d dry period. As a consequence, the impact of shortening the dry period on processing casein micelle-based dairy products such as cheese is expected to be limited. The lower β-casein fraction within the total casein fraction of cows with a 0 d dry period is not expected to affect cheese yield (Wedholm et al., 2006). The casein number, which contributes strongly to the cheese yield (Wedholm et al., 2006) tended to be lower in cows with a 0 d dry period than with a 30 d dry period ($P = 0.07$). Previous work, however, indicated a smaller or no numeric difference in casein numbers between cows with different dry period lengths (de Vries et al., 2015, de Vries et al., 2016). These results indicate that a possible difference in casein number cannot be noticed on the current experimental scale. Omission of the dry period resulted in a lower calcium concentration in serum, likely due to a higher casein content and a lower citrate concentration, compared with cows with a 30 d dry period. Previous work indicated a positive correlation between the calcium concentration in serum, and calcium activity (Bijl et al., 2013). Since the calcium concentration in serum and the citrate concentration in milk changed proportionally in the current work, the effect on calcium activity may be limited. Calcium activity is known to be an important factor in renneting of milk. Preliminary outcomes of renneting experiments done with the current samples indicate no differences in rennet coagulation time and curd firmness between milk samples of cows with either a 0 or a 30 d dry period. The calcium chloride concentration added is approximately a 5 to 10-fold the natural calcium activity in milk. Therefore, the addition of

calcium chloride before renneting of milk probably levels out possible effect of differences in calcium activity on renneting between milk from cows with either a 0 d or 30 d dry period. In conclusion, differences in milk composition between cows with a 0 d or 30 d dry period would not be expected to affect cheese processing quality of milk. Although the current work did not indicate a difference in casein number between cows with different dry period lengths, it is recommended to study the casein number on a larger scale in future work, since small differences may have an impact on the processing quality of milk, particularly on cheese yield.

Conclusion

Proportional changes in micellar casein content and micellar mineral concentrations indicated that the composition of casein micelles was not affected by dry period omission. A previous suggestion that dry period length could affect activation of plasminogen was not confirmed in the current study, due to low SCC in milk of cows with either a 0 d or a 30 d dry period. Plasmin activity did affect the β-casein fraction in casein micelles, but it did not seem to be the main cause of the lower β-casein fraction in milk of cows with a 0 d dry period. All in all, dry period omission had, apart from a small reduction of the β-casein fraction, no effect on casein micelle composition, and therefore it seems to have limited influence on the processing quality of milk.

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Chapter Five

Influence of dry period length of Swedish dairy cows on the proteome of colostrum

Ruben de Vries, Sjef Boeren, Kjell Holtenius,
Jacques Vervoort, Helena Lindmark-Månsson, Kasper Hettinga

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Abstract

The aim of this study was to evaluate the influence of applying a 4 wk instead of an 8 wk dry period to the dairy cow on the proteome of colostrum (first sample) and of transition milk (the fifth postpartum milk sample). From 12 Swedish Holstein (SH) and 12 Swedish Red (SR) cows, individual serum samples of colostrum and transition milk were analyzed. Samples were prepared by filter aided sample preparation and dimethyl labelling, and analyzed by liquid chromatography tandem mass spectrometry. Shortening the dry period resulted in upregulation of 18 proteins in colostrum and transition milk of SR, whereas statistically no differences were found for SH colostrum and transition milk. It is hypothesized that the upregulated proteins reflect a specific immune response in the SR samples that was reflected in colostrum as well as in transition milk. Upregulated proteins in colostrum seemed to reflect increased mammary epithelial cell proliferation in the periparturient period when a 4 wk dry period was applied. The proteome data indicate that a dry period of 4 wk to SR cows may not be sufficient for complete regeneration of the mammary epithelium.

Introduction

A dry period of 6 - 8 wk is known to maximise the milk yield of dairy cows in their successive lactation (Kuhn et al., 2005, van Knegsel et al., 2013). High milk yield generally aggravates a negative energy balance in early lactation. The negative energy balance is related to increased risk of metabolic and reproductive problems. Shortening the dry period is a strategy to ameliorate the negative energy balance in early lactation (Rastani et al., 2005, van Knegsel et al., 2014). A milk yield reduction in early lactation was observed after applying a 28 - 30 d dry period instead of a 56 - 60 d dry period (Annen et al., 2004, Rastani et al., 2005, van Knegsel et al., 2014). The milk yield over the entire lactation was decreased by shortening the dry period according to the study of Watters et al (2008), however in other studies no significant differences were found (Church et al., 2008). Colostrum yield was reduced when a 30 d (5.3 kg) instead of 60 d (7.7 kg) dry period was applied (Mayasari et al., 2015). Mastitis incidence was not influenced by shortening the dry period (Church et al., 2008, Watters et al., 2009). Complete omission of the dry period resulted in a stronger reduction in milk yield (van Knegsel et al., 2013) and elevated SCC in early lactation (de Vries et al., 2015), compared to a dry period of 30 d.

Prepartum repair and renewal of mammary epithelial cells was reported to occur to a high extent in a dry mammary gland (Capuco et al., 1997, Norgaard et al., 2008), as compared with a lactating mammary gland (Capuco et al., 1997). High numbers of renewed secretory epithelial cells at the beginning of lactation were suggested to be responsible for the high milk production of cows with a 60 d dry period (Kuhn et al., 2005). During a 60 d dry period of cows, secretory capacity of the epithelial cells reduced until day 25 in the dry period. In the last 35 d of the dry period, secretory activity of epithelial cells increased to a higher level than was the case for cows that were lactating until parturition (Capuco et al., 1997). Although direct experimental data are lacking, reduced milk yield and udder size indicate that mammary gland development seems to be diminished in cows with a shortened dry period as compared to a 60 d dry period (Pezeshki et al., 2010). Mammary epithelial cell regeneration resembles a wound healing process with phagocytosis of apoptotic cells (Stein et al., 2004). The apoptotic index of mammary epithelial cells is high in the first days after drying off and also during the first days of lactation, independent from the presence or absence of a preceding dry period (Collier et al., 2012). Removal of apoptotic cells is followed by formation of new cell-cell interactions, after which the barrier function of the epithelium is restored (Stein et al., 2007). Overall, it has been demonstrated that the dry period is an important period for the mammary epithelium to prepare for the successive lactation.

Colostrum quality is important as it is the first food source for the newborn calf, and colostrum is key in developing its immune system. Colostrum contains both major milk proteins, such as caseins, as well as over 200 other proteins with various functionalities (Le

et al., 2011, Zhang et al., 2015a). Antimicrobial and immunity related proteins were found to be more abundant in colostrum compared to mature milk (Le et al., 2011, Zhang et al., 2015a). Two days postpartum, the milk proteome showed a transition from colostrum-like towards a proteome comparable with mature milk (Zhang et al., 2015a). Most research related to variation of the milk proteome has focused on changes as a result of pathogen-induced mastitis. Besides proteins with direct antimicrobial activity such as cathelicidins and lactotransferrin (LTF), also proteins related to epithelial barrier integrity such as clusterin (CLU) and plasminogen (PLG) were found to be upregulated in mastitic milk (Danielsen et al., 2010, Ibeagha-Awemu et al., 2010). Variation in content of immune proteins that are important for the neonate has mainly be related to mastitis thus far, and to a minor extent to lactation stage, but limited work has been done on the effect of dry period length.

Shortening the dry period to 28 d was reported to not influence protein concentration of colostrum compared with a 56 d dry period (Rastani et al., 2005). The IgG concentration in colostrum was not affected by shortening the dry period to 30 d either (Annen et al., 2004, Watters et al., 2008, Mayasari et al., 2015), although IgG yield of cows with a 30 d dry period was lower compared to a 60 d dry period due to a reduced colostrum yield (Mayasari et al., 2015). Total omission of the dry period was shown to result in lower IgG concentration in colostrum (Mayasari et al., 2015), an effect that was only found for primiparous cows by Annen et al. (2004). Reduced IgG concentration in colostrum of cows without a dry period is hypothesized to be the result of the absence of protein accumulation prepartum. To our knowledge, no more detailed work on the colostrum proteome in relation to dry period length exists up to date. The aim of this study is to evaluate the colostrum and transition milk proteome of cows with a shortened dry period. Outcomes can be used to generate a better understanding of processes in the mammary gland in the periparturient period.

Materials and Methods

Experimental design, animals, sampling

The Uppsala Local Ethics Committee approved the experimental protocol (C178/12). For the current study, 12 Swedish Holstein (SH) and 12 Swedish Red (SR) cows in late lactation with proper udder health were used. They were blocked according to parity and dried off either 4 or 8 wk before expected parturition. The cows were housed at the Swedish Livestock Research Centre Lövsta in a loose house system with slatted floor and cubicles with rubber mats and shopped straw as bedding. Water was always available *ad libitum.* About a week before expected calving, the cows were moved to individual calving boxes with shopped straw as bedding. No intramammary antibiotics were used at drying off. Before dry-off, the cows were fed silage *ad libitum* and concentrate according to milk production. The week prior to dry-off, concentrate was withdrawn and during the dry off procedure the cows were fed 4 kg DM of silage and straw *ad libitum* and no concentrate. After dry-off, the cows were fed a blend of silage and straw *ad libitum* and concentrate was stepwise increased to 3 kg at parturition. Milk samples were taken at the first (colostrum) and the fifth milking (transition milk) occasion after calving while the cows were still kept in the calving boxes and milked in buckets. Samples were taken as aliquots of the entire milking, resulting in a total of 24 samples per breed. The milk yield was recorded at the sampling day. Samples were preserved with 0.03% Bronopol and stored at -20°C immediately after collecting. Analysis of protein, fat, lactose and somatic cell counts were done in house, using an infrared spectroscopy method (FTIR, Fourier Transform Instrument, FT 120, Foss Electric, Hillerød, Denmark). Colostrum samples were diluted 3 times with ultrapure water prior to FTIR analysis. All 48 individual samples were used for analysis of their protein composition by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Serum preparation

Colostrum samples were diluted 4 times with ultrapure water after thawing. All samples were centrifuged (1500 x *g*, 10 min, 10°C) and the fat layer was removed. Samples were ultracentrifuged (100,000 x *g*, 90 min, 30°C) using a Beckman L60 ultracentrifuge equipped with a 70 Ti rotor. A pellet of caseins and a transparent milk serum fraction were formed. Milk serum was collected and stored at -20°C prior to further analyses. Protein concentration of serum samples was determined by BCA assay (Zhang et al., 2015a). Serum samples were diluted to a protein concentration of 10 µg/µl using ultrapure water. One pooled serum sample was made for SH and 1 for SR, consisting of equal volumes of diluted milk serum of all 24 samples of either SH or SR colostrum and milk.

Filter Aided Sample Preparation

Filter Aided Sample Preparation (FASP) was performed according to Lu et al. (2011). Individual and pooled milk serum samples were diluted 10 times in SDT-lysis buffer (0.1 M Tris, 0.1 M DTT, 4% SDS) in low binding 2 mL tubes (Eppendorf, Germany). Samples were incubated at 95°C for 10 minutes and subsequently cooled down to room temperature. Samples were centrifuged (15,871 x *g*, 10 min) and the supernatant was diluted 10 times in 8 M urea, 0.1 M Tris/HCl (pH 8), 0.9% iodoacetamide in low binding 2 mL tubes (Eppendorf). Two hundred µl sample was transferred to a omega filter and centrifuged (15,871 x *g*, 30 min). One hundred µl 8 M urea in 0.1 M Tris/HCl pH8 was pipetted on top of the filter, and samples were centrifuged (15,871 x *g*, 30 min). This step was repeated 2 more times. One hundred and ten µl ABC (0.05 M NH_4HCO_3) was added on the filter, and samples were centrifuged (15,871 x *g*, 30 min). One hundred µl ABC containing 0.5 µg trypsin was added on the filter which was transferred into a new 2 mL low binding tube (Eppendorf). Samples were incubated overnight while mildly shaking. After overnight incubation, samples were centrifuged (15,871 x *g*, 30 min), and acidified to a pH value around 2 by adding 3.5 µl 10% trifluoroacetic acid.

Dimethyl labelling

Dimethyl labelling is a method that was proved for accurate milk protein quantification (Lu et al., 2011). Tryptic digests of individual colostrum and milk serum samples were labelled with a heavy label (cyanoborohydride with deuterated formaldehyde). Tryptic digests of pooled colostrum and milk serum samples were labelled with a light label (cyanoborohydride with normal formaldehyde). Dimethyl labelling was done according to Boersema et al. (2009), using C18+ stage tips that were prepared in house according to Lu et al. (2011). The volume of the labelled samples was reduced in a vacuum concentrator (Eppendorf Vacufuge) at 45°C for approximately 30 min until a volume of 15 µl or less was reached. Every heavy labelled individual sample was mixed with the light labelled pooled sample, and made up to 100 µl with 1 mL/l HCOOH.

LC-MS/MS

The technical details are identical to Zhang et al. (2015). Trypsin digested and dimethyl labelled colostrum and milk serum fractions were loaded (18 µl) on a 0.10 x 30 mm Magic C18AQ 200A 5 µm beads (Michrom Bioresources Inc., USA) pre-concentration column (prepared in house). The maximum column pressure was 270 bar. Pre-concentration was followed by elution onto a 0.10 x 200 mm Magic C18AQ 200A 3 µm beads analytical column (Michrom Bioresources Inc., USA). A gradient from 8% to 33% of acetonitrile in water with 0.5% acetic acid was applied at a flow rate of 0.5 mL/min for 50 min. Post-run column washing was done by increasing the percentage acetonitrile to 80% (with 20% water and 0.5% acetic acid in the acetonitrile and the water) in 3 min. The pre-concentration and the analytical column were

connected by a P777 Upchurch micro-cross. A stainless steel needle that fitted into the waste line of the micro-cross was used for applying a direct electrospray potential of 3.5 kV to the eluent. Full scan FTMS spectra in LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA) were measured in positive mode between an m/z of 380 and 1400. Of the four most abundant multiply charged peaks in the FTMS scan, CID fragmented MS/MS scans were recorded in data-dependent mode in the linear trap (MS/MS threshold = 5,000).

Protein identification and quantification

Maxquant 1.3.0.5 with Andromeda search engine (Cox and Mann, 2008) was used for analysing all MS/MS spectra from each run. Carbamidomethylation of cysteines was set as a fixed modification (enzyme = trypsin, maximally 2 missed cleavages, peptide tolerance 20 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation and de-amidation of asparagine or glutamine were set as variable modification for both identification and quantification. The bovine reference database for peptide and protein searches was downloaded as fasta files from Uniprot (http://www.uniprot.org/; accessed March 2014) with reverse sequences generated by Maxquant. Sequences of 31 common contaminant proteins were added including Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1C1 (P35527, human). A maximum of 2 missed cleavages were allowed and mass deviation of 0.5 Da was set as limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. The false discovery rate (FDR) was set to 1% on both peptide and protein level. The length of peptides was set to at least 7 amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least 1 unique.

Statistical analyses

Protein concentrations were corrected for dilution factors, since all serum samples were diluted to a protein concentration of 10 μg/μl. Differences in protein concentrations between cows with a 4 wk or an 8 wk dry period were based on dimethyl ratios between individual samples and the pooled sample. Dry period comparison was done with a two-sample t-test in Perseus 1.3.0.4 (Max Planck Institute of Biochemistry, 2012). A permutation-based false discovery rate of 0.05 was applied to correct for multiple testing. As there was 1 pooled sample for SH and 1 for SR, statistical analysis was done separately per breed. Proteins quantified in less than half of the samples were excluded from statistical analyses. Differences in milk yield, macronutrient composition and SCC between cows with either a 4 wk or an 8 wk dry period were tested using an independent samples T-test in SPSS 22 (IBM SPSS Statistics). This T-test was done within breeds (SH or SR) and sampling moments (colostrum or transition milk).

Results

Colostrum and milk composition

Five colostrum samples had SCC > 10^7 cells / mL, whereas the average SCC of the other samples was 2.5 x 10^6 cells / mL. The underlying cause of the high SCC is not clear, but it was not dry period related as these 5 samples were distributed over all groups: 2 SR 4 wk dry period, 1 SR 8 wk dry period, 1 SH 4 wk dry period, 1 SH 8 wk dry period. These samples had clearly distinct proteomes, although the limited sample number per breed (2 or 3) was not appropriate for further statistical analysis. If not mentioned differently, comparisons between dry periods is done after exclusion of cows that had SCC > 10^7 cells / mL in colostrum. Both colostrum and transition milk samples of these cows are left out. Although these samples were included in the pooled samples used as a reference for the dimethyl labelling-based quantification, these samples do not present a problem, as a structural change in the reference sample will not influence the comparison between individual samples. Colostrum yields did not differ between cows with a 4 wk and an 8 wk dry period (Table 5.1). Colostrum protein percentage of SH with a 4 wk dry period (15.5%) was higher than with an 8 wk dry period (11.7%). Lactose content in colostrum of SH with a 4 wk dry period (5.2%) was lower compared with SH with an 8 wk dry period (5.5%). SCC in colostrum of SH with a 4 wk dry period (5.0 x 10^6 cells / mL) was higher compared with SH with an 8 wk dry period (1.4 x 10^6 cells / mL). Colostrum composition of SR did not differ between cows with either a 4 wk or an 8 wk dry period (Table 5.1).

Proteome of cows with a 4 or an 8 wk dry period

In total, 222 proteins were quantified based on their dimethyl ratios in the current sample set. The number of quantified proteins was higher in transition milk of SH cows with a 4 wk dry period than with an 8 wk dry period (Table 5.1). Concentrations of 18 proteins were higher (FDR ≤ 0.05) in colostrum of cows with a 4 wk dry period compared with an 8 wk dry period (Table 5.2). Upregulated proteins in SR colostrum of 4 wk dry cows were 3 – 58 times more abundant compared with colostrum of 8 wk dry cows (Table 5.2). Differences in protein concentrations are visualized in Figure 5.1. Hierarchical clustering of colostrum proteomes of SH cows showed numerical differences in the same proteins between cows with either a 4 wk or an 8 wk dry period (Figure 5.2). Quantitatively however, the upregulation factors were lower than for SR (Table 5.2). Hence, no significant changes were found in the colostrum proteome of SH with either a 4 wk or an 8 wk dry period. Some of the proteins were not quantified for all samples in SH colostrum (Figure 5.2). However, replacing missing values by a minimum value (the lowest detected concentration of a protein) did also not result in significant differences between cows with a 4 wk or an 8 wk dry period. Colostrum of cows with a 4 wk dry period did not have any proteins with lower abundance compared

with an 8 wk dry period. In transition milk samples, 18 proteins were upregulated (FDR = 0.05) in milk of cows that had a 4 wk dry period compared with an 8 wk dry period. Of these 18 proteins, 10 were upregulated in their colostrum samples as well (Table 5.2, Figure 5.3). In transition milk of SR, the maximum upregulation was 14-fold (lactotransferrin (LTF)). Like in colostrum, concentrations of individual proteins in transition milk of SH did not differ between cows with either a 4 wk or an 8 wk dry period.

TABLE 5.1. Yield, macronutrient composition, SCC, and number of quantified proteins in colostrum and transition milk of individual Swedish Holstein and Swedish Red cows with either a 4 wk or an 8 wk dry period. Differences between dry period lengths, within breeds and sampling moment (colostrum or transition milk) are tested by an independent samples T-test. Values are presented as means with SEM

		Swedish Red			Swedish Holstein		
		4 wk dry N = 4	8 wk dry N = 5	SEM	4 wk dry N = 5	8 wk dry N = 5	SEM
Colostrum	Yield (kg/d)	5.5	10.0	2.0	6.2	7.2	1.0
	Fat (%)	5.5	5.8	1.1	5.9	10.7	2.3
	Protein (%)	15.1	12.6	1.4	15.5*	11.7	1.0
	Lactose (%)	5.3	5.3	0.2	5.2*	5.5	0.1
	SCC (x10^3 cells/mL)[1]	1838	1702	1020	4996*	1398	1221
	Proteins quantified	107	124	8.3	133	119	6.2
Transition milk	Yield (kg/d)	15.9	23.8	3.0	26.5	28.5	4.1
	Fat (%)	5.4	4.0	1.2	3.0	3.0	0.5
	Protein (%)	5.8	5.6	0.2	5.4	5.1	0.2
	Lactose (%)	6.4	6.5	0.1	6.7	6.6	0.1
	SCC (x10^3 cells/mL)[1]	1292	510	755	2524	997	1529
	Proteins quantified	127	123	3.9	143*	124	6.2

* Value is significantly higher at P <0.05 within a breed and sampling moment (colostrum or transition milk)

** Value is significantly higher at P <0.01 within a breed and sampling moment (colostrum or transition milk)

[1] T-test based on log-values

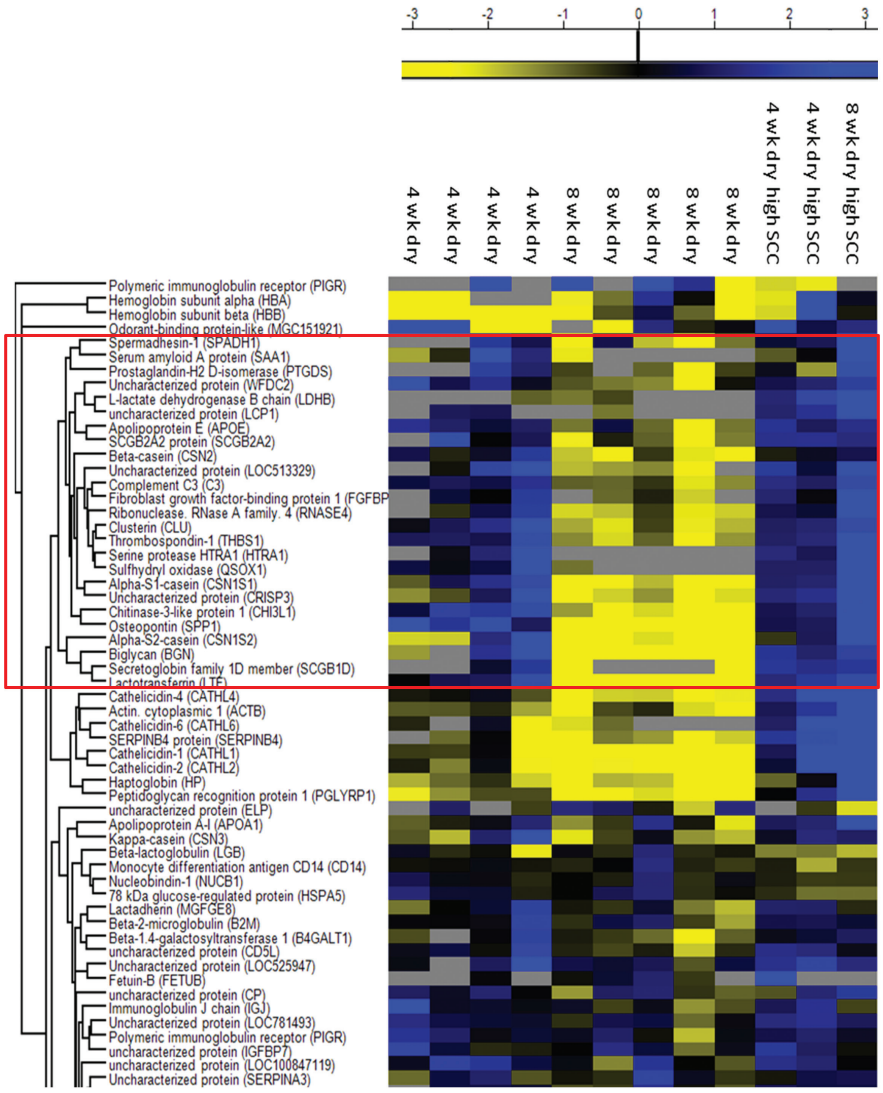

FIGURE 5.1. Hierarchically clustered dimethyl ratios of Swedish Red colostrum proteins (rows) shown as a heat map. Columns represent individual samples of cows with a 4 wk or an 8 wk dry period, ordered manually. The 3 far right columns represent colostrum samples with SCC > 10^7 cells/mL.

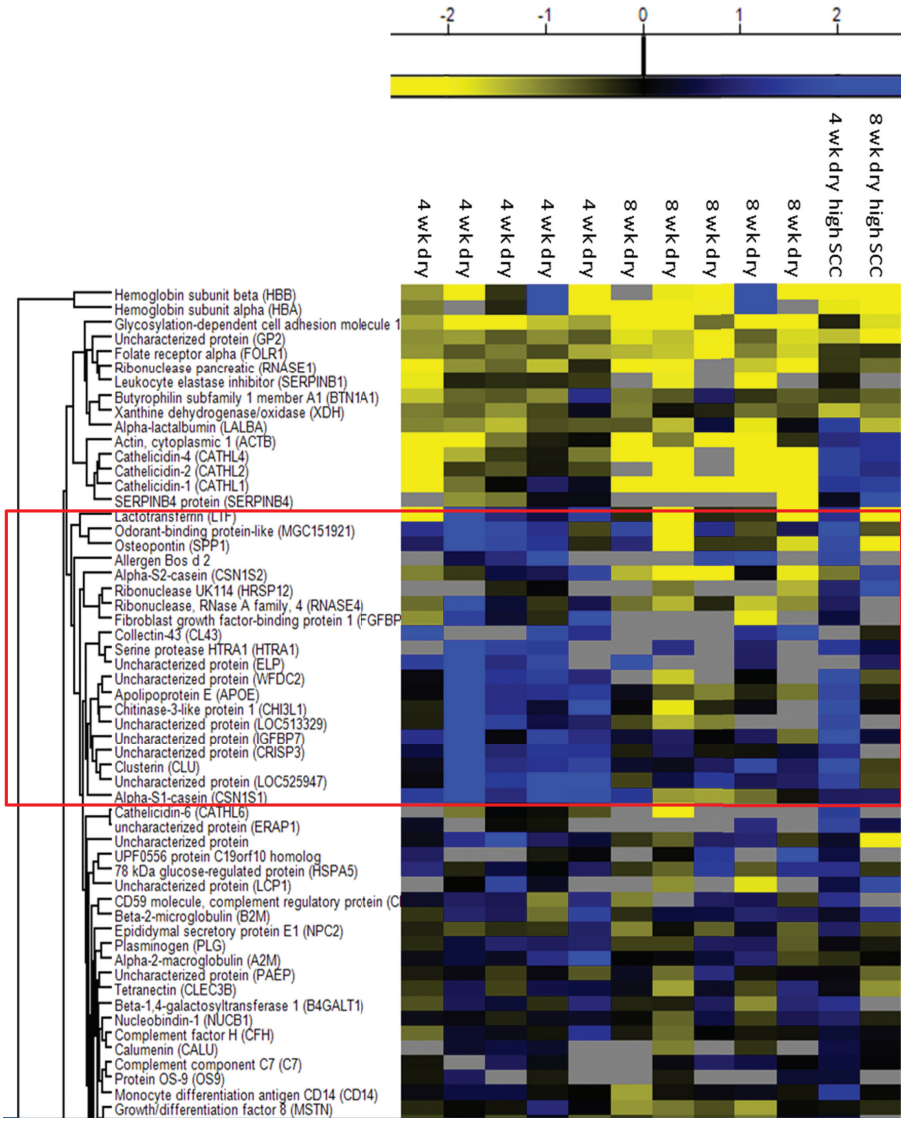

FIGURE 5.2. Hierarchically clustered dimethyl ratios of Swedish Holstein colostrum proteins (rows) shown as a heat map. Columns represent individual samples of cows with a 4 wk or an 8 wk dry period, ordered manually. The 2 far right columns represent colostrum samples with SCC > 10^7 cells/mL.

TABLE 5.2. Upregulation factors of proteins in either colostrum or transition milk of Swedish Red and Swedish Holstein cows with a 4 wk dry period compared with an 8 wk dry period. Values represent the protein concentrations in samples of cows with a 4 wk dry period relative to the concentrations in samples of cows with an 8 wk dry period, based on dimethyl ratios. The proteins that are presented are the proteins that were upregulated (FDR = 0.05) in colostrum of Swedish Reds with a 4 wk dry period compared with an 8 wk dry period.

Protein	Gene	Colostrum		Transition milk	
		SR (N = 4 + 5)	SH (N = 5 + 5)	SR (N = 4 + 5)	SH (N = 5 + 5)
Tissue regeneration					
Apolipoprotein E	APOE	3.4*	4.0	2.9	1.7
Biglycan	BGN	57.7*	ND	3.5	ND
Thrombospondin-1	THBS1	7.9*	ND	1.3	ND
Chitinase-3-like protein 1	CHI3L1	17.7*	6.1	3.2*	0.7
Clusterin	CLU	8.9*	2.0	6.4*	1.0
Host-defense					
Cathelicidin-2	CATHL2	6.0*	7.8	5.1	2.0
Cathelicidin-4	CATHL4	5.1*	5.1	5.6	2.3
Peptidoglycan recognition protein 1	PGLYRP1	3.4*	1.6	3.3	4.1
Ribonuclease. RNase A family. 4	RNASE4	6.0*	3.7	1.1	1.0
Mucosal protection					
Glycosylation-dependent cell adhesion molecule 1	GLYCAM1	4.0*	1.3	2.0*	3.1
Lactotransferrin	LTF	39.1*	12.9	13.5*	2.1
Osteopontin	SPP1	33.7*	9.6	4.1*	1.8
Uncharacterized protein	WFDC2	7.7*	5.8	8.7*	2.8
Transport					
Alpha-S1-casein	CSN1S1	18.5*	4.8	3.8*	1.2
Beta-casein	CSN2	6.2*	1.4	5.2*	1.1
Complement system					
Complement C3	C3	4.3*	1.7	2.0*	1.0
uncharacterized protein	CFI	3.4*	1.6	2.0*	1.1
Uncharacterized protein	LOC513329	15.3*	10.1	3.2	0.9

* Significantly higher in samples of cows with a 4 wk dry period compared with an 8 wk dry period (FDR = 0.05)

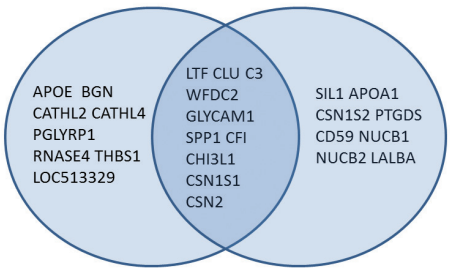

FIGURE 5.3. Left circle: Proteins upregulated in 9 colostrum samples of Swedish Red cows with a 4 wk dry period compared with an 8 wk dry period. Right circle: proteins upregulated in transition milk samples of the same cows with a 4 wk dry period compared with an 8 wk dry period. Proteins in the overlapping of the circles are upregulated in both colostrum and transition milk of cows with a 4 wk dry period.

Discussion

The current study shows that applying a dry period of 4 wk instead of 8 wk resulted in an upregulation of 18 proteins in colostrum of SR cows. Of the 18 upregulated proteins, 14 clustered together in a heat map of the quantified SR colostrum proteins (Figure 5.1, red frame), indicating a similar response for the proteins in the different cows. Of the proteins upregulated in colostrum, 10 were also upregulated in transition milk of these cows (Figure 5.3). For SH colostrum, no differences in individual protein concentrations were found between cows with a 4 or an 8 wk dry period. However, in a heat map of SH colostrum, 9 of the proteins that were significantly different in SR colostrum were clustered together (Figure 5.2, red frame), even though the differences were insignificant for SH. These proteins generally had numerically higher intensities in colostrum of SH with a 4 wk dry period than an 8 wk dry period, thus similar to SR. No difference in the proteome was found in the transition milk samples of SH between cows with a 4 or 8 wk dry period. Absence of dry period-induced proteome differences in transition milk is another indication that the colostrum proteome of SH was affected in a milder way by shortening the dry period than the colostrum proteome of SR (Table 5.2).

All proteins that were upregulated in colostrum of SR with a 4 wk dry period are known to play a role in immune responses, apart from the caseins. In general, the upregulated proteins can be divided into a group of antimicrobial proteins (e.g. cathelicidin-2 and -4 (CATHL2 and CATHL4)), peptidoglycan recognition protein 1 (PGLYRP1) and lactotransferrin (LTF)), and a group of proteins related to tissue regeneration (ao. apolipoprotein E (APOE), biglycan (BGN) and thrombospondin (THBS1)). No acute-phase proteins were upregulated in colostrum of SR cows with a short dry period. This indicates that shortening the dry period induced a preventive rather than an inflammatory immune response. Such a preventive immune response may be the result of incomplete regeneration of the mammary epithelium. Previous work has shown that a dry period of more than 30 days is needed for complete regeneration of the mammary epithelium of dairy cows (Capuco et al., 1997). It can be assumed that cows with a 4 wk dry period in the current study did not complete an optimal cycle of tissue regeneration of the mammary epithelium. Based on the study of Capuco et al. (1997) it is expected that cows with a 4 wk dry period still have increased cell regeneration in early lactation. Postpartum changes in protein composition in milk that may reflect mammary epithelial development have not been reported before. The current work indicates that applying a 4 wk dry period may result in a preventive immune response postpartum, possibly because of unfinished mammary epithelial cell regeneration.

Some of the proteins that were more abundant in colostrum when the dry period was shortened originate from epithelial cells (GLYCAM1, LTF), whereas others may originate both from epithelial as well as somatic cells (cathelicidins, SPP1). Due to practical limitations, samples in this study have been frozen and thawed prior to serum preparation,

as is commonly done in milk proteomics studies. Previous work has indicated that freeze-thawing of milk has limited or no influence on serum concentrations of proteins that are described in the current work (Zhang et al., 2016). Therefore, freeze-thawing of samples is highly unlikely to have affected the proteome comparison of cows with a 4 wk or an 8 wk dry period. Moreover, there was no difference in SCC between SR with a 4 wk and an 8 wk dry period, and no correlations were found between SCC and proteins such as cathelicidins. CATHL2, CATHL4 and PGLYRP1 have direct antimicrobial activity (Alonso-Fauste et al., 2012), and were more concentrated in colostrum of SR with a 4 wk dry period. Cathelicidin gene expression has been found in healthy mammary tissue, indicating its role in tissue maintenance (Kosciuczuk et al., 2014). From these findings it can be hypothesized that the mammary epithelium of SR cows with a 4 wk dry period undergoes more maintenance activity at parturition than that of SR cows with an 8 wk dry period. Alpha-caseins have been reported to contain antimicrobial peptides encrypted in their sequence (McCann et al., 2006), though upregulation of caseins in milk serum in relation to mastitis is highly variable over different studies (Danielsen et al., 2010, Alonso-Fauste et al., 2012, Smolenski et al., 2014). Casein-derived peptides used for casein identification in the current study comprise a major part of the amino acid sequence, indicating that intact caseins and not their breakdown products were measured. In general, upregulation of antimicrobial proteins in colostrum of cows with a shortened dry period seems to be a result of a tissue regeneration-related immune response rather than an actual infection.

The high colostrum concentration of LTF, compared with other antimicrobial proteins, indicates that this protein played an important role in the mammary gland of cows with a 4 wk dry period. Both in SH and SR, LTF was upregulated proportionally with SPP1, as was observed in previous work (Zhang et al., 2015b). Both LTF and SPP1 have various functionalities. LTF is known as an antimicrobial protein, but has also been related to cell proliferation (Nakajima et al., 2015) and wound healing due to its immune regulatory properties (Engelmayer et al., 2008, Tang et al., 2010). SPP1 has also been related to immune regulation, wound healing and mucosal protection (Sodek et al., 2006). Both the immune as well as cell regeneration-related functionality of LTF and SPP1 fit in the hypothesis that shortening the dry period of the cow results in an immune response due to altered mammary epithelial tissue, which indicates more tissue regeneration at parturition. The potential involvement of LTF and SPP1 in both an immune response and cell regeneration may indicate their key role around parturition in the mammary gland of cows with a shortened dry period, resulting in high concentrations in colostrum and transition milk. SPP1 has been described to be involved in protection of the mucosal layer (Sodek et al., 2006), which serves for the first line of defense against bacterial invasion (Sando et al., 2009). Glycosylation-dependent cell adhesion molecule 1 (GLYCAM1) is a mucin-like protein that is part of the mucosal layer (Dowbenko et al., 1993). Mucin was not detected since in milk it is solely present in the milk fat globule membrane and not in milk serum,

in contrast to GLYCAM1 (Hettinga et al., 2011). The uncharacterised protein WFDC2 is a protease inhibitor which in humans has been shown to also protect the mucosal layer (Tomazic et al., 2014). Increased concentrations of GLYCAM1, SPP1 and WFDC2 in colostrum of SR cows with a shortened dry period, indicating increased mucosal protection, seem to confirm an immune response due to altered mammary epithelial tissue. This is in line with the upregulation of antimicrobial proteins described in the previous paragraph. Proteins involved in protection of the mucosal layer were still upregulated in transition milk (Figure 5.3). Proteins with direct antimicrobial properties, on the other hand, were not upregulated anymore in transition milk (Figure 5.3). These results indicate that there may be increased protection of the epithelium when the dry period is shortened, although it seems that there is no persistent antimicrobial immune response.

The immune response that was observed in colostrum of SR cows with a short dry period involved the apoptosis-related proteins Clusterin (CLU), Chitinase-3-like protein 1 (CHI3L1) and SPP1 (Strange et al., 1995, Sodek et al., 2006, Singh et al., 2008). Previous work has shown that apoptotic indices of mammary epithelial cells did not differ between cows that had either a 0 d or a 60 d dry period during the first days of lactation (Collier et al., 2012). The apoptosis-related proteins that were upregulated in the current results have other functionalities as well, such as immune suppression (CLU) (Danielsen et al., 2010) and mucosal protection (SPP1) (Sodek et al., 2006). Hence, the upregulation of 3 apoptosis related proteins does not dismiss previous findings that apoptosis in early lactation is not influenced by dry period length.

Increased formation of new mammary epithelial tissue was reflected in the immune response in colostrum of SR cows with a 4 wk dry period. SR cows with a 4 wk dry period had higher colostrum concentrations of APOE, BGN and THBS1 (table 5.2). These proteins have been described to be involved in cell regeneration. APOE has been related to accelerated epithelial injury repair and muscle cell regeneration in mice (Nathan et al., 2010, Arnold et al., 2015). BGN, of which the concentration in colostrum is strongly affected by dry period reduction, has been suggested to be involved in cell growth and differentiation (Couchman and Woods, 1993). THBS1 had been related to accelerated re-epithelialisation in wounded corneal epithelia (Uno et al., 2004). Mammary involution has been described to be similar to a wound-healing process (Stein et al., 2007). Upregulation of APOE, BGN and THBS1, but also previously discussed LTF and SPP1, in colostrum indicate that a more active wound healing-like process is going on in the mammary gland around parturition when a 4 wk dry period is applied compared with an 8 wk dry period. Wound healing related proteins were not upregulated anymore in transition milk of cows with a shortened dry period. In transition milk, predominantly epithelial cell renewal and protection related proteins were upregulated. A comparable phenomenon was found for cows with a 0 d dry period, which had an increased cell proliferation index compared with cows that had a 60 d dry period at the second day postpartum (Annen et al., 2008). The current study indicated a more

pronounced proteome effect of shortening the dry period for SR than for SH. In previous work, it was indicated that Holstein or Jersey cows had differences in their milk proteome (Tacoma et al., 2016, Vincent et al., 2016). No detailed reports on the milk proteome of SR, or on the proteomic response of different breeds towards intervening in the lactation cycle were found. However, following the hypothesis of increased tissue regeneration of cows with a 4 wk dry period, it can be suggested that a dual purpose breed (SR) requires more time to regenerate its mammary epithelium between lactations than a pure milk breed (SH).

Conclusion

Increased concentrations of proteins in colostrum that are involved in host defence and cell regeneration seem to confirm previous findings that a dry period of 4 wk is not sufficient for complete regeneration of the mammary epithelium of cows. Compared with SR, SH cows showed a milder response towards shortening the dry period in colostrum and no response in transition milk, leading to the hypothesis that this breed needs less time for mammary epithelial cell regeneration during the dry period.

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Chapter Six

Blood glucose concentration relates to milk composition of dairy cows in early lactation

Ruben de Vries, Ariëtte van Knegsel,
Kasper Hettinga, Elsa Antunes-Fernandes

Manuscript in preparation

Abstract

The glucose concentration in blood differs among individual cows in early lactation. Previous work showed a relation between metabolic status of cows and milk protein synthesis after infusion of glucose and insulin. The aim of this study was to increase understanding of glucose use by dairy cows for the synthesis of lactose and major milk proteins in a natural situation. This was done by comparing milk and blood composition of 12 Holstein Friesian cows in early lactation. Milk samples were analyzed for macronutrient composition, protein composition, and the metabolome. Blood samples were analyzed for glucose and insulin concentrations. Greater glucose concentrations in blood were related to a decline in lactose yields relative to milk protein yields. Glucose derived metabolites such as UDP-hexoses and sugars, and metabolites involved in the Krebs cycle correlated positively with the β-lactoglobulin fraction and negatively with α_{s1}- and β-casein fractions in milk. These correlations indicated a relation between the metabolic status of the cow and milk protein composition. It was suggested that the blood glucose concentration is related to gene expression by the mTOR pathway in mammary epithelial cells and consequently affects milk protein composition. To our knowledge, this is the first work showing a relation between the glucose concentration of cows and milk protein composition in a natural situation without affecting the metabolism by for instance glucose or insulin infusions.

Introduction

During early lactation, energy intake of high producing dairy cows does not cover the energetic requirements for milk yield and body maintenance, and cows experience a negative energy balance (NEB) (de Vries and Veerkamp, 2000). The NEB is related with an increased incidence of metabolic diseases such as ketosis (Rastani et al., 2005, van Knegsel et al., 2013). Cows in severe NEB have lower concentrations of glucose (Andersen et al., 2005), and higher concentrations of ß-hydroxybutyrate (BHB) and non-esterified fatty acids (NEFAs) in blood plasma (Drackley et al., 1991, van Knegsel et al., 2007). Cows that had increased concentrations of blood NEFAs had increased C18:1 *cis*-9 concentrations in blood (Jorjong et al., 2014). This was in accordance with other studies reporting that cows in severe NEB had increased contents of long chain fatty acids in milk (Mather and Keenan, 1998, Rukkwamsuk et al., 2000, van Knegsel et al., 2014). In contrast with milk fatty acid composition, the milk protein profile and lactose production received little attention in relation to blood metabolite concentrations during NEB.

The majority of blood glucose being transported into mammary epithelial cells (MECs) is used as one of the sugars composing the disaccharide lactose (Cant et al., 2002). Further, glucose can be reduced to pyruvate through glycolysis (Abraham and Chaikoff, 1959), which will further be incorporated in the Krebs cycle for energy production in the form of ATP. Improving the metabolic status of cows by infusion of glucose or insulin, regulator of glucose uptake by the MEC, did however not necessarily result in an increased synthesis of lactose. Infusion of insulin was related to an increased protein content in milk (Mackle et al., 2000b). The same group found that insulin infusion resulted in a lower β-casein fraction in milk (Mackle et al., 1999). Insulin was reported to stimulate milk protein gene expression, with the strongest effect on the β-lactoglobulin gene (Menzies et al., 2009). Generally, the aforementioned studies showed that infusing glucose or insulin, and therefore improving the metabolic status of cows can stimulate the synthesis of milk proteins to a different extent for different proteins.

The metabolic status of the cow is reflected on milk metabolite composition. For instance, the incidence of ketosis, has been related to the ratio of glycerophosphocholine and phosphocholine in milk (Klein et al., 2012). Lu et al. (2013) showed that milk of cows in severe NEB had higher concentrations of for instance galactose-1-phosphate, than cows with better EB.

The influence of blood glucose concentration on milk protein synthesis has been reported, but most of the studies performed used infusion of insulin or glucose to alter blood glucose concentration (Mackle et al., 1999, Curtis et al., 2014) whilst the applicability for dairy farming in practice is limited. In addition, milk metabolomics may provide new insights in the role of blood derived components in synthesis of milk components. Therefore, the aim of this work is to increase knowledge on the relation between the glucose concentration in blood and the concentration of milk proteins as well as lactose yield during early lactation.

Materials and Methods

Experimental design and milk samples

The animal experiment and milk sampling procedure have been described previously (Van Hoeij, Submitted). The protocol was approved by the Institutional Animal Care and Use Committee of Wageningen University (Protocol number 2014125). The current experiment included 12 clinically healthy Holstein Friesian cows, housed at the Wageningen UR Dairy Campus (Lelystad, the Netherlands). Seven cows had a 0 d dry period and 5 cows had a 30 d dry period. Cows were in second or third parity, equally distributed over the dry period groups. Milk samples were taken during the morning milking at 44 ± 13 days in milk (DIM), similar for both groups. Milk fat, protein, and lactose content and somatic cell count (SCC) were measured at milk control station Qlip (Zutphen, the Netherlands) following ISO 9622. Milk samples for further analysis were frozen at -20°C immediately after collecting.

Blood collection and analyses

Blood samples were collected at the same days as milk samples. The blood sampling and analysis protocol was described earlier (Van Hoeij, Submitted). Blood (10 mL) was collected after the morning milking. Blood plasma was isolated by centrifugation (3000 x g, 15 min, 4°C). Glucose concentrations in blood plasma were measured using kit no. 61269 (BioMerieux, Marcy l'Etoile, France). Insulin concentrations were measured using kit no. PI-12K (EMD Millipore Corporation, Billerica, MA, USA).

Milk protein composition

Milk protein composition was determined by reversed phase-high performance liquid chromatography (RP-HPLC), according to De Vries et al. (2015). In short, milk samples were dissolved (1:3 v/v) in a solution consisting of 8 M urea, 0.1 M BIS-TRIS, 5.37 mM sodium citrate and 19.5 mM dithiothretiol dissolved in MilliQ water (Millipore, Billerica, MA) (pH 6.8). After incubation for 60 min, samples were defatted after centrifuging, using an Eppendorf 5430 R centrifuge (20,817 x *g*, 5 min, 4°C). Defatted samples were further diluted in a solution of 6 M urea in MilliQ water containing 0.1 % trifluoroacetic acid (TFA) (pH 2). Milk proteins were separated by an Ultimate 3000 LC module equipped with an Aeris widepore 3.6 µm XB-C18 column (250 x 4 mm) (Phenomenex, Utrecht, the Netherlands). A gradient of 33 - 47% acetonitrile (ACN) containing 0.1% TFA in milliQ water containing 0.1% TFA was applied during 36.5 min, followed by a linear decrease to the starting condition of 33% ACN (0.1% TFA) during 0.5 min until the end of the run at 41 min (de Vries et al., 2015). A flow rate of 0.25 mL/min was applied for 24 min, linearly increasing for 3 min until 0.40 mL/min, which was maintained until the end of the run at 41 min. Chromatograms were

obtained after absorbance measurement by a UV-vis detector at 214 nm. Data processing was done with Dionex Chromeleon 7.1.2 (Thermo- Scientific, Waltman, MA). Milk protein fractions are expressed as percentage of the surface of individual proteins of the total protein surface in the RP-HPLC chromatograms.

Milk metabolome

The metabolite profile of the milk samples was determined by ^{1}H-NMR. The procedure is described in detail by Lu et al. (2013). In short, milk samples were thawed overnight at 4°C and subsequently ultracentrifuged at 117,500 x *g* and 4°C (75 min) to obtain milk serum. This was further centrifuged at 16,000 x *g* and 4°C (20 min) for removal of remaining fat. The obtained milk serum (175 µL) was thoroughly mixed with phosphate buffer (175 µL; 0.3 M, pH 6.0, and 1 mM of 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP; Sigma-Aldrich, Germany)).

Nuclear magnetic resonance Bruker spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit (Bruker, Rheinstetten, Germany) was used to obtain spectra (one-dimensional nuclear Overhauser enhancement spectroscopy (1-D-NOESY)) for all milk serum samples. Baseline corrections, alignment, and calibration were done to internal TSP (δ = 0.00 ppm) for all milk serum spectra. To assign milk serum non overlapping metabolite resonances, comparisons were made with published literature (Lu et al., 2013, Antunes-Fernandes et al., 2016) and the Human Metabolome Database version 2.0 online library (http://www.hmdb.ca). The peak area of each assignment is relative to the calibration standard TSP, resulting in a relative peak area in arbitrary units that was used for statistical analyses.

Statistical analysis

A general linear model was used for comparison of blood glucose and insulin concentrations, energy balance, milk yield and milk component concentrations among dry period lengths and parities (Proc GLM, SAS 9.3, SAS institute, SAS Inc., Cary, NC). Fixed factors in the model were dry period length (0 d or 30 d) and parity (second or third), and the interaction between dry period length and parity. Pearson correlations were determined by Proc corr in SAS. Differences were considered significant if $P < 0.05$.

Results

Table 6.1 shows an overview of milk yield, milk macronutrient composition, milk protein composition, blood glucose and insulin concentrations, and energy balance (EB) of the cows. Cows with a 0 d dry period had a higher milk protein content (3.76%) than cows with a 30 d dry period (3.15%). Milk yield, fat content, lactose content, blood glucose, blood insulin and energy balance were not affected by dry period length (DPL) (Table 6.1). Cows with a 0 d dry period had a lower α-lactalbumin fraction (3.3%) in milk protein than cows with a 30 d dry period (4.1%) ($P < 0.01$). Cows with a 0 d dry period had a lower β-casein fraction in milk protein (32.5%) than cows with a 30 d dry period (34.0%) ($P < 0.01$). Milk yield correlated negatively with the EB of the cows (R = -0.61, $P = 0.04$) and with the glucose concentration in blood (R = -0.65, $P = 0.02$). Milk yield correlated positively with the lactose yield (R = 0.98, $P < 0.01$). Lactose yield correlated negatively with the glucose concentration in blood (R = -0.63, $P = 0.03$). The EB of the cows correlated positively with the glucose concentration in blood (R = 0.65, $P = 0.02$). There was no correlation between EB and blood insulin concentration (R = 0.38, $P = 0.20$). Also the blood glucose concentration did not correlate with the insulin concentration in blood (R = 0.45, $P = 0.13$).

TABLE 6.1. Milk yield, milk composition, milk protein fractions[1], blood constituents and energy balance of cows (N = 12) in early lactation, the correlation coefficient (R) of the correlation with the glucose concentration in blood, and the *P*-value of the difference between cows with a 0 d or a 30 d dry period.

	Mean	Min	Max	Std Error	Glucose[2]	*P*-value DP
Milk yield (kg/d)	36.2	28.3	42.8	1.5	-0.65*	0.24
Fat (%)	4.47	3.85	4.95	0.11	0.01	0.13
Protein (%)	3.50	2.93	3.95	0.11	0.51	<0.01
Lactose (%)	4.66	4.45	4.88	0.03	0.08	0.27
SCC (x1000 cells/mL)[3]	77	13	281	23	-0.58*	0.89
α-lactalbumin (%)	3.6	2.8	4.4	0.1	-0.55	<0.01
β-lactoglobulin (%)	7.9	5.5	9.5	0.4	0.47	0.22
α_{s1}-casein (%)	26.0	23.9	28.1	0.3	-0.22	0.66
α_{s2}-casein (%)	17.4	15.7	19.4	0.4	-0.08	0.14
β-casein (%)	33.1	31.7	34.5	0.3	-0.30	<0.01
κ-casein (%)	11.9	9.7	13.1	0.3	0.40	0.81
Blood glucose (mmol/L)	3.99	3.65	4.38	0.07	-	0.91
Blood insulin (μU/mL)	13.79	8.77	21.52	1.21	0.45	0.97
Energy balance (kJ/$kg^{0.75}$·d)	-36.5	-210.2	148.7	34.6	0.65*	0.96

* $P < 0.05$

[1] Protein fractions are expressed in percentage of the sum of all proteins

[2] the Pearson correlation coefficient (R) of the vertically oriented variable with glucose

[3] GLM based on the log-value of SCC

Both blood glucose and the major milk protein fractions α-lactalbumin, β-lactoglobulin, α_{s1}-casein and β-casein correlated with metabolite concentrations in milk (Table 6.2). None of the metabolites correlated with the α_{s2}-casein fraction in milk. Eleven metabolites (sugar A, lactate, ethanol, methylmalonate, alanine, acetate, N-acetylsugar A, N-acetylsugar B, oxaloacetate, succinate and malonate) correlated, all positively, with the κ-casein fraction in milk (Supplementary table 6.1). Nine metabolites (sugar A, lactate, ethanol, methylmalonate, alanine, acetate, N-acetylsugar A, malonate and hippurate) correlated with blood glucose, as well as with fractions of α-lactalbumin, β-lactoglobulin, α_{s1}-casein and β-casein (Figure 6.1). All correlations between β-lactoglobulin or κ-casein fractions and concentrations of metabolites that correlated with blood glucose were positive. Correlations between α-lactalbumin, α_{s1}-casein or β-casein fractions and metabolites that correlated with blood glucose were all negative. Correlation coefficients of UDP-hexoses and sugars with blood glucose concentrations and milk protein fractions are presented in Table 6.2. Correlations between all metabolite concentrations in milk, and glucose concentrations in blood as well as protein fractions in milk can be found in supplementary table 6.1.

TABLE 6.2. Correlations (R) between sugar metabolite concentrations[1] in milk and blood glucose, lactose yield, protein content, and individual protein fractions[2]

	UDP-hexose A	UDP-hexose B	UDP-hexose C	Sugar A	Sugar B	Sugar C
Energy balance (kJ/kg$^{0.75}$·d)	0.34	0.29	0.31	0.56	-0.23	-0.29
Blood glucose (mmol/L)	0.61*	0.47	0.59*	0.75**	-0.16	-0.29
Insulin (µU/mL)	-0.38	-0.30	-0.24	0.25	-0.71*	-0.74**
Lactose yield (kg/d)	-0.46	-0.42	-0.48	-0.78**	0.45	0.50
Milk protein %	0.63*	0.53	0.54	0.73**	-0.33	-0.46
α-lactalbumin (%)	-0.56	-0.33	-0.46	-0.64*	0.34	0.48
β-lactoglobulin (%)	0.81**	0.85***	0.83***	0.66*	0.11	0.02
α_{s1}-casein (%)	-0.79**	-0.76**	-0.75**	-0.65*	-0.13	-0.05
α_{s2}-casein (%)	0.19	-0.02	0.04	0.39	-0.30	-0.37
β-casein (%)	-0.58*	-0.50	-0.43	-0.86***	0.53	0.62*
κ-casein (%)	0.55	0.53	0.45	0.67*	-0.36	-0.42

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

[1]Metabolite concentrations in arbitrary units

[2]Protein fractions are expressed in percentage of the sum of all proteins

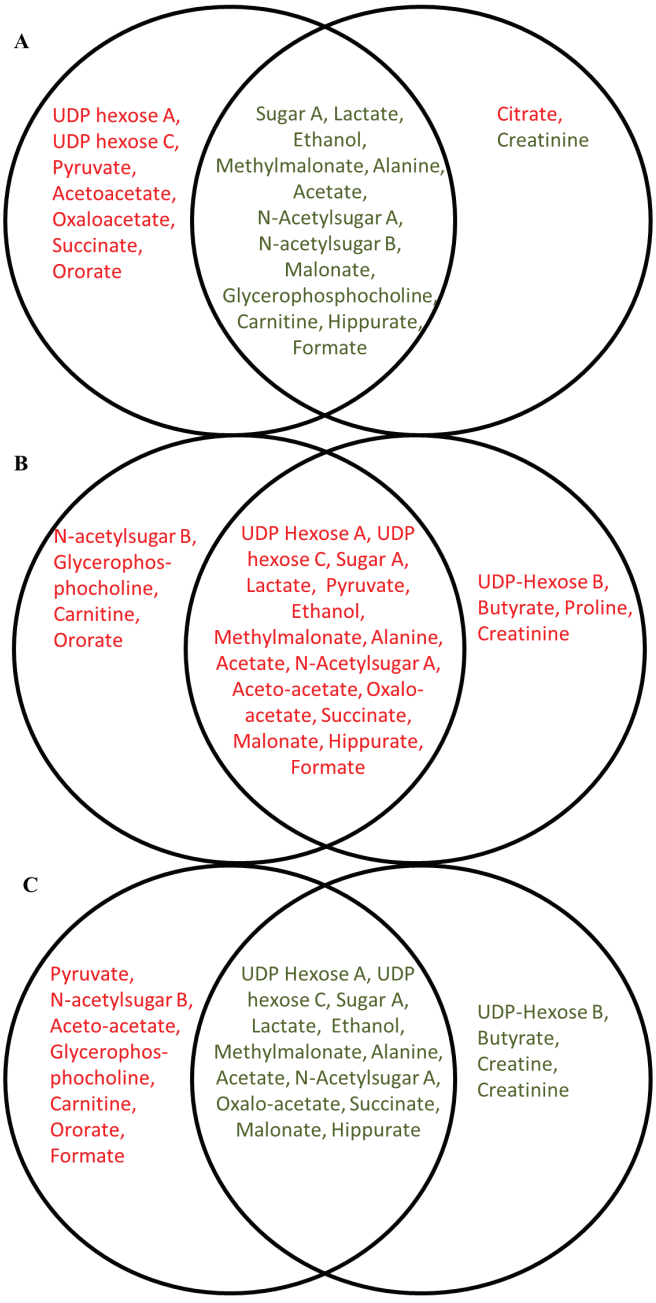
A
UDP hexose A, UDP hexose C, Pyruvate, Acetoacetate, Oxaloacetate, Succinate, Ororate
Sugar A, Lactate, Ethanol, Methylmalonate, Alanine, Acetate, N-Acetylsugar A, N-acetylsugar B, Malonate, Glycerophosphocholine, Carnitine, Hippurate, Formate
Citrate, Creatinine
B
N-acetylsugar B, Glycerophos-phocholine, Carnitine, Ororate
UDP Hexose A, UDP hexose C, Sugar A, Lactate, Pyruvate, Ethanol, Methylmalonate, Alanine, Acetate, N-Acetylsugar A, Aceto-acetate, Oxalo-acetate, Succinate, Malonate, Hippurate, Formate
UDP-Hexose B, Butyrate, Proline, Creatinine
C
Pyruvate, N-acetylsugar B, Aceto-acetate, Glycerophos-phocholine, Carnitine, Ororate, Formate
UDP Hexose A, UDP hexose C, Sugar A, Lactate, Ethanol, Methylmalonate, Alanine, Acetate, N-Acetylsugar A, Oxalo-acetate, Succinate, Malonate, Hippurate
UDP-Hexose B, Butyrate, Creatine, Creatinine

FIGURE 6.1.

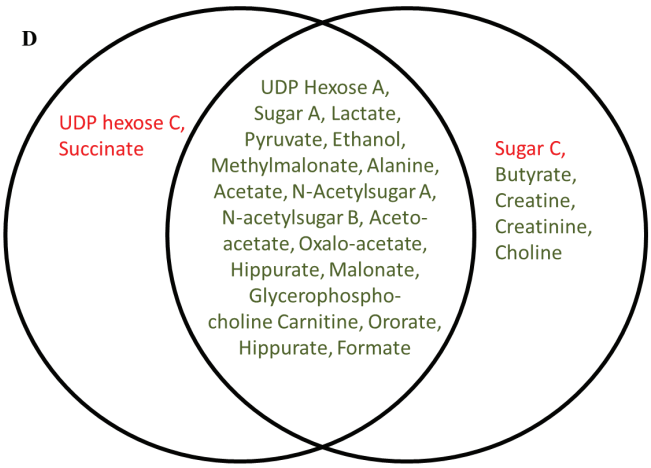

FIGURE 6.1. Metabolites that correlated ($P < 0.05$) with either the glucose concentration in blood (left circles), or with the major milk protein fractions α-lactalbumin (A), β-lactoglobulin (B), α_{s1}-casein (C) or β-casein (D) (right circles). Metabolites in the intersection of 2 circles correlated with both glucose and the corresponding protein fraction. Positive correlations are shown in red while negative correlations are shown in green.

Discussion

The aim of this work was to increase knowledge on the relation between glucose concentration in blood and milk protein concentrations and lactose production during early lactation. The current work included cows with a dry period of either a 0 d or 30 d, in order to induce variation in EB and glucose concentration in blood. Based on previous work, it was expected that DPL was a major factor contributing to the variation in these variables (Andersen et al., 2005, van Knegsel et al., 2014). However, EB and blood glucose concentration were not affected by DPL in the current work. Although milk yield, blood glucose concentration, and EB were not affected by DPL, their interrelations were according to expectation. In the current work, cows with a relatively high EB had a high blood glucose concentration (Table 6.1), as was reported before (Gross and Bruckmaier, 2015). The EB in early lactation is dependent on the milk yield (van Knegsel et al., 2014), as was reflected in the current work by a negative correlation between milk yield and energy balance (EB).

Most of the blood derived glucose entering the MECs is used for lactose synthesis (Cant et al., 2002). Lactose yield is the driving force for milk yield, as lactose regulates the osmolality of milk (Linzell and Peaker, 1971). This was also observed in the current work, as milk yield and lactose yield were positively correlated. The lactose yield correlated negatively with the glucose concentration in blood ($R = -0.63$, $P = 0.03$). A relatively high blood glucose concentration may be an indirect effect of a low lactose synthesis, due to a low milk yield, and consequently a good EB. The other way around, a high glucose concentration may also be the cause of a low lactose yield. It has been shown that the amount of glucose used for lactose synthesis reduced after infusion of glucose with insulin (Mackle et al., 2000b). Other work on the other hand reported an increased lactose yield after glucose infusion (Curtis et al., 2014). As the lactose yield did not correlate with the blood insulin concentration in the current work, it seems most likely that a high lactose yield results indirectly in a low blood glucose concentration in the current work. Previously, milk metabolites have been used to better understand the cellular processes in milk synthesis. For instance, sugar phosphates in milk were suggested to be intermediates of glucose metabolism and lactose synthesis (Antunes-Fernandes et al., 2016). In the present study, the milk metabolites UDP-hexose A and C and sugar A correlated positively with the blood glucose concentration. Of these metabolites, sugar A is the only one that correlated with the lactose yield. This correlation between sugar A and the lactose yield was negative, which may indicate depletion of sugar A for synthesis of lactose. Sugar B and sugar C however did not correlate with lactose yield (Table 6.2). Glucose may be involved in cellular processes other than lactose synthesis, such as synthesis of fatty acids or triacylglycerides (Dijkstra et al., 1996), which might explain that not all glucose derivative metabolites in milk were related to lactose yield. Besides, concentrations of UDP-hexoses in milk may increase due to leakage from the MEC in case of apoptosis (Lu et al., 2013). The current outcomes are in line with previous findings that

glucose in the mammary gland is only partly used for lactose synthesis by dairy cows, which in the current study was indicated by the limited correlations between glucose derivatives and lactose yield. Also the previous finding that cows with relatively high blood glucose concentration have a relatively low lactose yield was confirmed, most likely because a high lactose yield results in a low EB and subsequently a low glucose concentration in blood.

Glucose can be converted into pyruvate, which is used in MECs for either lactose synthesis or for further utilisation in the Krebs cycle (van Knegsel et al., 2005). Cows in a NEB, that have a high number of MECs use their available glucose predominantly for lactose synthesis (van Knegsel et al., 2005). Cows without a dry period have lower number of MECs in early lactation due to limited prepartum regeneration of MECs (Capuco et al., 1997, Collier et al., 2012). Therefore, DP omission resulted in a reduced milk production in early lactation with increased protein content (Schlamberger et al., 2010, van Knegsel et al., 2014). This seems to be in agreement with the current outcome that a low lactose synthesis, and thus low milk yield, is related to a high glucose concentration in blood, whereas the protein yield was not related to the blood glucose concentration. The latter resulted in a high milk protein content at a high blood glucose concentration due to a relatively low milk yield. Previous work showed a positive effect of glucose infusion on milk protein content (Toerien et al., 2010). In the current work, blood glucose concentration and milk protein content did not correlate with each other (R = 0.46, P = 0.13). Derivatives of glucose metabolism however correlated positively (UDP-hexose A, sugar A), or tended to correlate positively (UDP-hexose B and C) with the protein content in milk (Table 6.2). Not only milk metabolites that were derived from glucose but also the milk metabolites pyruvate, acetoacetate, oxaloacetate and methylmalonate, which are all involved in the Krebs cycle, correlated positively with protein content. These results indicate that an increase of the blood glucose concentration of the cow can stimulate ATP production via glycolysis and the Krebs cycle. This increased ATP production may subsequently result in increased protein synthesis, leading to an increased protein content in milk.

An important regulator of protein synthesis in cells is the mammalian target of rapamycin (mTOR) pathway, which is responsible for phosphorylation processes (Richter and Sonenberg, 2005) and thereby influencing mRNA translation (Wang and Proud, 2006, Wullschleger et al., 2006). In cows, the mTOR pathway is dependent on several factors, such as lactation stage, nutrient availability, and metabolic status of the cow (Bionaz et al., 2012, Curtis et al., 2014). The mTOR pathway is a regulator of protein synthesis, mainly at the initiation and elongation stages of translation (Wang and Proud, 2006). However, an attempt to stimulate the mTOR pathway by supplementation of glucose showed no effect on milk protein content (Curtis et al., 2014). The authors did not find a stimulatory effect of glucose supplementation on the glucose concentration in blood, which may be essential for stimulation of the mTOR pathway. The mTOR pathway can be inhibited by upregulation of the AMPK pathway due to ATP deficiency, limiting the process of milk protein synthesis

(Sarbassov et al., 2005, Wang and Proud, 2006). It can be hypothesized that increased ATP formation due to increased glucose concentrations in blood has the opposite, inhibitory, effect on the AMPK pathway, and can thereby stimulate the mTOR pathway, leading to increased protein synthesis. The current work indicated that milk protein content is strongly related to metabolites involved in the energy cycle of MEC. If cows with a low milk yield have a low number of MECs, as is the case for cows without a dry period (Collier et al., 2012), the increased milk protein content of cows with an improved metabolic status seems to be caused by increased availability of energy per MEC.

Not only the milk protein content, but also milk protein composition, i.e. the proportion of different protein fractions in milk, was related to the milk metabolome. The α_{s1}-casein, β-casein and α-lactalbumin fractions in milk correlated negatively and the β-lactoglobulin fractions correlated positively with glucose derivatives in milk such as UDP-hexose A and Sugar A (Table 6.2). Differential synthesis of milk proteins may be the result of different gene expression regulation by the mTOR pathway, which is energy dependent. Both energy supply, for instance by starch infusion, and insulin concentration have been shown to influence translational regulation by the mTOR pathway (Wullschleger et al., 2006, Rius et al., 2010). Other studies showed that an increased glucose metabolism due to infused insulin resulted in upregulated translation of milk protein genes (Mackle et al., 1999, Menzies et al., 2009). Menzies et al. (2009), who studied gene expression after insulin infusion in dairy cows, indicated upregulation of milk protein genes, with the greatest stimulation of the β-lactoglobulin gene. Mackle et al. (1999) reported lower relative abundance of β-casein, and a tendency (P = 0.06) for a higher relative abundance of β-lactoglobulin in milk after stimulation of the glucose metabolism by insulin infusion. Outcomes from both sources seem to be in agreement with the current work, in which the relative abundance of β-lactoglobulin increases with an increased blood glucose concentration, whereas the β-casein fraction decreased with an increased glucose concentration. In addition, the α_{s1}-casein fraction also decreased with an increased blood glucose concentration in the current work. This study only provides blood glucose concentrations, but we do not have actual data on the amount of blood glucose that is used by MEC for synthesis of milk components. However, glucose derivatives that were measured in milk correlated with milk protein fractions (Table 6.2). No correlations between protein fractions and insulin concentrations in blood were found though. Insulin was reported to have an indirect rather than a direct effect on the protein synthesis, as this effect may be mediated by insulin growth factors (Mackle et al., 2000a). Besides, the insulin response that was found after infusion as in the work of Mackle et al. (2000a) is likely to be stronger than in a natural situation such as in the current work. These two aspects may have contributed to the absence of a correlation between the insulin concentration in blood and protein fractions in the current work. All in all, the current outcomes are in line with the finding that the metabolic status of dairy cows is related with milk protein synthesis, possibly due to differential regulation of expression of

milk protein genes by the mTOR pathway. In addition to previous work, the current outcomes indicate that not only infusive interventions, but also natural variability in metabolic status of cows during early lactation can be reflected in milk protein composition. Similarities were found in the protein fractions that were positively (β-lactoglobulin) or negatively (β-casein) related with increased glucose concentration as in studies in which cows were infused with insulin in their blood stream.

Conclusions

The current study showed that glucose-derived milk metabolites such as UDP-hexoses, sugars and milk metabolites that are involved in the Krebs cycle correlate with protein composition of early lactation milk. The positive correlation between these metabolites and the glucose concentration in blood indicated that the glucose metabolism of the cow is related to milk protein composition. The β-lactoglobulin fraction increased, whereas fractions of α-lactalbumin, and α_{s1}- and β-casein decreased with increasing glucose concentration. To our knowledge, this is the first work indicating relations between blood glucose concentration of cows and milk protein composition in a natural situation.

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SUPPLEMENTARY TABLE 6.1. Correlations of all quantified metabolites with glucose concentrations in blood, and the six major milk protein fractions, expressed as correlation coefficients (R), with significance levels (*P*). α-LA = α-lactalbumin, β-LG = β-lactoglobulin, CN = casein.

	Glucose		α-LA		β-LG		α_{s1}-CN		α_{s2}-CN		β-CN		κ-CN	
	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*
UDPhexose A	0.61	0.03	-0.56	0.06	0.81	< 0.01	-0.79	< 0.01	0.19	0.56	-0.58	0.05	0.55	0.06
UDPhexose B	0.47	0.13	-0.33	0.30	0.85	< 0.01	-0.76	< 0.01	-0.02	0.96	-0.50	0.10	0.53	0.08
UDPhexose C	0.59	0.04	-0.46	0.13	0.83	< 0.01	-0.75	< 0.01	0.04	0.89	-0.43	0.17	0.45	0.15
Sugar A	0.75	< 0.01	-0.64	0.02	0.66	0.02	-0.65	0.02	0.39	0.21	-0.86	< 0.01	0.67	0.02
Sugar B	-0.16	0.62	0.34	0.28	0.11	0.73	-0.13	0.68	-0.30	0.34	0.53	0.08	-0.36	0.24
Sugar C	-0.29	0.36	0.48	0.11	0.02	0.95	-0.05	0.89	-0.37	0.24	0.62	0.03	-0.42	0.17
Lactate	0.58	0.05	-0.64	0.02	0.86	0.00	-0.71	0.01	0.19	0.55	-0.75	< 0.01	0.62	0.03
Butyrate	0.54	0.07	-0.52	0.09	0.87	0.00	-0.75	< 0.01	0.10	0.76	-0.65	0.02	0.61	0.04
Pyruvate	0.62	0.03	-0.55	0.06	0.74	0.01	-0.55	0.06	0.15	0.64	-0.70	0.01	0.53	0.08
Ethanol	0.70	0.01	-0.69	0.01	0.81	< 0.01	-0.75	0.01	0.23	0.47	-0.74	0.01	0.69	0.01
Methylmalonate	0.71	0.01	-0.63	0.03	0.89	< 0.01	-0.75	< 0.01	0.09	0.77	-0.68	0.02	0.67	0.02
Alanine	0.66	0.02	-0.71	0.01	0.84	< 0.01	-0.70	0.01	0.24	0.46	-0.76	< 0.01	0.61	0.04
Acetate	0.63	0.03	-0.71	0.01	0.82	< 0.01	-0.64	0.03	0.18	0.58	-0.72	0.01	0.60	0.04
Nacetylsugar A	0.69	0.01	-0.68	0.02	0.79	< 0.01	-0.61	0.03	0.18	0.58	-0.72	0.01	0.60	0.04
Nacetylsugar B	0.64	0.02	-0.80	< 0.01	0.39	0.21	-0.43	0.16	0.46	0.14	-0.78	< 0.01	0.66	0.02
Nacetylsugar C	0.00	1.00	-0.08	0.81	0.21	0.51	-0.25	0.44	0.09	0.77	0.22	0.49	-0.34	0.27
Acetone	0.27	0.40	0.02	0.94	0.55	0.06	-0.49	0.10	-0.33	0.29	-0.09	0.78	0.41	0.19
Acetoacetate	0.67	0.02	-0.61	0.03	0.75	< 0.01	-0.55	0.06	0.13	0.70	-0.69	0.01	0.57	0.06
Oxaloacetate	0.62	0.03	-0.49	0.10	0.82	< 0.01	-0.68	0.02	0.01	0.97	-0.63	0.03	0.66	0.02
Proline	0.40	0.20	-0.27	0.39	0.67	0.02	-0.53	0.08	0.09	0.77	-0.51	0.09	0.30	0.34
Succinate	0.59	0.04	-0.47	0.12	0.84	< 0.01	-0.77	< 0.01	0.02	0.95	-0.55	0.07	0.63	0.03
Oxoglutarate	0.33	0.29	-0.15	0.64	0.47	0.12	-0.51	0.09	-0.22	0.49	-0.14	0.66	0.53	0.07
Citrate	-0.01	0.98	0.60	0.04	-0.02	0.95	-0.14	0.65	-0.48	0.12	0.42	0.18	0.10	0.75
Creatine	0.48	0.11	-0.30	0.34	0.56	0.06	-0.59	0.04	0.15	0.65	-0.58	0.05	0.58	0.05
Creatinine	0.50	0.09	-0.68	0.02	0.81	< 0.01	-0.65	0.02	0.26	0.42	-0.74	0.01	0.51	0.09
Phosphocreatine	-0.13	0.69	0.23	0.46	0.28	0.37	-0.27	0.40	-0.25	0.43	0.32	0.32	-0.20	0.54
Malonate	0.64	0.03	-0.82	0.00	0.77	0.00	-0.62	0.03	0.23	0.46	-0.75	0.01	0.65	0.02

SUPPLEMENTARY TABLE 6.1. Correlations of all quantified metabolites with glucose concentrations in blood, and the six major milk protein fractions, expressed as correlation coefficients (R), with significance levels (*P*). α-LA = α-lactalbumin, β-LG = β-lactoglobulin, CN = casein. (continued)

	Glucose		α-LA		β-LG		α_{s1}-CN		α_{s2}-CN		β-CN		κ-CN	
	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*	R	*P*
Choline	0.35	0.26	-0.35	0.26	0.19	0.56	-0.16	0.62	0.26	0.42	-0.68	0.01	0.55	0.07
Phosphorylcholine	-0.24	0.46	0.32	0.31	0.03	0.92	0.00	1.00	-0.29	0.36	0.55	0.06	-0.45	0.14
Glycerophosphoholine	0.60	0.04	-0.75	< 0.01	0.55	0.06	-0.42	0.17	0.31	0.33	-0.65	0.02	0.45	0.14
Acetylcarnitine	0.33	0.30	0.02	0.95	0.25	0.43	-0.35	0.27	-0.23	0.47	-0.10	0.76	0.53	0.08
Betaine	0.44	0.15	0.05	0.87	0.15	0.64	-0.34	0.28	0.13	0.69	-0.06	0.86	0.07	0.83
Carnitine	0.62	0.03	-0.67	0.02	0.39	0.21	-0.46	0.14	0.50	0.10	-0.71	0.01	0.49	0.10
N-acetylsugar D	-0.10	0.76	-0.07	0.84	0.08	0.80	-0.10	0.77	0.11	0.73	0.27	0.40	-0.44	0.15
Galactose 1phosphate	0.31	0.32	0.08	0.81	0.43	0.16	-0.54	0.07	-0.29	0.37	0.15	0.64	0.26	0.41
Orotate	0.58	0.05	-0.57	0.05	0.25	0.43	-0.39	0.21	0.48	0.11	-0.61	0.04	0.47	0.12
Hippurate	0.60	0.04	-0.64	0.02	0.79	< 0.01	-0.58	0.05	0.08	0.81	-0.59	0.04	0.51	0.09
N-acetylsugar E	0.37	0.23	-0.51	0.09	0.55	0.06	-0.48	0.12	0.27	0.40	-0.28	0.39	0.03	0.94
Formate	0.66	0.02	-0.66	0.02	0.67	0.02	-0.52	0.08	0.22	0.49	-0.72	0.01	0.58	0.05

Chapter Seven

General discussion

During the last 2 decades, increasing attention has been paid to find the optimal dry period of dairy cows. Cows with a traditional dry period of approximately 60 days generally experience in a severe negative energy balance after calving. It was suggested that improving the energy balance by shortening or omitting the dry period could improve the metabolic status and fertility of cows. Cow health and milk production were the main issues that have been studied in relation to shortening or omitting the dry period, whereas only limited studies had indicated the impact on milk composition. Milk composition in relation to dry period length can increase knowledge about the status of the mammary epithelium and the synthesis of milk components. Next to that, high quality milk is essential for the production of high quality milk products for human consumption. Therefore, in this thesis the effect of shortening or omitting the dry period on milk composition was evaluated.

This thesis about the relation between dry period length of dairy cows and milk composition and quality comprises two main research questions: *how is milk composition influenced by applying different dry period lengths to cows with regard to the processing quality of milk?* And, *how does milk composition of cows with different dry period lengths relate to the lactation physiology of the cow?* Chapter 2, 3 and 4 provide data that indicate the effect of dry period length on processing quality of milk. Chapter 5 and 6 focus on the relation between dry period length and mammary gland physiology. However compositional data in chapter 2, 3 and 4 are also used as an indication of processes going on in the mammary gland. In this chapter, firstly, new insights from the different chapters about mammary gland physiology are discussed (section 7.1). Secondly, implications of applying different dry period lengths for milk processing quality are evaluated (section 7.2). Thirdly, the contribution of this work to the opportunities and threats for different actors in the dairy chain of applying different dry period lengths in practice are discussed (section 7.3). Finally, the overall conclusions of this thesis are addressed in section 7.4.

The main conclusions from the different chapters were:

Chapter 2.

- ► Cows with a 0 d dry period have an increased glycosylated κ-casein fraction in milk at 2 weeks before calving.
- ► Cows with a 0 d dry period have a reduced β-casein fraction in early lactation compared with cows with a 60 d dry period, possibly as a result of increased plasmin activity.

Chapter 3.

- ► Multiparous cows with a 4 wk dry period have higher plasmin activity in early lactation milk than cows with an 8 wk dry period.
- ► Increased plasminogen activation by activators from somatic cells may indicate increased regeneration of mammary epithelial cells during early lactation of multiparous cows with a 4 wk dry period.

Chapter 4.

- Plasmin activity in milk of cows with a 0 d dry period tended to be higher compared with cows with a 30 d dry period because of a lower milk yield.
- At low somatic cell count, dry period length does not affect plasminogen activation.
- Casein and mineral composition of casein micelles is relatively constant, and not related to dry period length, whereas the calcium concentration in milk serum increases with increasing protein content and decreasing citrate concentration.

Chapter 5.

- None of the concentrations of the low abundant proteins in milk were reduced by shortening the dry period, indicating no negative effect of shortening the dry period on colostrum quality.
- Cows with a 4 wk dry period showed a proteomic immune response in colostrum, which indicated increased proliferation of mammary epithelial cells.

Chapter 6.

- Differences in metabolic status of cows in early lactation, which can be induced by dry period omission, were related to differences in milk protein composition.
- The relation between the blood glucose concentration and milk protein composition was similar in a natural situation as in studies using glucose and insulin infusions to alter the glucose metabolism.

7.1 New Insights in Mammary Gland Physiology

Cows with a short or with no dry period have a lower milk yield than cows with a conventional dry period. Dry period length also influences milk composition on macronutrient level, but also within the fat fraction (van Knegsel et al., 2014) and the protein fraction (this thesis). The physiological background of changes in the fat fraction in early lactation milk of cows with different dry period lengths is well understood (Jorjong et al., 2014). However the mechanism underlying differences in milk protein composition in relation to dry period length have not been reported so far. In this work, shortening or omitting the dry period was related to a reduction in milk yield and β-casein fraction in milk, and an increase in milk protein content and plasmin activity in milk. In this section, the relation between these changes and the physiology of the mammary epithelium is discussed.

Regeneration of epithelial cells

The dry period is commonly known as a period during which regeneration of the mammary epithelium of the cow takes place (Capuco et al., 1997, Collier et al., 2012). When the dry

period is shortened or omitted, lactogenesis and regeneration of the mammary epithelium may overlap. Cows that did not have a dry period had less prepartum proliferation of mammary epithelial cells (MECs), resulting in a lower number of active MECs in their successive lactation compared with cows that had a conventional dry period (Collier et al., 2012). This lower number of active mammary epithelial cells in early lactation of cows that did not have a dry period results in a lower milk yield postpartum (Annen et al., 2007, Annen et al., 2008). In the current work, dry period omission was indeed related to a lower postpartum milk yield, in comparison to a 60 d dry period (Chapter 2). Regeneration of MECs has not been described for cows with a 30 d dry period. It can be assumed that numbers of active MECs will be lower for these cows than of cows with a 60 d dry period since the proliferation index is relatively high during the entire dry period (Collier et al., 2012). Indeed, cows with a 28-30 d dry period had a lower milk yield than cows with a 56-60 d dry period (Chapters 2 and 3), in accordance to previous findings (van Knegsel et al., 2013).

Enzymatic activity in the mammary epithelium

Plasmin activity in milk is a result of transcellular transport of plasmin, or its precursor plasminogen, from blood to milk (Silanikove, 2016). Plasminogen is transported in Golgisomes to milk, in the presence of casein micelles. Plasminogen is activated by either tissue-type activators, that are mainly associated with casein micelles, or urokinase-type activators that originate from somatic cells (Ismail and Nielsen, 2010). Hence, the plasmin activity in milk is dependent on the amount of plasmin and plasminogen that is transported from blood to milk, and the amount of plasminogen that has been activated. Consequently, an increase in plasmin activity can indicate a number of processes in the mammary epithelium. Firstly, it can reflect an increased casein content in milk, since plasminogen and casein are transported together. Secondly, increased plasmin activity can reflect an increase in somatic cell count (SCC) in milk. Thirdly, plasmin activity may reflect physiological processes in the mammary epithelium such as proliferation of mammary epithelial cells (Politis, 1996). In the current work, shortening or omitting the dry period was related to an increase in, or a tendency towards, an increased plasmin activity. In this section, 2 questions are evaluated. Firstly, does shortening or omitting the dry period by itself induce an increase in plasmin activity in the successive lactation? Secondly, are differences in plasmin activity related to the status of the mammary epithelium of cows with different dry period lengths?

The current work indicated that multiparous cows with a 4 wk dry period had increased plasmin activity in milk compared with cows with an 8 wk dry period, but no increased plasminogen activity (Chapter 3). These cows with high plasmin activity in milk also had high SCC. Dry period reduction from 30 to 0 d resulted in only a tendency towards higher plasmin activity, which was proportional to plasminogen activity (Chapter 4). All cows in the latter study had low SCC. Dry period omission may induce an increase in SCC (van Knegsel et al.,

2014), although SCC was not affected by shortening the dry period to 30 d (Bernier-Dodier et al., 2011, van Knegsel et al., 2014). If SCC exceeds 300,000 cells/mL milk, it is related to increased activation of plasminogen (Politis et al., 1989), which was observed in the current work as well. All in all, shortening or omitting the dry period may result in an increased plasmin activity, proportional to the casein content in milk. However, increased SCC, which can be induced by dry period omission, may result in an increased plasminogen activation on top of increased plasmin activity. In the current work, plasmin activity increased with decreasing milk yield between early and mid-lactation. Milk yield declined proportionally between early and mid-lactation for cows with different dry period length (Schlamberger et al., 2010, van Knegsel et al., 2014). Therefore, the suggestion that plasmin activity is higher in milk of cows without a dry period due to the inverse relation with milk yield can be made for both early and mid-lactation.

High plasmin activity in milk of cows that had high SCC (Chapter 3) indicated a role related to mammary health. Plasminogen activators from SCC have been related to proliferation of MEC. In primiparous cows, dry period omission did not affect MEC proliferation in early lactation (Annen et al., 2007). The effect of shortening the dry period on MEC proliferation in multiparous cows has not been reported. It is known that regeneration of MEC is slower for multiparous cows compared with primiparous cows (Miller et al., 2006). Several studies reported that the milk yield of multiparous cows is not affected by shortening the dry period (Annen et al., 2004, Pezeshki et al., 2007, Klusmeyer et al., 2009), in contrast to other work that show a drop in milk yield when the dry period is shortened, regardless of parity (van Knegsel et al., 2014). It can be hypothesized that multiparous cows require a high number and activity of MEC, whereas the formation of MEC during a dry period occurs in a lower rate compared with primiparous cows. Subsequently, multiparous cows may have increased postpartum MEC proliferation after a shortened dry period, which is reflected by increased activation of plasminogen into plasmin. Future work is needed to test this hypothesis, with focus on the actual MEC proliferation and the mechanism of plasminogen activation.

All in all, controlling SCC in milk of cows with a short or no dry period is important with regard to controlling plasmin activity. Further work is needed to assess the interactions between dry period length, parity and herd effects as risk factors for high SCC. When SCC can be kept low, shortening or omitting the dry period does not alter the ratio between plasmin activity and casein content in milk. Besides plasmin activity, also free fatty acid (FFA) concentrations were measured in the milk samples that were described in chapter 3. However, no difference was found in FFA concentrations between early lactation milk samples of cows with a dry period of either 4 or 8 weeks. Spontaneous lipolysis due to physical damage of the milk fat globule membrane is the major cause for the presence of FFAs in milk (Deeth, 2006). The absence of a relation between lipolysis and plasmin activity of cows with a shortened dry period indicates that plasmin activity is unlikely to be related to physical damage of the epithelial cell membrane. With regard to dry period length, most

physical damage to the MEC membrane may take place in the last weeks of lactation of cows without a dry period, due to a high cell turnover (Capuco et al., 1997, Annen et al., 2007). Therefore it is recommended to analyse FFA concentrations in the last weeks of lactation of cows without a dry period, which has not yet been included in the current work.

Milk synthesis in relation to energy status of the cow

The lower number of active MECs of cows without a dry period may be responsible for the lower milk production in early lactation (Collier et al., 2012). Due to the lower milk production, the metabolic status of the cow is improved (van Knegsel et al., 2014), which is reflected in a higher glucose concentration in blood of cows with a short or no dry period (van Knegsel et al., 2013). In chapter 6, it was suggested that a higher glucose concentration in blood was not only related to an increased protein content in milk, but also to milk protein composition. In chapter 2, only the β-casein fraction in early lactation milk was affected by applying a dry period of 0 d instead of 30 d, as was the case in chapter 4. However, in chapter 6, also other major milk protein fractions such as α_{s1}-casein and β-lactoglobulin were related to the blood glucose concentration of the cow. It is important to consider that dry period omission itself does not affect milk protein fractions, but it can reduce the number of active MECs, improve the energy balance and increase the blood glucose concentration in early lactation. The current work indicates that variability in glucose concentration in blood, and a reduced number of MECs, have an indirect effect on milk protein composition. The β-casein fraction in early lactation milk was related to both the blood glucose concentration (Chapter 6) and plasmin activity (Chapter 4). The effect of plasmin on β-casein was particularly found in mid-lactation, whereas no correlation between β-casein and plasmin was found in early lactation. The difference in the β-casein fraction in early lactation milk of cows with different dry period lengths was thus predominantly related to a difference in glucose concentration in blood, since the differences in metabolic status between individual cows are relatively large. In mid-lactation, when cows generally are in a positive energy balance, plasmin activity seems to be the major factor affecting the β-casein fraction in milk. In chapter 4, it was addressed that cows with a 0 d dry period tended to have a lower casein number than cows with a 30 d dry period. Part of this tendency can be explained by the positive correlation between glucose metabolism and the β-lactoglobulin fraction in milk (Chapter 6). However, a more large scale experiment using a conventional method to determine the casein number would be needed to determine whether dry period omission indeed has an effect on casein number.

7.2 Implications for Milk Processing

The current work showed that dry period length influenced macronutrient composition, casein composition, plasmin activity and mineral composition of milk. Besides, cows without a dry period have increased milk protein content and glycosylated κ-casein fraction in the last weeks of lactation. The cheese making process is sensitive for differences in casein composition and contents, and differences in mineral contents. Plasmin activity may affect the quality of dairy products during long term storage. Two dairy products that undergo long term storage are ultra-high temperature (UHT) milk, and cheese, in which proteolytic activity plays a major role for texture and flavour formation during ripening. Therefore, UHT milk and cheese are considered as important products with regard to the compositional differences in milk of cows with different dry period length. The current work has focused on late lactation and early lactation milk composition. In practice, bulk milk in the dairy plant contains milk from cows in various lactation stages. In this section, an estimation of the impact of different compositional parameters that are affected by dry period length is done on the processing characteristics of bulk milk.

In modern large-scale dairy processing, milk from large numbers of cows in different lactation stages and from different farms are mixed. This way, the composition of bulk milk is very constant, which is important for sensitive processes such as cheese making. For instance, the proportion of individual proteins is known to influence the cheese yield per amount of milk, and the amount of cheese solids per 100 g milk protein (Wedholm et al., 2006). In the current work, milk composition of individual cows, and of specific moments during lactation, was analysed. In order to be able to estimate the effects of differences in milk composition on cheese and UHT milk processing, a number of assumptions has to be made. Firstly, differences in milk composition are recognized on tanker level in the dairy plant. Milk from an individual tanker can be used in a specific process, based on the composition of the milk. Secondly, it is assumed that all milk comes from farms that do not apply a dry period to all cows. Thirdly, it is assumed that farms use complete randomized calving during the year, so that lactation stage effects can be ignored on bulk level.

Macronutrient contents and yields

The milk yield of cows reduces with progressing lactation, and milk protein content increases (Ostersen et al., 1997). In the current work, milk protein content of cows without a dry period increased up to 6.1% at two weeks before calving (Chapter 2). Milk protein content of cows with a 4 wk dry period was 4.1% at 5 weeks before calving, which only tended (P = 0.06) to be higher than at 10 weeks before calving (Chapter 3). Hence, the main concentration effects seem to take place after 5 weeks before calving. The cheese yield per kg milk is strongly dependent on the casein content in milk (Wedholm et al., 2006). The milk protein content of cows without a dry period was higher compared with cows

with a conventional dry period during the first 20 weeks of lactation (Schlamberger et al., 2010), but no differences were found later in lactation. However, a field study indicated that during the first 305 days in milk (DIM), cows without a dry period had on average a 6.6% higher protein content than cows with a conventional dry period. The total cheese yield per cow depends on the total casein yield over the entire lactation. In contrast to the protein content, the average protein yield until 305 DIM of cows with a 0 d dry period was on average lower (33 kg, 9.4%) compared with cows with a conventional dry period (Steeneveld et al., 2013). This, however, does not include the protein yield of the last 60 days of the lactation of cows without a dry period. Assuming a protein content of 4.2% in 16.9 kg milk/d between 60 and 30 days prepartum, and 6.1% in 9.1 kg milk/d between 30 and 0 days prepartum (Chapter 2), cows with a 0 d dry period yield an additional 38 kg protein during the last 60 days of their lactation. Hence, the protein loss during the first 305 d in lactation of cows without a dry period is compensated by the additional protein yield in the last 60 d before calving. In bulk milk from farms on which no dry period is applied, a high protein content will obtain a higher cheese yield per kg milk due to the increased protein content. In contrast to cheese yield, UHT milk yield is proportional to the milk yield, and not to protein yield. As the protein content in UHT milk is not standardised, it will be higher when no dry period is applied to cows.

In the current work, cows with different dry period lengths had similar fat content in milk, as was found in a systematic review by van Knegsel et al., (2013). Both cheese and UHT milk have a standardized fat content. Therefore, milk fat content does not seem to play a major role in the processability of milk in relation to dry period length. However, a 82 kg lower fat yield in the first 305 d of lactation of cows without a dry period (Steeneveld et al., 2013) is not fully compensated by the additional 40 kg fat in the last 60 d before calving (van Knegsel et al., 2014). A loss in fat yield results in an economic loss for dairy farmers, as milk fat is one of the constituents of milk that determines the milk price for the farmer. All in all, of the macronutrients, the lower fat yield seems to be the major economic concern for farmers when no dry period is applied. On a milk tanker level, milk of cows without a dry period may obtain a higher cheese yield. Since UHT milk, like other liquid dairy products, is not standardised to protein content, it may be disadvantageous to use milk of cows without a dry period for this application.

Plasmin activity and β-casein

Cows without a dry period had higher postpartum plasmin activity, as it changed proportionally with the milk protein content. Additionally, dry period reduction could increase SCC, which may could be related to increased activation of plasminogen into plasmin. In dairy products, plasmin activity may alter product quality due to proteolysis

of caseins during long term storage. Besides, plasmin may affect casein fractions prior to processing. Reduction of casein fractions may result in altered processing properties of milk. Plasmin activity increased proportionally with the casein content in milk when cows did not have a dry period. Therefore it is not expected that dry period omission affects plasmin-induced breakdown of casein in a concentrated casein product such as cheese.

The current work indicated that dry period omission resulted in a reduced β-casein fraction in casein micelles, which in early lactation was related to the blood glucose concentration of the cow and not to plasmin activity. At a proportional change of casein content and plasmin activity, plasmin activity is not expected to affect casein composition. Wedholm et al. (2006) reported no significant influence of the β-casein fraction within the total casein fraction on cheese yield per kg milk. In the current work, however, the reduced β-casein fraction of cows without a dry period was found in total protein instead of total casein, and a lower casein number cannot be excluded. The casein number is one of the major compositional factors in milk affecting cheese yield (Wedholm et al., 2006). However, the difference in protein content on bulk milk level between cows without a dry period or cows with a conventional dry period are higher (6.6%, Steeneveld et al., 2013) than the difference in casein number (-2.6% in early lactation, not significant). It can be concluded that based on casein content, milk of cows without a dry period is suitable for cheese making. However, reductions in casein number are strongly reflected in a reduction of cheese yield per amount of protein (Wedholm et al., 2006). Therefore, it is recommended to do a large scale study about the casein number in bulk milk of cows without a dry period.

Plasmin is thought to be one of the main factors causing instability of UHT milk during long term storage. The potential role of plasmin activity in UHT milk depends on the temperature profile of the UHT treatment. Although plasmin has a certain heat stability, no activity was found after preheating at 95°C for 180 s followed by UHT treatment (direct steam infusion, 150°C, >0.2 s). After preheating at 72°C for 180 s followed by UHT treatment, however, residual plasmin activity was 31% of the plasmin activity in raw milk (Rauh et al., 2014). Hence, the effect of the temperature profile seems to have a larger effect on plasmin activity in UHT milk than differences in plasmin activity in raw milk that can be induced by dry period length.

Glycosylated κ-casein

The current work showed an increase in the glycosylated κ-casein fraction in late lactation milk of cows without a dry period. At 10 days postpartum, cows without a dry period also had a greater glycosylated κ-casein fraction in milk than cows with a conventional dry period. This effect however was not found later in lactation. It was hypothesised that increased glycosylated κ-casein would result in a reduced casein micelle size, as is the case in middle

lactation (Bijl et al., 2014). In house follow-up experiments indicated that casein micelle size and renneting properties remained constant until 4 weeks pre-calving. At 2 weeks pre-calving, however, casein micelle size and renneting properties appeared to vary widely between individual cows. The underlying mechanism of this variation should be subject of further studies. Considering a total lactation yield of 10,633 kg (Schlamberger et al., 2010), and an average yield of 9.1 kg/d during the last 4 weeks of lactation, the contribution of these last 4 weeks is 2.4% of the total yield. Assuming 6.0% of glycosylated κ-casein in the remaining 97.6% of the bulk milk of cows without a dry period, the glycosylated κ-casein fraction in total protein may increase from 6.0 to 6.15% on tank level when none of the cows receives a dry period. In cheese processing, curd firmness increases proportionally with the N-acetyl neuraminic acid content of κ-casein, a measure for the glycosylation of the κ-casein fraction (Robitaille et al., 1993). Therefore, even though the contribution of the glycosylated κ-casein fraction in late lactation is relatively small on bulk level, it may enhance the cheese making properties of milk from cows without a dry period. However, the increase in protein content of at least 6.6% in bulk milk (Steeneveld et al., 2013) is likely to have a much larger contribution than the estimated 2.5% increase in only the glycosylated κ-casein fraction.

Calcium concentration

Milk of cows without a dry period had a lower calcium concentration in early and mid-lactation milk serum compared with cows with a 30 d dry period, which was related to a higher milk protein content and a lower citrate concentration in milk. The calcium concentration in milk serum correlates positively with the concentration of free calcium ions (calcium activity) in mid-lactation milk (Bijl et al., 2013). Calcium activity contributes to the coagulation of casein micelles during renneting of milk. Calcium activity was not analysed in the current work. During cheese making, an excess of calcium chloride is added to milk prior to the renneting process. This added calcium is expected to level out differences in calcium activity of milk from cows with different dry period.

Wrapping up: processing of milk

On bulk level, dry period omission had a major effect on protein content of milk. Other compositional differences, such as the increased glycosylated κ-casein fraction, had a lower impact on bulk level since the effect is specific for one lactation stage. The increased protein content seems to make bulk milk from cows without a dry period suitable for cheese production. The increase in protein content of bulk milk from cows without a dry period has much more impact on the casein content in milk than the relatively small potential reduction in casein number. Indications for a reduced casein number in milk of cows without a dry period should be subject for further investigation however, as it may affect the cheese yield per 100 g protein. The small increase in glycosylated κ-casein fraction in bulk milk may

have a minor positive contribution to the curd formation of milk from cows without a dry period. Plasmin activity was not expected to affect the cheese making properties of milk from cows without a dry period, since plasmin activity is proportional to the casein fraction of milk. Milk from cows without a dry period seems economically less suitable for UHT milk processing. Firstly, dry period omission results in a lower milk yield with an increased milk protein content, which results in a lower amount of product with a higher milk protein content. Secondly, increased plasmin activity may affect stability of UHT milk when no sufficient temperature profile is applied.

7.3 Dry Period Management in Practice

The aim of shortening or omitting the dry period of dairy cows is to improve the health of dairy cows, without loss of milk quality and profitability. The current work contributes to finding an optimal dry period length by providing a detailed overview of the milk composition of cows with a dry period of either 0, 30 or 60 d. One of the questions that has recently been put forward is whether there is an optimal dry period length for all cows, or whether an individual approach is more suitable (van Hoeij et al., 2016).

The current work showed higher plasminogen activation in early lactation milk of multiparous cows with a short dry period. Although not all studies are in line with each other, multiple studies showed that primiparous cows showed a decrease in milk yield as a result of shortening the dry period from 56 to 35 d, whereas milk yield of multiparous cows was not affected by shortening the dry period (Annen et al., 2004, Pezeshki et al., 2007, Klusmeyer et al., 2009). Primiparous and multiparous cows did not only show a difference in milk yield response towards shortening the dry period, but parity also influenced the metabolic status after shortening the dry period. Primiparous cows with a 35 d dry period had lower non-esterified fatty acid (NEFA) concentrations in blood than primiparous cows with a 56 d dry period, which reflects lower mobilisation of body reserves (Pezeshki et al., 2007). This indicates a positive effect of dry period reduction on the metabolic status of primiparous cows, whereas no effect of dry period length on NEFA concentrations was found for multiparous cows. Metabolic problems during early lactation are related to cows with a high body condition, since these cows tend to mobilise more of their own body reserves and consequently end up in a lower negative energy balance (Rukkwamsuk et al., 1999). Hence, for milk production and optimising the metabolic status of cows, a customised dry period seems to be beneficial, taking factors such as parity and body condition into consideration.

From a milk composition perspective, the choice for a customised dry period length for dairy cows is supported by plasmin activity data (Chapter 3) and the proteome of colostrum (Chapter 5). It turned out that parity and the breed (Swedish Red or Swedish Holstein) of cows may affect the composition of milk and colostrum in relation to dry period length.

Besides, herd effects may play a role, since the parity effect on plasmin activity, and the breed effect on the colostrum proteome were both found in the same herd with cows that had a relatively high SCC. This is supported by the finding that in another herd, there was no interaction between dry period length and parity on the β-casein fraction in milk. The current results do not favour a short dry period for multiparous cows, because of the risk of increased plasmin activity in milk after calving. Possibly a herd effect on SCC, which can contribute to activation of plasminogen, interacts with the parity effect on plasmin activity. The outcome that multiparous cows may have higher plasmin activity in milk after a short dry period is contrasting with other milk production characteristics such as the limited drop in milk yield, which are favourable for applying a reduced dry period to multiparous cows (Pezeshki et al., 2007). Proteome results in chapter 5 indicated that Swedish Red cows had higher concentrations of cell proliferation related proteins as a result of shortening the dry period than Swedish Holsteins. It is suggested that this breed effect was related to a different cell proliferation rate of a dual purpose breed (Swedish Red), compared with a milk breed (Swedish Holstein). Shortening the dry period did not result in concentration reduction of any of the low abundant proteins in colostrum, and therefore there seems to be no disadvantage for the cow or its calf. Milk production characteristics did not differ between breeds in response to dry period reduction (Chapter 3), so also for the farmer, Swedish Holsteins and Swedish Reds seem equally suitable for applying a shortened dry period. In conclusion, based on milk composition analyses, multiparous cows from herds with a high average SCC may be less suitable for dry period reduction from a milk quality perspective. The current work showed no other arguments for applying a customised dry period based on milk composition.

Late lactation milk: problem or opportunity?

Milk obtained in the last 4 weeks before calving of cows without a dry period was shown to have a small effect on bulk level, for example by increasing the protein content and the glycosylated κ-casein fraction (Chapter 2). In addition, IgG production is increased prepartum (Guy et al., 1994), which in cows with a dry period results in accumulation of IgG in the mammary gland that will eventually end up in colostrum (Mayasari et al., 2015). Cows with no dry period may secrete some of this IgG prepartum in late lactation milk, which is likely to be the cause of the reduced IgG concentration in their colostrum (Mayasari et al., 2015). Both IgG and glycosylated κ-casein are functional components of milk. The glycosylated macropeptide of κ-casein was reported to have both prebiotic and antimicrobial properties (O'Riordan et al., 2014). The additional IgG that is secreted in prepartum milk may be used by farmers for additional feeding of their calves since the IgG concentration in colostrum of cows without a dry period is lower than in colostrum of cows that did receive a dry period (Mayasari et al., 2015). The increased IgG concentration in prepartum milk may however also be used in products for human consumption for specific functional dairy products. In the

milk processing discussion (section 7.2), only milk processing at large scale was considered. For small scale, on-farm cheese production, a more flexible milk collection may create opportunities for late lactation milk of cows without a dry period. The different protein content and composition of this milk may result in distinct processing properties. Before utilising late lactation milk of cows without a dry period, an in depth analysis of the variability of milk components and the economic feasibility of their application should be done.

7.4 Conclusions

- Shortening or omitting the dry period of cows with good mammary health obtains milk with a higher protein content with little differences in protein composition. The tendency towards a lower casein number in milk of cows without a dry period was the major factor that may influence processing of milk, particularly with regard to cheese yield.
- The casein composition of milk is related to both plasmin activity in milk and the metabolic status of cows. The blood glucose concentration is particularly important in early lactation, whereas the contribution of plasmin activity is greater in mid-lactation when all cows are in positive energy balance.
- Low abundant protein concentrations in colostrum, and plasminogen activation in early lactation milk indicate that a dry period of 4 weeks is not sufficient for all cows to regenerate their mammary epithelium.
- Apart from controlling plasmin activity in milk, milk composition analyses did not obtain further arguments for applying a customised dry period to cows.

7.5 Recommendations

- The current work showed a tendency towards a lower casein number in milk of cows without a dry period. This reductions in casein number may cause a lower cheese yield. Therefore it is recommended to study the effect of dry period omission on casein number in a large scale study, using a method better suited to study this parameter.
- A study on the influence of a 0 d dry period on the colostrum proteome can obtain further insight in the role of mammary involution prepartum and mammary epithelial cell proliferation postpartum.
- Further investigation of compositional changes in the last days before calving for both its functional properties and the insights in preparation for a new lactation.
- The suggested effect of dry period omission on cheese processing should be evaluated in practice. Such a study can be done by using tank milk, to evaluate dilution effects of changes in milk composition that were reported in the current work on bulk level.

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Summary

A dry period of dairy cows is historically seen as a period during which the cow can restore its body condition and regenerate its mammary epithelium in order to be high yielding in the successive lactation. **Chapter 1** of this thesis provides an overview of the current knowledge about dry period length of dairy cows, and the effect of dry period length on milk composition. This thesis aimed at evaluating the influence of dry period length on milk composition and milk quality. Milk composition parameters indicate the processing quality of milk for the dairy plant, and may also reflect the physiological condition and energy status of the cow.

The aim of the first study in this thesis was to evaluate the effect of shortening or omitting the dry period of dairy cows on milk casein composition (**Chapter 2**). For this study, milk samples were collected of 90 cows with either a dry period of 0, 30 or 60 d in early lactation. Milk was sampled 6 and 2 wk prepartum, and 2, 6 and 12 wk postpartum. Milk was analyzed for casein (CN) composition and isoforms of κ-CN. Milk of cows with a 0 d dry period had a higher milk protein content than milk of cows with a 60 d dry period. In milk from cows with a 0 d dry period, the glycosylated κ-CN fraction in late lactation increased from 8% to 12% between 6 and 2 wk prepartum. In early lactation, the β-CN fraction was reduced in milk of cows with a 0 d dry period. A low β-CN fraction was associated with high SCC and greater parity, indicating that it was a result of proteolytic activity. It was concluded that the relation between dry period length and proteolytic activity should be further studied.

Therefore, the influence of shortening the dry period of cows on plasmin activity and casein composition in milk was evaluated (**Chapter 3**). Swedish Holstein and Swedish Red cows, 45 in total, were assigned to a dry period of either 4 or 8 weeks. Milk samples were taken 10 and 5 weeks prepartum, and 6 and 12 weeks postpartum and analyzed for plasmin activity and casein composition. Cows with a 4 weeks dry period had 61% higher plasmin activity in postpartum milk than cows with an 8 weeks dry period. Cows of third or greater parity tended to have a stronger increase in plasmin activity as a result of applying a short dry period than second parity cows. Although the α_{s1}- and β-CN fractions declined with increasing plasmin activity, no dry period effects were found on casein composition. Based on postpartum differences in plasmin activity, it was concluded that particularly multiparous cows require more than 4 weeks between lactations for recovery of the mammary epithelium.

In **Chapter 4**, the influence of applying a dry period of either 0 or 30 days to dairy cows on micellar casein composition, plasmin activity and mineral composition of early and mid-lactation milk was analyzed. Milk samples of 18 cows with a dry period of either 0 or 30 days were collected during early lactation (5-8 wk postpartum) and mid lactation (20-28 wk postpartum). Cows with a 0 d dry period had a higher milk protein content than cows with a 30 d dry period, and tended to have a lower casein number in milk. Milk of cows with a dry

period of 0 d had a lower β-casein fraction in casein micelles. The lower β-casein fraction in milk of cows without a dry period did not seem to be a result of proteolytic activity. Micellar magnesium and phosphorus concentrations were proportional to the casein content. It was concluded that dry period omission resulted in an increase of protein content that was proportional to micellar mineral concentrations and plasmin activity in milk. Therefore, dry period omission did not affect casein micelle composition, apart from a small reduction of the β-casein fraction.

Whereas milk composition is important in the dairy plant, the composition of colostrum can provide new insights in the status of the mammary gland around parturition. The aim of the fourth study in this thesis was to evaluate the influence of applying a 4 wk instead of an 8 wk dry period to dairy cows on the proteome of colostrum (first sample) and transition milk (the fifth postpartum milk sample) (**Chapter 5**). From 12 Swedish Holstein (SH) and 12 Swedish Red (SR) cows, the proteome of individual serum samples of colostrum and transition milk were analyzed by liquid chromatography tandem mass spectrometry. Shortening the dry period resulted in upregulation of 18 proteins in colostrum and transition milk of SR, whereas statistically no differences were found for SH colostrum and transition milk. Upregulated proteins in colostrum seemed to reflect increased mammary epithelial cell proliferation in the periparturient period when a 4 wk dry period was applied. The proteome data indicate that a dry period of 4 wk to SR cows may not be sufficient for complete regeneration of the mammary epithelium.

As there is large variation in energy balance between cows during early lactation, possibly induced by different dry period lengths, the relation between blood composition and milk composition in early lactation was studied (**Chapter 6)**. Blood glucose and insulin concentrations were compared with milk macronutrient and milk protein composition, and metabolite concentrations in milk. Greater glucose concentrations in blood were related to a decline in lactose yields relative to milk protein yields. Glucose derived metabolites such as UDP-hexoses and sugars, and metabolites involved in the Krebs cycle correlated positively with the β-lactoglobulin fraction and negatively with α_{s1}- and β-CN fractions in milk. These correlations indicated a relation between the metabolic status of the cow and milk protein composition.

In the general discussion (**Chapter 7)** an integrative view of the outcomes of the different studies is provided. The casein composition of milk is related to both plasmin activity in milk and the metabolic status of cows. The tendency towards a lower casein number in milk of cows without a dry period may influence the processing quality of milk, particularly with regard to cheese yield. It is recommended to study the effect of dry period omission on casein number in a large scale study, using a method better suited to study this parameter. It was concluded that shortening or omitting the dry period of cows with good mammary health obtains milk with a higher protein content with little differences in protein composition.

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Tot slot wil ik mijn familie bedanken, in het bijzonder mama en Peter, die onvoorwaardelijk klaar staan. Wat zou het mooi zijn geweest als we het einde van mijn PhD-tijd met zijn allen hadden kunnen vieren.

Curriculum vitae

Ruben de Vries was born on the 24th of August 1988 in Almelo, the Netherlands. He obtained his high school diploma at the Reggesteyn college in Nijverdal in 2007. In the same year, he started his bachelor in food technology at Wageningen university, which was finished with the project *in vitro* fermentation of human and caprine oligosaccharides. In 2010, he started his master food technology, with specialisation dairy science and technology at Wageningen university. He finalised his study with a graduation project on milk composition in relation to casein micelle size in milk of individual cows at the department of Product Design and Quality Management of Wageningen university. This was followed by an internship at the Food Structuring group of Royal FrieslandCampina corporate research (Deventer, the Netherlands). After obtaining his MSc degree in 2012, he started a double degree PhD research project at the Swedish University of Agricultural Sciences, Uppsala, Sweden, and Wageningen university. Outcomes of this PhD project were merged in this thesis, entitled: 'Dry period length of dairy cows – milk composition and quality'.

List of publications

Peer-reviewed papers

- de Vries, R., M. Brandt, A. Lundh, K. Holtenius, K. Hettinga, and M. Johansson. 2016. **Short communication: Influence of shortening the dry period of Swedish dairy cows on plasmin activity in milk.** J Dairy Sci 99(11):9300-9306.
- de Vries, R., A. van Knegsel, M. Johansson, H. Lindmark-Månsson, T. van Hooijdonk, K. Holtenius, and K. Hettinga. 2015**. Influence of shortening or omitting the dry period of Holstein-Friesian cows on casein composition of milk**. J. Dairy Sci. 98(12):8678-8687.
- Bijl, E., R. de Vries, H. van Valenberg, T. Huppertz, and T. van Hooijdonk. 2014**. Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of κ-casein.** Int. Dairy J. 34(1):135-141.
- Johansson M., Å. Lundh, R. de Vries, and K. Svennersten Sjaunja. **Composition and enzymatic activity in bulk milk from dairy farms with conventional or robotic milking systems.** Submitted for publication.
- de Vries, R., S. Boeren, K. Holtenius, J Vervoort, H Lindmark-Månsson, K Hettinga **Influence of dry period length of Swedish dairy cows on the proteome of colostrum.** Submitted for publication.
- de Vries, R., A. van Knegsel, K. Hettinga, E. Antunes-Fernandes. **Blood glucose concentration relates to milk composition of dairy cows in early lactation.** Submitted for publication.

Conference abstract

- de Vries, R., A. van Knegsel, M. Johansson, H. Lindmark-Månsson, T. van Hooijdonk, K. Holtenius, and K. Hettinga. 2015**. Effect of shortening or omitting the dry period of Holstein-Friesian cows on casein composition of milk**. 9th NIZO dairy conference, September 30 – October 2, Papendal, the Netherlands.

Overview of training and education activities

Discipline specific activities	Country	Year
International conferences		
15th International Congress on Production Diseases (ICPD), Uppsala, Sweden		2013
9th NIZO dairy conference, Papendal, the Netherlands		2015
Courses and workshops		
Milk protein analyses, Milk Genomics Initiative	NL	2012
Advanced proteomics, VLAG, Biochemistry	NL	2013
Biomarkers in Dairy Science, Estonian University if Life Sciences	EE	2013
Construction of trial protocols for controlled clinical trials, TCM	SE	2015
Food Stability, NOVA, University of Copenhagen	DK	2015
General courses		
Scientific Writing in Food Science, Food in Focus	SE	2013
Applied Statistics, VLAG	NL	2014
SAS Programming, Institute for Animal Genetics	SE	2014
Project and time management, WGS	NL	2015
Organising and supervising thesis projects, WGS	NL	2015
Systematic approaches to reviewing literature, WGS	NL	2015
Ethics and Philosophy in Food Science, VLAG	NL	2016
Essentials in Scientific writing and presenting, WGS	NL	2016
PhD carrousel workshops, WGS	NL	2013
Optionals		
Preparation of PhD research proposal		2012
PhD study trip Thailand, Singapore		2014
Organisation committee PhD study trip		2014
Teaching and didactic training		
Supervised 7 MSc students and 2 BSc students		
Teaching assistant in the course 'Dairy Technology'		2013, 2014
Teaching assistant in the course 'Advanced Fermentation Science'		2014, 2016

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