UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXV

S.S.D. AGR/19

Understanding the Dry-Off in Dairy Cows: Insights into Metabolism and Inflammation

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

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Matriculation n: 4915301

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Abstract

The dry-off is recently becoming a more and more important phase of the lactation cycle. It represents a potentially stressful event for dairy cows because it includes several changes in animal management and physiology. Inflammatory events in this phase seem to have a carryover effect on the ensuing lactation. Moreover, the high milk yield still achieved by modern cows in late gestation can affect the adaptation to the non-lactating period and might impair future lactation performance and health. Furthermore, the public demand to reduce antibiotic use in livestock and the spread of selective dry-cow therapy raise additional concerns about this phase.

In the present thesis, the effect of pre-existing systemic inflammatory conditions at dry-off on the adaptation to the subsequent periparturient period has been investigated. Then, a selective dry-cow therapy approach was evaluated, particularly focusing on the immunometabolic responses and the implications on the ensuing early lactation. Moreover, to optimize this strategy, the supplementation of a nutraceutical (*Aloe arborescens* Mill.) has been tested with a similar approach. Furthermore, circulating leukocyte gene expression was analyzed at the turn of the dry-off, to understand what happens at a molecular level in this phase. In the same study, cows with different milk production in late lactation were compared, confirming the detrimental effect of high milk yield on the inflammatory response. Several strategies have been proposed over the years to gradually reduce milk yield before dry-off, promoting at the same time the beginning of the mammary involution process. Thus, we evaluated the effects of a feed restriction strategy on performance and metabolism.

This work provides a comprehensive overview of the dry-off, analyzes the effects of the selective dry-cow therapy, the high milk yield in this phase, and possible nutritional strategies implementable to optimize this critical phase in an integrated way.

Keywords: metabolism; inflammation; dry period; mammary gland involution; transition period

Chapter 1: The Challenge of Drying-off High-Yielding Dairy Cows

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1.1 Abstract

Along the lactation cycle of dairy cows, the dry-off is recently acquiring ever more consideration. This practice is necessary for the remodeling of mammary gland, but represents a stressful event, incorporating several changes in daily routine, diet, and metabolism. Moreover, the increasing milk yield still achieved by modern cows in late gestation exacerbates the need for relevant adaptations in the days immediately following the dry-off and might jeopardize the success of the dry period, with potential negative effects on the future lactation. Productions over 15 kg/d are an additional risk factor for udder health, delay mammary involution, and worsen metabolic stress and inflammatory response. Furthermore, the urge to reduce antibiotic use in livestock raises further threats and concerns about this phase.

Several strategies have been proposed over the years to cope with this challenge, aiming to gradually reduce milk yield before dry-off, promoting at the same time the start of mammary involution. Among them, the most common are based on a feed or nutrient restriction, a decrease in milking frequency, or administration of prolactin inhibitors. These practices have different abilities to reduce milk yield through different mechanisms and entail several implications on udder health, animal welfare, behavior, endocrine status, metabolism, and inflammatory conditions.

The present review aims to provide a comprehensive overview of the dry-off in high-yielding cows, the effects of the high milk yield in this phase, and the possible strategies implementable by farmers and veterinarians to optimize this critical phase in an integrated way.

Keywords*:* metabolism, welfare, intramammary infection, mastitis

1.2 Introduction

The most widely investigated phase of the lactation cycle in dairy cows is the transition period, because it is usually considered the most critical one (Drackley, 1999). However, recent advances anticipated the origins of cow's adaptation to calving as well as the onset of early lactation diseases, resulting in an extension to a wider time frame of the classical transition period, even before the dry-off (Trevisi and Minuti, 2018; Mezzetti et al., 2021).

The dry-off consists of the cessation of milking at the end of lactation to prepare the cow's metabolism and the udder for the maximization of milk production in the following lactation. The dry period, which is the nonlactating period between dry-off and calving, represents an important phase of the cow's production cycle, with relevant implications for cows' health and physiology, animal welfare, and sustainability of dairy production. During this period, cows complete the restore of the body condition and renew mammary epithelial cells (Capuco et al., 1997). The mammary gland undergoes a process of active involution, where, through a series of coordinated changes in the

mammary gland morphology, the integrity of tight junctions, and the composition of mammary secretions, returns to a nonlactating state (Zhao et al., 2019).

Traditionally, cows were dried off on a scheduled day, about 6-8 weeks before expected calving, through abrupt cessation of milking and a long-acting antibiotic treatment. In recent years, milk yield per cow significantly increased, and is becoming more and more common to find cows with still high production in late gestation. Therefore, the transition from lactation to a nonlactating state is even more challenging. Moreover, antimicrobial resistance has become a relevant concern for animals but also for possible implications for human health. Consequently, blanket dry cow therapy (DCT) has been questioned. This antibiotic treatment is used to cure existing intramammary infections (IMI) and prevent the development of new ones during the dry period, which is, in particular at its beginning and end, one of the times at highest risk (Bradley and Green, 2004). Therefore, selective DCT, consisting of treating with antibiotics only subjects at higher risk, is becoming widespread around the world with favorable results in antibiotic use reduction without major side effects in terms of SCC increase and mastitis incidence in the subsequent lactation (Kabera et al., 2021; Krattley-Roodenburg et al., 2021). Nevertheless, drying-off cows still producing a huge amount of milk without the support of antibiotic action might represent a threat.

In order to cope with this multifaceted challenge, several strategies were proposed, and literature about milk cessation methods, selective DCT, and their implications on animal physiology, metabolism, health, and future lactations is rapidly growing. However, available literature should be re-evaluated to harmonize these findings and provide an integrated dry-off approach. Therefore, this literature review aims to summarize the key aspects regarding the dry-off and the recent findings on the strategies to optimize the dry-off phase with a perspective on the outcomes in the subsequent lactation.

1.3 The Physiology of The Dry-Off

The dry-off is a stressful event for dairy cows. Suddenly, milking is stopped (often abruptly), and diet and group are changed. Therefore, cows implement a series of physiological mechanisms to adapt to the new conditions. The most important one is the active involution of the mammary gland, which starts within 2 days after dry-off and ends in about 3 weeks (Hurley, 1989). Moreover, the energy density of the diet is reduced, increasing forage inclusion in the ration, to foster the regression of galactopoietic activity and match the lower requirements of the dry period. In this phase, the fetus becomes the priority over the mammary gland (Dingwell et al., 2001). At the same time, rumen papillae decrease in length and size to adapt to the more fibrous and less digestible diet (Dieho et al., 2016). The regrouping and the need to establish a new social structure could add psychological stress,

particularly in weaker and subordinate animals (von Keyserlingk et al., 2008). Common markers of stress, such as blood cortisol and fecal glucocorticoids increase after dry-off (Bertulat et al., 2013; Putman et al., 2018) and this condition can have different duration in accordance with the ability to adapt to the previous changes.

The withdrawal of milk removal leads to the accumulation of milk in the udder, a consequent engorgement of cisternal ducts and alveoli, and an increase in intramammary pressure, which triggers the involution process (Wilde et al., 1997). The prolactin synthesis stops when cows are no longer milked, promoting apoptosis of mammary epithelial cells. Besides, milk residuals and hormonal assets can take part in the process of inhibiting milk synthesis, with β-casein and serotonin being two main candidates for this role (Collier et al., 2012; Shoshani and van Straten, 2022). The immune system is overwhelmed in this phase by the variety of functions needed. Mammary epithelium tight junction permeability increases, resulting in an exchange of components between milk and interstitial fluid (Stelwagen et al., 1994). Mammary secretion composition changes, with higher levels of SCC and components of blood origin (e.g. lactoferrin, immunoglobulins, albumin), altered ion concentration, and decreased citrate (Zhao et al., 2019). Conversely, substances of milk origin, such as lactose, are increased in the bloodstream. Thus, variations in these parameters can be used as markers of involution. In the first stages of involution, epithelial cells undergo programmed cell death and neutrophils start to infiltrate the gland (Atabai et al., 2007). The clearance of apoptotic cells and milk fat globules is mainly performed by viable epithelial cells. Later, epithelial cell apoptosis increases, the alveolar luminal structure collapses, and macrophages and lymphocytes infiltrate the gland to phagocytize death cells and milk debris. Therefore, the immune system is likely engulfed by the removal of milk components and apoptotic cells and is less active in preventing bacterial infections and mastitis. High pressure and udder engorgement impair the teat canal closure process and the formation of the protective keratin plug on the teat sphincter, increasing the risk of milk leakage (Dingwell et al., 2001). Since cows suffering from milk leakage at dry-off have an increased probability of pathogen entrance (Dingwell et al., 2004), the latter becomes a risk factor for the development of new IMI in the first month of lactation (De Prado-Taranilla et al., 2020). After completion of involution, the combination of morphological, physiological, and immune modifications drastically reduces the risk of IMI (Bradley and Green, 2004). In particular, the antimicrobial activity of lactoferrin, immunoglobulins, leukocytes, and milk pH contribute to creating a hostile environment for bacterial growth.

The effects of the several changes and the involution process can be observed in blood (Putman et al., 2018; Mezzetti et al., 2020). Following dry-off, metabolic adaptations are required. Plasma NEFA increased after dry-off due to the drop in energy and nutrient intake, and urea

concentration decreased as a result of the changes in requirements, intake, and rumen fermentation. The effects on glucose and BHB are inconsistent, likely depending on milk yield at dry-off and the magnitude of diet change. At the same time, liver enzymes (GGT and bilirubin), positive acute-phase proteins (ceruloplasmin and serum amyloid A), and nitrogen species (nitrate, nitrite, and nitric oxide) levels increased, whereas negative acute-phase proteins (cholesterol and retinol) and antioxidant species (FRAP, thiol groups, tocopherol, and β-carotene) levels concentrations decreased (Mezzetti et al., 2020). These alterations are consequences of the absorption of molecules (mainly form milk residuals) that trigger an inflammatory and immune response. Leukocytes count in blood decreases after dry-off, due to the migration toward the mammary gland (Sordillo and Nickerson, 1988; Atabai et al., 2007) and their activation could account for the release of pro-inflammatory stimuli (i.e. cytokines) into the blood and the consequent inflammatory response taking place in this phase. The depletion of the antioxidant systems is likely related to the inflammatory response and the intense degradation of milk residuals in the mammary gland immediately after the milking cessation. This inflammatory response is usually mild, if there are not ongoing intramammary infections, and can be observed in the increase in haptoglobin and serum amyloid A levels, which can be related to the process of tissue remodeling (Odensten et al., 2007; Dancy et al., 2019). Moreover, blood calcium greatly increases, as a consequence of either reduced milk demand or milk accumulation in the mammary gland, which compromises mammary tight junctions integrity, increasing paracellular transport of calcium into the blood (Putman et al., 2018; Mezzetti et al., 2020).

At the animal level, other measurements can be assessed to evaluate the stress caused by dry-off. In particular, behavioral records can highlight the impact of dry-off on animal welfare. Lying represents a priority for dairy cows and a decrease in the time dedicated to it might suggest a welfare impairment. A consistent decrease in lying time was observed after dry-off (Zobel et al., 2013), especially in primiparous cows (Chapinal et al., 2014; Rajala-Schultz et al., 2018), suggesting that milking cessation and the consequent milk accumulation into the udder cause discomfort and limit the willingness to lay down. Similarly, although the magnitude of the drop is lower than at calving, the decrease in rumination time observed for up to 4 d after dry-off can be interpreted as a proxy of stress (Abuelo et al., 2021), since rumination time after dry-off, in contrast, would be expected to rise as a consequence of the higher fiber content of the diet.

1.4 The Importance of the Dry Period and Its Optimal Length

Considering all the potential concerns and difficulties of dry-off and subsequent dry period, one possible solution is to omit or shorten it. The main aim of this strategy is to improve energy

balance in early lactation shifting milk production from the post-calving period to the last weeks of gestation (van Knegsel et al., 2013). Additionally, cows have to face fewer diet and group changes.

Andersen et al. (2005) compared a 7-week dry period with continuous milking through late gestation in cows with peak milk yield greater than 45 kg/d. These authors reported a consistent decrease in milk production in the following early lactation, but better metabolic balance in cows which skipped the dry period. Moreover, no dry period led to reduced antibody concentration in colostrum (Mayasari et al., 2015). On the other hand, shortening the dry period (up to about 30 days) can potentially balance the side effects of the different dry period strategies. Compared to a 6-8 weeks nonlactating period, a short dry period resulted in slight milk loss, but improved milk protein and energy balance (van Knegsel et al., 2013). A short dry period also seems to improve peripartum energy balance and ruminal adaptation compared with a 60 days dry period (Jolicoeur et al., 2014). Moreover, cows with a short dry period (28 d) experienced fewer metabolic changes than cows with a dry period of 90 d (Weber et al., 2015). Various hypotheses have been proposed to explain the milk loss caused by shortening dry period length (Pezeshki et al., 2010) but the most likely is related to the effects of dry period on cell turnover and replacement of senescent mammary epithelial cells (Capuco et al., 1997). This process is completed in 25 days, which hence represents the minimum length requirement of dry period. However, short or omitted dry period can also influence udder health. Shortening the dry period did not seem to affect udder health, without negative effects on the odds of mastitis (van Knegsel et al., 2013). However, most of the trials available in the literature were carried out using blanket DCT, which could have affected the results (Kok et al., 2019). Cows selectively treated at dry-off might benefit from a longer dry period. In fact, the dry period is an important phase for curing intramammary infections (IMI), even subclinical (i.e. low level of SCC but presence of some strains of microbes) because, alongside the antibiotic effect, there is the physiological clearing of many bacteria during the mid-dry period.

1.5 The Impact of High Milk Yield at Dry-Off

The continuous improvements in management, nutrition, and genetics allowed for greatly improve milk yield and persistency of lactation. However, one side effect of these advances is the level of production at dry-off (increased 2-3 times in the last 4-5 decades), which makes challenging the transition to the dry period. The need to reduce milk production before stopping the milking routine was already felt 70 years ago. In those times, gradual dry-off was performed to reduce the risk of mastitis caused by high intramammary pressure (Oliver et al., 1956), but, with the spread of antibiotic therapy at dry-off, it was gradually replaced by abrupt dry-off. However, milk yields at dry-off were below 10 kg/d (Natzke et al., 1975). Around 20 years ago, (Dingwell et al., 2001) brought

back the question and, nowadays, this topic is more and more discussed. Production at dry-off can easily exceed 25 kg/d and selective DCT application is steadily increasing. The actual effect of drying-off without the antibiotic therapy on a cow producing a huge amount of milk is unknown but this practice is typically avoided to preserve animal health and welfare. No study investigated the effects of high milk yield at dry-off in cows not receiving antibiotic DCT. However, in a survey conducted a few years ago in the Netherlands, high milk yield at dry-off was one of the main reasons provided by farmers for using antibiotic DCT in cows with low SCC (Krattley-Roodenburg et al., 2021).

High milk yield at dry-off leads to increased milk accumulation in the udder in the subsequent days, which, in turn, causes augmented intramammary pressure and enhances the risk of milk leakage (Dingwell et al., 2004). These factors together delay the formation of the keratin plug, which acts as a protective layer against pathogen entrance into the teat canal. Therefore, high milk yield at dry-off represents a significant risk factor for developing new IMI during the dry period and Rajala-Schultz et al. (2005) reported an increased risk of new IMI at calving for production over 12.5 kg/d. Moreover, mammary involution seems to be slower and more stressful in high-yielding cows. After dry-off, they have higher intramammary pressure and milk leakage, as well as a greater increase in fecal glucocorticoid concentration, interpreted as an indirect measure of stress (Bertulat et al., 2013). Cows producing 25-35 kg/d at dry-off showed signs of distress, increased vocalizations, delayed involution, and milk neutrophilia, whereas cows producing less than 14 kg/d had a smoother transition to dry period (Silanikove et al., 2013). In another study, cows with milk yield greater than 15 kg/d during the week preceding dry-off have more marked severe metabolic changes and an increased and longer inflammatory response around dry-off compared with low-producing subjects (Mezzetti et al., 2020).

The huge amount of milk accumulated into the udder after milking cessation can also provoke pain or discomfort associated with the engorged udder, as suggested by the reduced lying time observed in cows with high milk yield at dry-off (Chapinal et al., 2014; Rajala-Schultz et al., 2018). Moreover, they had shorter lying bouts and daily lying time after dry-off. The sacrifice of a high-priority activity suggests a detrimental effect of abrupt dry-off on the welfare of high-yielding cows. Therefore, strategies to reduce milk yield at dry-off and its negative effects are needed.

1.6 Approaching the Dry-Off: How to Reduce Milk Production?

Abrupt cessation of milking is a practical solution in commercial farms but may represent a threat to udder health and welfare in cows still producing a generous amount of milk (Zobel et al., 2015; Vilar and Rajala-Schultz, 2020). Thus, to ensure cow health a target of about 15 kg/d has been suggested (Vilar and Rajala-Schultz, 2020). Therefore, different drying-off practices have been

proposed to gradually reduce milk yield in the period closest to dry-off to improve cows' comfort, welfare, and health. These methods mainly include gradual milk cessation, diet changes, or a combination of both. The main effects of these approaches are summarized in Table 1.1. The greatest challenge is to develop a gradual cessation method able to reduce milk production at safe levels without affecting cows' metabolism, and immune system welfare (Zobel et al., 2015). Gradual dry-off strategies are common in countries where selective DCT is mandatory (Vilar et al., 2018; Krattley-Roodenburg et al., 2021) but not where blanket DCT is prevalent (Bertulat et al., 2015), indicating the need for systems to reduce milk yield when antibiotic use has to be limited.

Table 1.1. Effects of different methods to reduce milk yield before dry off on dry matter intake (DMI), behavior, milk composition, blood hormones, and metabolites in high-yielding dairy cows

Strategy	Effect	Reference
Milking	↑ Lying time	(Rajala-Schultz et al., 2018)
	↓ Udder engorgement	(Larsen et al., 2021)
	↑ Haptoglobin	(Martin et al., 2020)
Feeding	↓ DMI	(Ollier et al., 2014, 2015; Larsen et al., 2021; Jermann et al., 2022)
	↑ Rumination time	(Franchi et al., 2022b)
	↓ Udder engorgement	(Larsen et al., 2021)
	↑ SCC, albumin, lactoferrin	(Ollier et al., 2014)
	↑ Citrate, Na^+; ↓ K^+	(Ollier et al., 2014; Jermann et al., 2022)
	↑ Milk fat	(Jermann et al., 2022)
	↓ Insulin, prolactin, glucagon; ↓ IGF-1	(Ollier et al., 2014, 2015; Jermann et al., 2022)
	↑ NEFA, BHB, urea; ↓ Glucose, amino acids	(Ollier et al., 2014, 2015; Jermann et al., 2022)
Milk×Feed	↓ DMI	(Dancy et al., 2019)
	↑ Rumination time	(Dancy et al., 2019)
	↓ Udder engorgement, milk leakage	(Zobel et al., 2013; Larsen et al., 2021)
	↑ Milk fat, protein, lactose and SCC	(Odensten et al., 2005; Dancy et al., 2019)
	↓ Insulin; ↑ Cortisol	(Odensten et al., 2005, 2007)
	↑ Glucose, NEFA; ↓ Urea, BHB	(Odensten et al., 2005; Dancy et al., 2019)
	↑ Serum amyloid A, haptoglobin	(Odensten et al., 2005, 2007)
	↓ Heart rate	(Odensten et al., 2007)
	Altered rumen VFA	(Odensten et al., 2005)
Prolactin inhibitors	↓ DMI	(Ollier et al., 2013, 2014, 2015; Larsen et al., 2021)
	↓ Rumination time	(Franchi et al., 2022b)
	↓ Udder engorgement	(Larsen et al., 2021)
	↑ SCC, albumin, lactoferrin	(Ollier et al., 2013, 2014)
	↓ Bacterial count	(Ollier et al., 2015)
	↓ Prolactin	(Ollier et al., 2013, 2014, 2015)
	↑ Glucose, BHB	(Ollier et al., 2014, 2015)

In selective DCT, the use of teat sealant is recommended. The function of teat sealant is to close the teat canal immediately after dry-off, preventing the entrance of bacteria. Its use at dry-off has a protective effect on the development of IMI, both alone and in combination with antibiotic treatment (McParland et al., 2019). In high-yielding cows, teat sealants are even more beneficial. These animals have high intramammary pressure, delayed formation of the keratin plug, increased probability of milk leakage, and, thus, more favorable conditions for the development of IMI.

Milking can be stopped abruptly or gradually. Gradual milk cessation can be performed by reduced milking frequency or intermittent milking. The first strategy consists of switching from the usual 3x/2x milkings/d schedule to 1x around a week before dry-off or can also be applied through intermittent milking (e.g., 1x milking/d on d 1, 2, 3, and 5 and then dry-off at d 5). Through this strategy is possible to achieve up to a 40% reduction in milk yield (Fig. 1.1). The reduction in milking frequency inhibits milk synthesis and promotes apoptosis by local stimuli, due to prolonged milk accumulation in the udder between milkings (Wilde et al., 1997). The main concern about this practice is the increase in intramammary pressure which can affect cow's comfort and result in milk leakage. The latter is a risk factor for IMI after dry-off. However, Gott et al. (2016) did not observe negative effects on milk leakage, and Rajala-Schultz et al. (2018) did not notice any change in lying behavior before and after dry-off in cows milked 1x compared with 3x (with 1x cows passing through the parlor despite not being milked and thus matching time standing for milking). Regarding udder health at calving, no differences were observed between milk cessation methods in SCC and IMI (Gott et al., 2017), but, although 1x milking was beneficial for primiparous cows, multiparous cows had increased odds of IMI at calving with gradual milking cessation compared with abrupt dry-off (Gott et al., 2016). It is important to consider that, during the week leading to dry-off, milk is still actively produced and might exacerbate the engorgement, resulting also in tissue damage and pain (Bertulat et al., 2013). Moreover, cows, in particular if high-producing, have a strong motivation to be milked, and suddenly changing their routine could have negative implications on welfare (Zobel et al., 2013). In automatic milking systems, it is also possible to tailor the process to each cow, setting different protocols of milking paired or not with restrictions in concentrate allowance and allowing an actual gradual milking cessation (Martin et al., 2020; France et al., 2022).

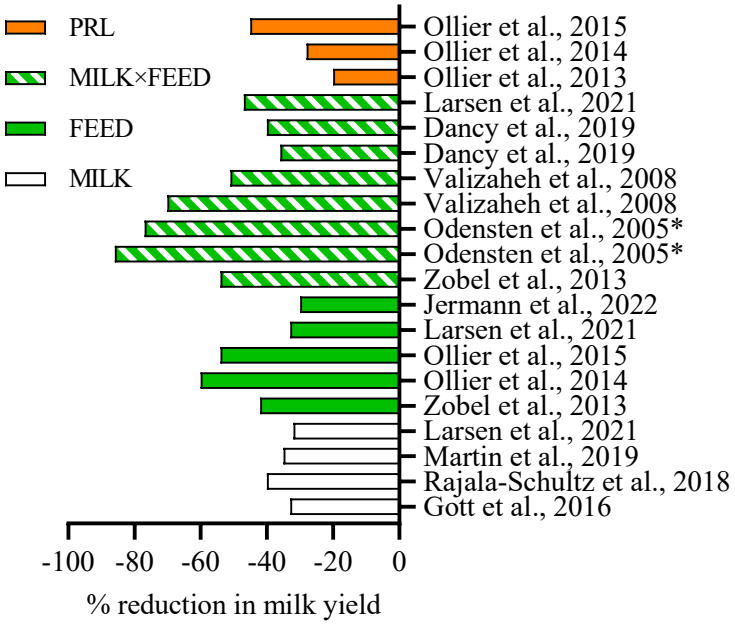

Figure 1.1. Decrease in milk yield (%) achieved from the beginning of the dry-off procedure to the day before the last milking with different strategies (MILK = reduction in milking frequency; FEED = feed or nutrient restriction; MILK×FEED = reduction in milking frequency paired with changes in feeding; PRL = one or two injections/day of prolactin inhibitor, before dry off) in studies available in the literature involving dairy cows with milk yield higher than 15 kg/d at the beginning of treatments.
* Reduction achieved at the last milking after a day of no milking.

Several dietary strategies with diversified intensities have been tested in recent years. Feed restriction can be qualitative (e.g. reducing concentrates inclusion in the diet, diluting the lactation TMR with straw, or feeding forages only) or quantitative (limiting the intake allowance; Leduc et al., 2021). Milk reduction achievable depends on the severity of the restriction, varying from 40 to 60% (Fig. 1.1). The reduced amount of energy (and even amino acids) available from the diet can provoke a plunge in yields in just a few days. Marked feed restriction can cause also deep hormonal changes, with a decrease in circulating insulin, IGF-1, leptin, and prolactin, and an increase in GH, progesterone, and cortisol concentrations, promoting a catabolic state and redirecting nutrients to vital organs (Leduc et al., 2021), similarly to what happens in early lactation. Moreover, the changes in IGF-1 and prolactin, together with the decreased mammary blood flow and nutrient uptake (Guinard-Flament et al., 2007), can be suggestive of an anticipated mammary involution, as supported by the changes noted in milk and mammary secretion composition (Ollier et al., 2014). However, if the restriction is too severe, cows show signs of hunger, increasing vocalizations (Franchi et al., 2019), in particular when only low-quality hay is provided (Valizaheh et al., 2008), which suggests an impairment of welfare. Besides, cows that underwent a more marked restriction at dry-off had increased plasma cortisol, the primary marker of stress (Odensten et al., 2007). Moreover, the sudden

decrease in energy availability depress glucose availability, the main energy source of activated immune cells (Kvidera et al., 2017), and increases blood concentrations of BHB and NEFA (Ollier et al., 2014, 2015; Jermann et al., 2022), which are known to have an inhibiting effect on leukocytes (Ingvartsen and Moyes, 2013; Minuti et al., 2020). In fact, PBMC proliferation and IL-4 production were reduced in cows fed only hay before dry-off (Ollier et al., 2014). An active and efficient immune system is pivotal in this phase, to promote mammary involution (Zhao et al., 2019) and prevent new IMI.

Long-term effects on productivity in the ensuing lactation are still unclear. Gott et al. (2017) reported no association between milk yield and SCC in the subsequent lactation and milk cessation method, and Herve et al. (2019) did not observe any carryover effect on milk production at refeeding in mid-lactation cows that were feed restricted, despite an elevated rate of mammary epithelial cells exfoliation. Different stresses in early dry period (e.g. heat stress) impair milk production in the subsequent lactation, as a result of altered mammary involution process (Fabris et al., 2019). Moreover, the effects of a certain degree of undernutrition in this stage of gestation on the offspring are unclear. Stressors during the early dry period are known to affect offspring's performance. Early dam's transient undernutrition can impair the ovarian and cardiovascular systems of the offspring, despite no effects on birth weight and growth (Mossa et al., 2013). In beef cattle, late gestation nutrient restriction reduced offspring birth weight and compromised glucose regulation in early life (Maresca et al., 2018; López Valiente et al., 2022). However, there is a lack of information on the effects of the dam's transient nutrient restriction in dairy newborn calves, both directly (through in utero effect) and indirectly (through colostrum). Thus, severe feed restriction in this phase can be a double-edged sword, with possible positive short-term effects on involution and udder engorgement but unknown long-term effects.

The combination of milking and feeding restriction showed the best results, merging the effects of a moderate feed restriction and the reduction of milking frequency (Tucker et al., 2009; Larsen et al., 2021; France et al., 2022). These two strategies are addictive, but they effectively reduce yield with different feedback mechanisms (Guinard-Flament et al., 2007). With feed restriction, milk yield decreases because of reduced arterial glucose provision to the mammary gland due to a combination of reduced plasma glucose, arterial mammary blood flow, and mammary glucose uptake. Cows milked 1x with a moderate feed restriction during the week preceding dry-off had a 10 kg (~ 40%) decrease in milk yield, paired with higher milk fat, protein, and SCC, and increased daily rumination time compared with the pre-drying period (Dancy et al., 2019). Interestingly, in this period they also had higher plasma glucose and lower BHB. Similar results were observed by (Odensten et al., 2005). In their study, cows were milked 1x and fed either only straw or silage. During the dry-off

period, both groups had higher milk fat and protein concentrations, lower milk lactose and plasma BHB before dry-off compared with the preceding days. Moreover, straw-fed cows had an increase in NEFA concentration, whereas silage-fed cows had only a moderate increase in NEFA and an increase in glycemia. These results suggested that reducing milking frequency decreased glucose mammary uptake, which was not compensated by a mild decrease in dietary energy, thus limiting the negative effects of undernutrition (e.g. BHB concentration). Other effects reported in Table 1.1, such as the changes in feeding behavior, hormonal status, and rumen fermentation, are likely more related to the feed restriction.

To summarize, the feeding way might be more effective in terms of yield reduction but both methods have their side effects. With the feeding way, yield is lowered by a decrease in nutrient availability to the mammary gland rather than by a mechanism that involves increasing udder pressure and reducing mammary glucose uptake. However, restricting feeding increases feeding motivation and vocalizations, which are signs of hunger (Franchi et al., 2019, 2021), even though improved udder-related response, indicating reduced odds of experiencing pain and/or discomfort (Franchi et al., 2022a). On the other hand, milking 1x can cause greater milk leakage before dry-off compared with cows milked 2x and can impair cow's comfort. Nevertheless, if the feeding level is reduced together with milking, no differences were observed in udder engorgement and milk leakage after dry-off (Larsen et al., 2021), alongside the benefits on energy balance.

Other possible ways are treatments targeted to inhibit prolactin secretion, promote involution, or depress feed intake. Treatments with acidogenic boluses, aimed at reducing intake (Maynou et al., 2018), or metalloproteinase-9, which should promote involution (Parés et al., 2021), did not show the ability to effectively reduce milk yield, whereas prolactin inhibitors and casein hydrolysate infusions promoted mammary involution both if infused before or at dry-off. Casein hydrolysate is a local regulator of mammary gland function and provokes the loss of tight junction integrity. Its infusion into the udder causes changes in mammary secretions similar to those caused by involution (increased levels of Na^+ and K^+, immunoglobulins, and lactoferrin), promoted a local inflammatory response, had positive effects on IMI prevention and subclinical mastitis cure during the dry period and on milk production in the subsequent lactation (Shamay et al., 2003; Shoshani and van Straten, 2022). Prolactin is the hormone that promotes lactation, and its inhibition depresses milk synthesis. Daily single or double infusions of dopamine agonists (quinagolide or cabergoline), performed for a few days around dry-off, decreased prolactin and milk synthesis and likely promoted involution, as suggested by the changes in milk SCC, albumin, Na^+-to-K^+ ratio, and citrate-to-lactoferrin ratio, that are markers of membrane integrity (Ollier et al., 2014). Moreover, the drop in milk yield was obtained with only a limited decrease in feed intake and without metabolic disturbances, promoting a protective

effect against a *S. agalactiae* challenge (Ollier et al., 2015). A single injection of cabergoline at dry-off reduced also the risk of milk leakage, even though caused a drop in feed intake for approximately 24 h the day after the infusion (Larsen et al., 2021). Therefore, prolactin inhibitor infusions can be a viable solution to decrease milk yield and promote involution, despite some adverse effects on feed intake and feeding behavior, that require further investigations (Larsen et al., 2021; Franchi et al., 2022b). However, it is not authorized in some parts of the world for serious adverse events (including recumbency and deaths) associated with the use.

Delaying the moment of dry-off can be an alternative way to obtain cows producing less milk, without the additional labor and costs of the other practices. Previously, results about short and omitted dry-off strategies were reported. Another option could be to shift the moment of the first artificial insemination in order to obtain a longer lactation period and stop the milking at a later stage of the lactation curve, in particular in the most productive animals and primiparous cows, that have persistent lactation curve (Burgers et al., 2021).

1.7 Conclusions

The dry-off is a critical but important phase of the lactation cycle. The average milk yield at dry-off has increased in recent years and represents a threat to the outcome of the dry period. Several methods have been proposed to reduce production before milking cessation, including feed restriction, gradual milking, and prolactin inhibitors. Reducing milking frequency leads to milk accumulation in the udder, which can cause discomfort and increased risk of milk leakage. Feed restriction is effective in quickly achieving target yields but entails huge metabolic implications and welfare concerns. Combining the two strategies can mitigate the side effects of both and improve the capacity of terminating milk synthesis. Extending the lactation period could represent another viable solution, especially for primiparous cows. Nevertheless, reducing milk yield before dry-off is necessary for cows with still large production (over 15 kg/d), in particular when applying selective dry cow therapy approaches. Therefore, developing additional and alternative ways to dry-off high-yielding cows, without side effects, is still needed. In order to assess their effectiveness, the characterization of the metabolic, inflammatory, and behavioral (i.e. welfare) responses is required.

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Objectives of This Dissertation

The objective of this dissertation was to deeply explore the dry-off phase in dairy cows and to evaluate its impact on the ensuing lactation. In this context, the present works aimed to investigate:

- the effect of pre-existing systemic inflammatory conditions at dry-off on the adaptation to the subsequent calving and early lactation performance;
- the effect of a selective dry-cow therapy approach on inflammatory, metabolic, and immune system responses;
- the possibility to improve the inflammatory conditions in cows not receiving intramammary antibiotics at dry-off with the supplementation of a nutraceutical and its carryover effects at calving;
- the response to dry-off in terms of circulating leukocyte gene expression, comparing different production levels;
- the effects of a reduced nutrient density at dry-off on metabolism and future lactation performance.

Chapter 2: Plasma Albumin-to-Globulin Ratio Before Dry-off as a Possible Index of Inflammatory Status and Performance in the Subsequent Lactation in Dairy Cows

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2.1 Abstract

The dry-off of dairy cows represents an important phase of the lactation cycle, influencing the outcome of the next lactation. Among the physiological changes, the severity of the inflammatory response can vary after the dry-off, and this response might have consequences on cow adaptation in the transition period. The plasma protein profile is a diagnostic tool widely used in humans and animals to assess the inflammatory status and predict the outcome of severe diseases. The albumin-to-globulin ratio (AG) can represent a simple and useful proxy for the inflammatory condition. In this study, we investigated the relationship between AG before dry-off and inflammation, metabolic profile, and performance of 75 Holstein dairy cows.

Blood samples were collected from –62 (7 days before dry-off) to 28 days relative to calving (DFC) to measure metabolic profile biomarkers, inflammatory variables, and liver function. Daily milk yield in the 1st month of lactation was recorded. Milk composition, body condition score, fertility, and health status were also assessed. AG calculated one week before dry-off (–62 DFC) was used to retrospectively group cows into tertiles (1.06 ± 0.09 for HI, 0.88 ± 0.04 for IN, and 0.72 ± 0.08 for LO). Data were subjected to ANOVA using the PROC MIXED program in SAS software.

Differences among groups observed at –62 DFC were almost maintained throughout the period of interest, but AG peaked before calving. According to the level of acute-phase proteins (haptoglobin, ceruloplasmin, albumin, cholesterol, retinol-binding protein), bilirubin, and paraoxonase, a generally overall lower inflammatory condition was found in HI and IN than in the LO group immediately after the dry-off but also after calving. HI cows had greater milk yield than LO cows, whereas no differences were observed in milk composition. The somatic cell count (SCC) reflected the AG ratio trend, with higher values in LO than IN and HI either before dry-off or after calving. Fertility was better in HI cows, with fewer days open and services/conception than IN and LO cows.

Overall, cows with high AG before dry-off showed an improved adaptation to the new lactation, as demonstrated by a reduced systemic inflammatory response and increased milk yield than cows with low AG. In conclusion, the AG ratio before dry-off might represent a rapid and useful proxy to evaluate the innate immune status and likely the ability to adapt while switching from the late-lactation to the nonlactating phase and during the transition period with emphasis on early lactation.

Keywords: dry-off, acute-phase protein, inflammation, transition period

2.2 Introduction

The dry period is the period before calving in which the animal is not milked, traditionally, for approximately 6 to 8 weeks (Dix Arnold and Becker, 1936). Marked changes and adaptations occur in this timeframe, during which cows are subjected to two important transitions: from late lactation to dry period and from the latter to the new lactation. Many studies have focused on the periparturient period (Drackley, 1999; Trevisi et al., 2012a; Huzzey et al., 2015), but previous conditions might also influence performance, metabolism, and inflammatory status around calving. For instance, the mammary gland in early lactation appears to be affected by both status leading up dry-off (Whist and Østerås, 2006; Pantoja et al., 2009) and dry period management (Green et al., 2008). Moreover, milk yield at dry-off affects intramammary infections in the following lactation (Rajala-Schultz et al., 2005), and the milk cessation method influences future milk yield and somatic cell count (SCC) (Gott et al., 2017). However, the influence of inflammatory conditions at dry-off on the following lactation is poorly studied. The dry period may be necessary to allow the regeneration of the mammary epithelium to guarantee optimal milk production in the next lactation (Capuco et al., 1997). However, it triggers inflammatory and metabolic "dysregulation" conditions that could negatively affect the cows' health and performance over the short (dry period), altering plasma acute-phase proteins, liver enzymes, and antioxidant species (Mezzetti et al., 2020), and long term (early next lactation), influencing SCC (Gott et al., 2017).

Blood proteins, classified into albumin and globulin, have several functions in dairy cow metabolism. They are synthesized by the liver and, to a lesser extent, the immune system (Eckersall, 2008). Albumin is the most abundant constituent of plasma protein (35–50% of total protein; Eckersall, 2008). It is the most osmotically active plasma protein, and its main functions are in general binding and as a carrier (Eckersall, 2008). Globulin is a broad class of plasma proteins with different functions and can be divided into 3 main fractions (α, β, and γ). Inflammation and pathological states can lead to a shift in blood protein concentrations (Fleck, 1989). Albumin is a negative acute-phase protein (–APP). A decrease in blood concentrations can result from reduced liver synthesis due to inflammatory status (Bertoni et al., 2008). Globulin includes the main positive acute-phase proteins (+APP), i.e., haptoglobin, ceruloplasmin, and serum amyloid A. The blood levels of these proteins increase due to inflammatory conditions (Bionaz et al., 2007; Ceciliani et al., 2012). Therefore, the plasma protein profile analysis and interpretation represent a diagnostic tool in humans (O'Connell et al., 2005) and animals (Lumeij, 1987; Tothova et al., 2016). Abnormal profiles help to identify disease processes in animals that require further investigation. The albumin-to-globulin ratio (AG) represents a synthetic index of the blood protein profile, widely used in clinical biochemistry to identify dysproteinemias (Eckersall, 2008) and in humans as a pretreatment prognostic indicator (He

et al., 2017; Lv et al., 2018). Reference values for AG in healthy dairy cows were reported by Kaneko et al. (2008) and Alberghina et al. (2011). Values lower than those proposed may indicate physiological imbalances, such as inflammation, immunodeficiency, liver or kidney diseases (Eckersall, 2008).

This study aimed to assess the utility of AG before dry-off as a prognostic indicator of the outcome of the subsequent lactation. We investigated the effects of inflammatory status before dry-off, measured by the AG, on milk production, blood biomarkers, and fertility during the dry period and subsequent lactation.

2.3 Materials and Methods

2.3.1 Animal management

The research was conducted at the Università Cattolica del Sacro Cuore dairy farm (San Bonico, Piacenza, Italy) following Italian laws on animal experimentation and ethics (Prot. N- 44021 of 5.08-2013 in agreement with D. Lgs n.116, 27/01/1992 and authorization N 1047-2015-PR in agreement with D. Lgs. n. 26, 04/03/2014). This farm milks on average 70 Holstein cows, with an average milk production of 10,500 Kg/lactation. Over a period of four years, 75 different Holstein dairy cows ($n = 75$, parity 3 ± 2, median $\pm$ interquartile range), without clinical diseases, were regularly monitored from the drying-off through the next lactation.

Table 2.1. Ingredients and chemical composition of TMR fed during the study

Item	Dry period	Early lactation
Ingredient, % of DM		
Grass hay	47.2	2.1
Alfalfa hay	-	21.0
Corn silage	26.6	37.1
Ground corn	-	12.6
Barley	-	8.4
Wheat straw	14.1	-
Soybean meal	5.5	12.1
Sunflower meal	5.8	4.3
Hydrogenated fat	-	0.8
Vitamin and mineral supplement	0.8	1.6
Chemical composition		
NE_L, Mcal/Kg	1.25	1.59
CP, % of DM	12.2	15.8
NSC, % of DM	19.0	36.8
NDF, % of DM	56.0	35.8

During late lactation, cows were housed in a freestall barn with cubicle resting areas and fed lactation TMR delivered once daily (8:30). Fifty-five days before expected calving, cows were dried-off after final milking with mammary antibiotic treatment (Mamyzin A; Haupt Pharma Latina S.r.l, Italy). After dry-off, cows were moved to a straw-bedded pen, and for the first 10 days, they were fed ryegrass hay and water *ad libitum* and vitamin and mineral supplementation.

Diets were formulated according to NRC (2001); ingredients and chemical composition are shown in Table 2.1. During the dry period, the diet was fed as TMR once a day (10:00). On calving day, cows were moved to the postpartum pen, where they received the lactation diet. During lactation, cows were milked twice a day (2:00 and 14:00), and milk yield was recorded. All cows involved in this study were subjected to hoof-trimming 30 days before and after calving. In addition, cows were vaccinated against rota and coronaviruses approximately 30 days before the expected calving day.

2.3.2 Blood samples and metabolic profile

Blood samples were collected at –62 ± 5 (7 days before dry-off), –48 ± 5 (7 days after dry-off), –28 ± 3, –14 ± 3, –3 ± 1, 7 ± 1, 14 ± 1, 28 ± 1 day from calving (DFC) by jugular venipuncture into Li-heparin treated evacuated tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) before the morning feeding. The samples were processed as described by Calamari et al. (2016): the packed cell volume was immediately determined on whole blood aliquots by centrifugation, and plasma was stored at –20°C for subsequent biochemical profile analysis.

The following variables were measured using analysis methods described in Calamari et al. (2016): glucose, cholesterol, urea, calcium, phosphorus, magnesium, zinc, ceruloplasmin, total protein, albumin, globulin, aspartate amino transferase-glutamate oxaloacetate transaminase (AST-GOT), gamma-glutamyl transferase (GGT), bilirubin, haptoglobin, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), creatinine, reactive oxygen metabolites (ROM), paraoxonase, retinol, tocopherol, and β-carotene. Analytical methods, intra- and inter-assay coefficients of variation are reported in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.14345717; Cattaneo et al., 2021). Plasma concentrations of albumin, cholesterol, and bilirubin at 7 and 28 DFC were used to calculate the liver functionality index (LFI) according to Bertoni and Trevisi (2013).

2.3.3 Bodyweight, BCS, health status, and performance

Bodyweight was measured with a single walk-in scale before dry-off. The same operator determined BCS with a 1–5 points method (ADAS, 1986) at the same scheduled time point of blood sampling (from –62 to 28 DFC).

Health status was monitored daily throughout the entire period, and problems were recorded. Retained placenta was diagnosed when fetal membranes were not expelled within 24 h after calving (Beagley et al., 2010), metritis and endometritis were declared according to Sheldon et al. (2006),

and mastitis was diagnosed by visual assessment of each quarter milk and SCC analysis of suspicious cases. Ketosis was declared when blood BHB was greater than 1.4 mmol/L (Duffield, 2000). Displacement of the abomasum was by veterinary diagnosis. The veterinarian evaluated lameness during hoof-trimming operations and after farm operators reported suspicious cases.

Milk yield and conductivity were recorded automatically daily from 4 to 30 DFC by the Afimilk system (SAE Afikim, Israel). Daily milk yield was recorded 10 days before dry-off, and composite milk samples were collected and analyzed to assess SCC. After calving, between 15 to 35 DFC, a composite milk sample from each cow was collected, and milk composition (SCC, fat, protein, and lactose) was assessed. Fat, protein, and lactose were analyzed by mid-infrared assay (Milko Scan 133B, Foss Electric, Denmark) and SCC by an automated cell counter (Fossomatic 180, Foss Electric, Denmark), according to Foss FTIR prediction models (Foss Analytical). Somatic cell count was expressed as a linear score (somatic cell score, SCS; Wiggans and Shook, 1987).

The number of artificial inseminations (AI), days open, and pregnancy diagnosis results were recorded. Culled cows and the reason for culling were noted. The fertility status index (FSI) was calculated according to Esslemont and Eddy (1977). This index, calculated at group level, uses pregnancy risk to first service, number of AI/pregnancy, days open, and culling risk to summarize the overall fertility performance. Cows culled before the AI were excluded from the calculation.

2.3.4 Animal grouping and statistical analysis

The objective of the study was to assess the relationship between the severity of inflammatory before DO (evaluated through AG) and the overall cows' condition in dry period and early lactation. Sample size was calculated using a power analysis ($\alpha = 0.05$, and $\beta = 0.2$), assuming an expected AG of 0.9 and a standard deviation of 0.2. Calculated sample size (n = 23 per group) was increased of two subjects per group to consider potential exclusion of some cows. Therefore, 75 dairy cows were enrolled and retrospectively ranked into categorical tertiles based on the plasma AG calculated 7 days before dry-off (–62 DFC). Tertiles division was chosen because provides a good separation among groups with different AG. The three groups were defined as: low, cows in the 1st tertile (LO, n = 25; AG between 0.52 and 0.82); intermediate, cows in the 2nd tertile (IN, n = 25; AG between 0.83 and 0.94); high, cows in the 3rd tertile (HI, n = 25; AG between 0.95 and 1.26).

All statistical analyses were performed using the statistical software package SAS 9.3 (SAS Institute Inc., Cary, NC). Data were tested for normality by the Shapiro-Wilk test. When the distribution was not normal, data were normalized by a logarithmic transformation (bilirubin, haptoglobin, NEFA, BHB, GOT, and tocopherol). Results are presented as back-transformed data. Data about cows' characteristics and milk composition were analyzed with ANOVA using a mixed model (MIXED procedure of SAS), considering only the fixed effect of AG. Plasma biomarkers and

milk production were subjected to ANOVA using a repeated measures mixed model (REPEATED statement in the MIXED procedure of SAS). The model included the fixed effect of group (G; LO, IN, and HI), the effect of time (T; –62, –53, –28, –14, –3, 7, 14, 28 days from calving for plasma biomarkers; weeks from calving for milk production), and the interaction group × sampling day (G*T); cows nested within the groups were included as the random effect. The covariance structure (compound symmetry, autoregressive order, toeplitz, or spatial power) with the lowest AICC (Littell et al., 1998) was included in the MIXED model (Supplemental Table S2; https://doi.org/10.6084/m9.figshare.14345717; Cattaneo et al., 2021). Incidence of diseases was analyzed with χ^2 test (FREQ procedure of SAS). The pair-wise comparison was performed using the LSD (least significant difference) test when the F-test of one of the factors was statistically significant at $P \leq 0.05$.

2.4 Results

Table 2.2. Parity, mature equivalent milk yield, PFT, dry period length, total milk yield in the previous lactation, milk yield and SCS at dry off, body condition score (BCS) and body weight at the enrollment of 75 Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n = 25) albumin-to-globulin ratio (AG) before dry-off. Values presented as least squares means and greatest standard error

	AG				*P*-value
Item	HI	IN	LO	SEM[1]	*G*[2]
Parity	2.4[c]	3.0[a,b]	4.3[a]	0.2	<0.01
Mature equivalent milk yield, Kg/lactation	12094	11889	12420	331.3	0.52
PFT index[3]	1680	1526	1512	81.3	0.27
Dry period lenght, d	53.8[b]	54.5[b]	59.3[a]	1.3	0.01
Days in milk, d	359	346	353	13.7	0.81
Total milk yield, Kg/lactation	10,778	10,383	11,712	555	0.22
Milk yield at dry-off, Kg/d	16.8	19.0	15.7	1.7	0.34
SCS at dry-off, units	2.5[b]	3.3[a,b]	3.9[a]	0.3	0.01
BCS, units	2.6	2.6	2.6	0.1	0.95
Body weight, Kg	642	644	658	13.0	0.62

[a-b] Values in the column with different superscript differ ($P < 0.05$) for the albumin-to-globulin ratio effect
[1] Greatest standard error of mean (SEM)
[2] Overall effect of albumin-to-globulin ratio
[3] Productivity-Functionality-Type index: genetic selection index for Italian Holstein adopted from February 2002 (ANAFIJ)

Characteristics of cows involved in the study are reported in Table 2.2. The three resulting groups' average calving dates were similar, and cows in each group were distributed along the study period. The three groups differed for parity ($P < 0.01$). The dry period length was longer in LO than in other groups ($P = 0.01$). Mature equivalent milk yield, Productivity-Functionality-Type index

(PFT), milk production in the previous lactation and at dry-off, previous lactation length, BCS, and body weight at dry-off did not differ among groups ($P > 0.05$).

2.4.1 Plasma protein profile

Figure 2.1 shows the AG pattern during the study. Overall, the mean AG value was 0.92 ± 0.19, with a peak around calving (1.09 ± 0.19 at –3 DFC). Before dry-off (–62 DFC), the average AG was 0.89 ± 0.16. When groups were formed, the mean values were 1.06 ± 0.09 for HI, 0.88 ± 0.04 for IN, and 0.72 ± 0.08 for LO. Furthermore, the differences observed before dry-off were maintained during the whole period, with an average of 1.06 ± 0.16 for HI, 0.91 ± 0.13 for IN, and 0.79 ± 0.17 for LO ($P < 0.01$).

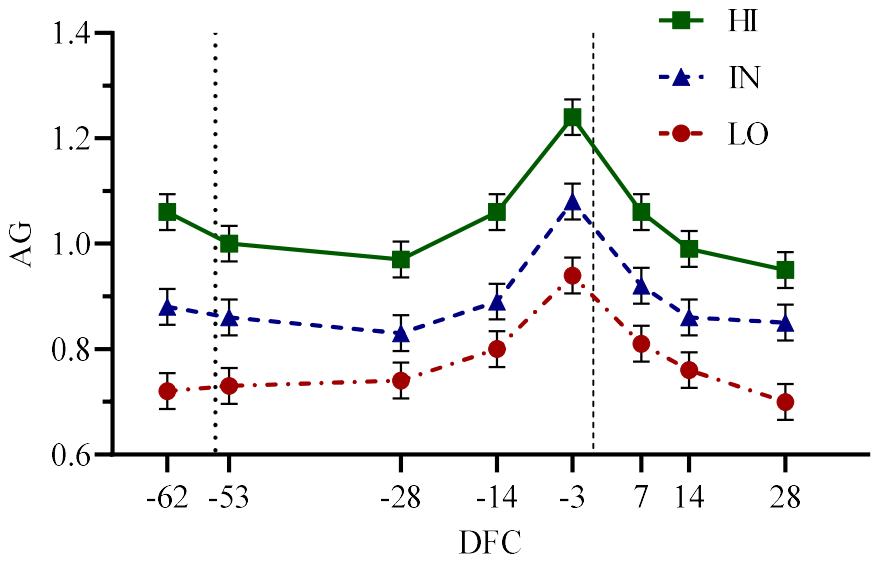

Figure 2.1. Time pattern of albumin-to-globulin ratio (AG) in Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n =25) AG before dry-off, from –62 to 28 days from calving (DFC). Values presented as least squares means ± standard error

[a–b] Differences ($P \leq 0.05$) between HI, IN, and LO groups at each time point relative to calving.

The total protein trend mirrored that of AG, reaching the lowest concentration at –3 DFC (68.88 ± 5.77 g/L), with marked differences among groups (G; $P < 0.01$). The difference among groups was higher during the dry period than in the first week after calving (G*T; $P < 0.01$). Moreover, globulin showed a similar pattern, with a nadir at –3 DFC (33.53 ± 5.86 g/L), resulting in an interaction effect (G*T; $P < 0.01$), whereas the nadir of albumin was observed at 7 DFC (34.10 ± 3.01 g/L).

2.4.2 Plasma biomarkers

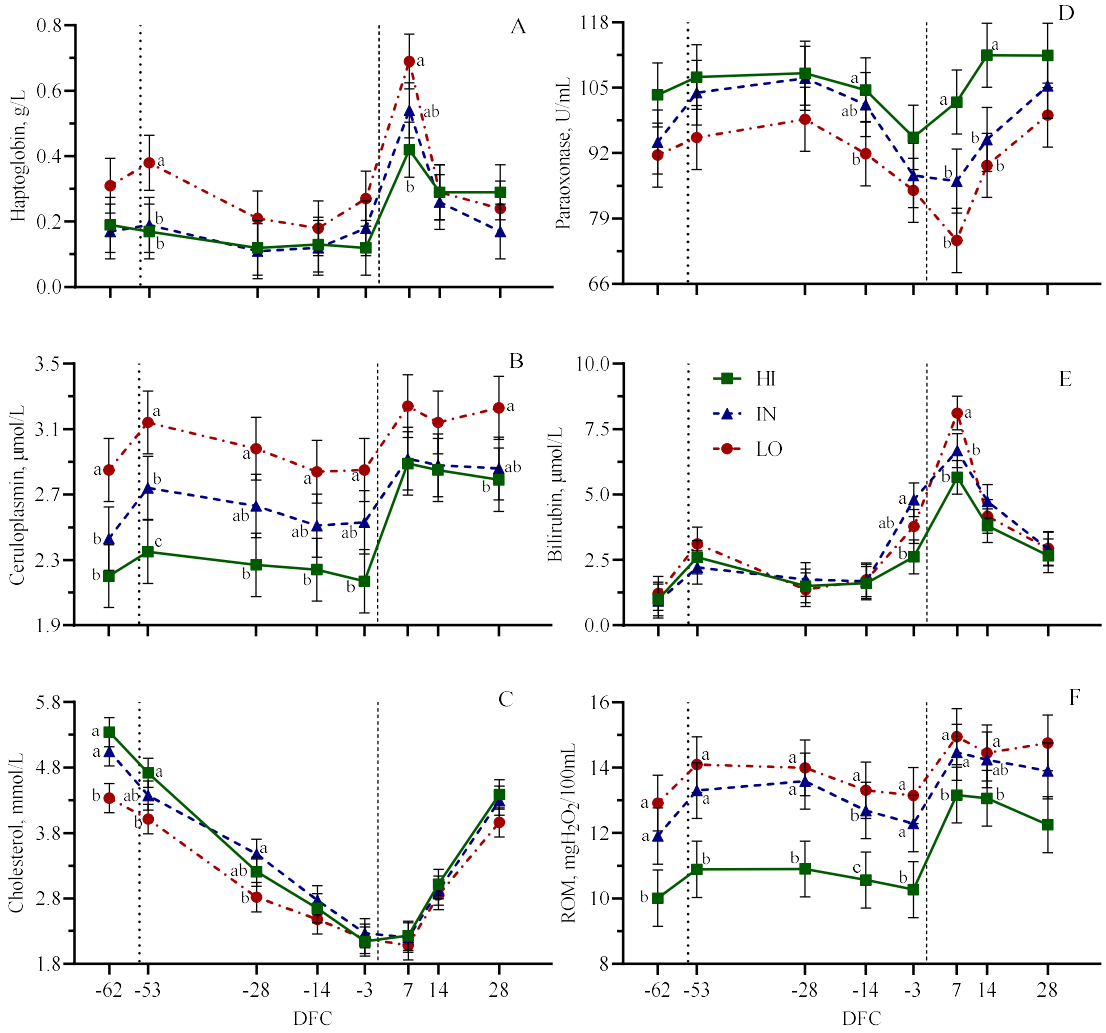

Figure 2.2. Time pattern of plasma inflammation biomarkers in Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n =25) albumin-to-globulin ratio (AG) before dry-off, from –62 to 28 days from calving (DFC). Values presented as least squares means ± standard error
a–b Differences (P ≤ 0.05) between HI, IN, and LO groups at each time point relative to calving.

The least-squares means of plasma biomarkers are summarized in Table 2.3. Positive acute-phase proteins revealed marked differences among groups. Haptoglobin was greater in LO than other groups until 14 DFC (G; $P < 0.01$; Table 2.3). The greatest difference was observed at 7 DFC between LO and HI groups (G*T; $P < 0.01$; Fig. 2.2A). Overall, the LO group showed the highest concentrations of ceruloplasmin throughout the entire study period (G; $P < 0.01$; Table 2.3). Before calving, ceruloplasmin was 2.93 ± 0.1 for LO, 2.56 ± 0.1 for IN, and 2.23 ± 0.1 for HI cows (Fig. 2.2B).

Table 2.3. Plasma biomarkers from –62 to 28 days relative to calving in 75 Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n = 25) albumin-to-globulin ratio (AG) before dry-off. Values presented as least squares means and greatest standard error

	AG				*P*-value		
Biomarker[1]	HI	IN	LO	SEM[2]	G[3]	T[4]	G*T[5]
Hematocrit, L/L	0.32[a]	0.32[ab]	0.31[b]	0.01	0.03	<0.01	0.10
Glucose, mmol/L	4.00	4.01	4.02	0.10	0.83	<0.01	0.38
Urea, mmol/L	3.42[ab]	3.64[a]	3.39[b]	0.29	0.09	<0.01	0.39
NEFA, mmol/L	0.41	0.45	0.39	0.08	0.38	<0.01	0.21
BHB, mmol/L	0.64	0.64	0.59	0.14	0.64	<0.01	0.32
Creatinine, µmol/L	100.7	99.7	97.8	2.9	0.36	<0.01	0.38
Calcium, mmol/L	2.57[a]	2.55[ab]	2.52[b]	0.04	0.03	<0.01	0.29
Phosphorus, mmol/L	1.77	1.77	1.71	0.13	0.61	<0.01	0.38
Magnesium, mmol/L	1.03[a]	1.01[ab]	0.98[b]	0.04	0.01	<0.01	0.05
Zinc, µmol/L	13.6[a]	12.8[ab]	12.1[b]	0.62	<0.01	<0.01	0.05
Cholesterol, mmol/L	3.33[a]	3.30[a]	3.00[b]	0.22	0.04	<0.01	0.01
Ceruloplasmin, µmol/L	2.43[b]	2.67[b]	3.02[a]	0.19	<0.01	<0.01	0.27
Total protein, g/L	72.7[c]	75.7[b]	79.4[a]	1.4	<0.01	<0.01	<0.01
Albumin, g/L	37.0[a]	35.7[b]	34.3[c]	0.6	<0.01	<0.01	0.32
Globulin, g/L	35.7[c]	40.0[b]	45.1[a]	1.4	<0.01	<0.01	<0.01
AG	1.06[a]	0.91[b]	0.79[c]	0.03	<0.01	<0.01	0.03
GOT, U/L	84.6	87.4	81.0	6.7	0.15	<0.01	0.94
GGT, U/L	24.5	23.4	26.1	2.2	0.25	<0.01	0.57
Bilirubin, µmol/L	2.62[b]	3.20[ab]	3.28[b]	0.65	0.09	<0.01	0.01
Haptoglobin, g/L	0.20[b]	0.21[b]	0.30[a]	0.08	<0.01	<0.01	0.62
Paraoxonase, U/mL	106.9[b]	99.0[ab]	91.6[a]	6.40	0.02	<0.01	0.02
ROM, mgH_2O_2/100mL	104.8[a]	97.2[b]	90.3[b]	0.9	<0.01	<0.01	0.80
Retinol, µg/100mL	52.0[a]	45.5[b]	43.4[b]	4.4	0.01	<0.01	0.44
Tocoferol, µg/mL	2.36	2.28	2.36	0.37	0.95	<0.01	0.11
β-carotene, mg/100mL	0.19	0.19	0.19	0.03	0.83	<0.01	0.24

[a-c] Values in the same row with different superscript differ (P < 0.05) for the interaction between albumin-to-globulin ratio and days from calving

[1] GOT = glutamic oxaloacetic transaminase; GGT = γ-glutamyltransferase; NEFA = non esterified fatty acids; BHB = β-hydroxybutyrate; ROM = reactive oxygen metabolites

[2] Greatest standard error of the mean (SEM).

[3] Overall effect of albumin-to-globulin ratio

4 Overall effect of days from calving (–62, –53, –28, –14, –3, 7, 14, and 28 d).

[5] Interaction between albumin-to-globulin ratio and days from calving

Negative acute-phase proteins revealed different trends among groups. Overall, the LO group's cholesterol was lower than in both IN and HI groups (G; $P = 0.04$; Table 2.3). The greatest differences were observed before and immediately after dry-off: the LO group had lower cholesterol than HI and IN groups before dry-off and only compared with the HI group immediately after at –53 DFC (G*T; $P < 0.01$; Fig. 2.2C). Paraoxonase concentrations were layered during the entire study,

with the highest values in HI and the lowest in LO (G; $P = 0.02$; Table 2.3). The greatest differences were observed at 7 and 14 DFC, when the HI group had greater concentrations than LO (G*T; $P < 0.01$; Fig. 2.2D), and at 14 DFC, with HI cows having a higher level than IN ($P < 0.01$).

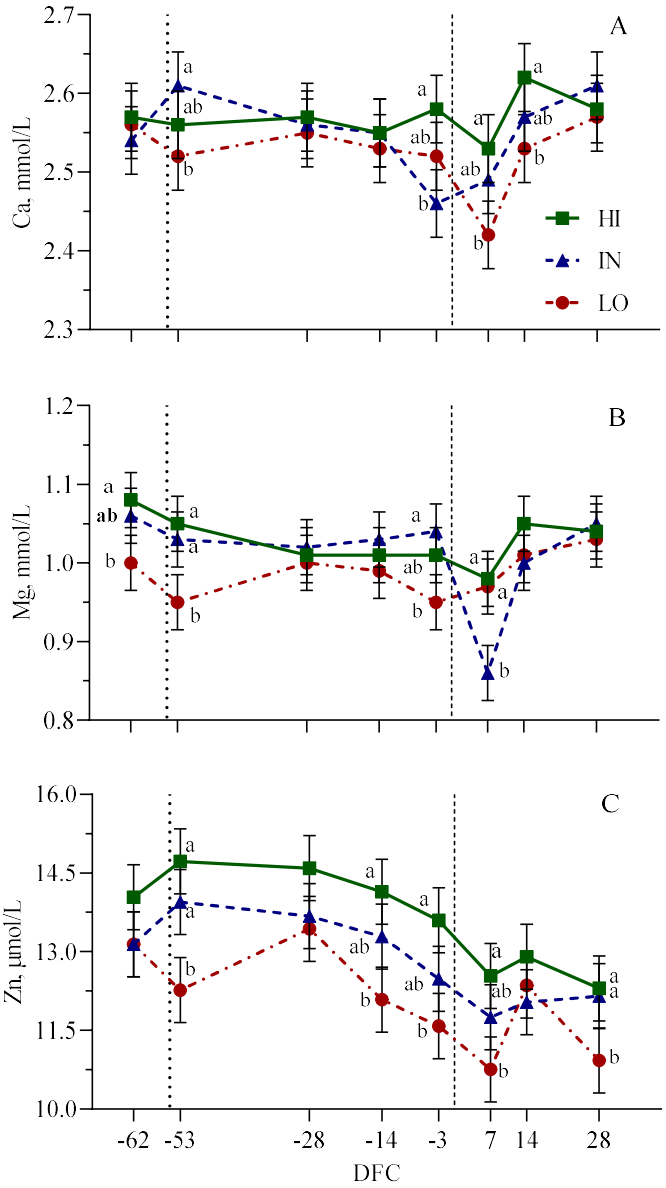

Figure 2.3. Time pattern of plasma mineral biomarkers in Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n =25) albumin-to-globulin ratio (AG) before dry-off, from –62 to 28 days from calving (DFC). Values presented as least squares means ± standard error
a–b Differences ($P \leq 0.05$) between HI, IN, and LO groups at each time point relative to calving.

Bilirubin was different only around calving, resulting in an interaction effect (G*T; $P < 0.01$; Fig. 2.2E). At –3 DFC, HI had lower levels than the IN group ($P < 0.01$), whereas LO reached the highest value at 7 DFC, resulting in greater bilirubin than both IN and HI groups ($P < 0.05$). Furthermore, the HI group had lower ROM concentrations until calving than other groups (G*T; $P <$

0.01; Fig. 2.2F). Overall, retinol was higher in HI than other groups (G; $P = 0.01$; Table 2.3), with a marked difference from IN at –53 and 7 DFC ($P < 0.01$). Tocopherol and β-carotene, instead, were not different among groups.

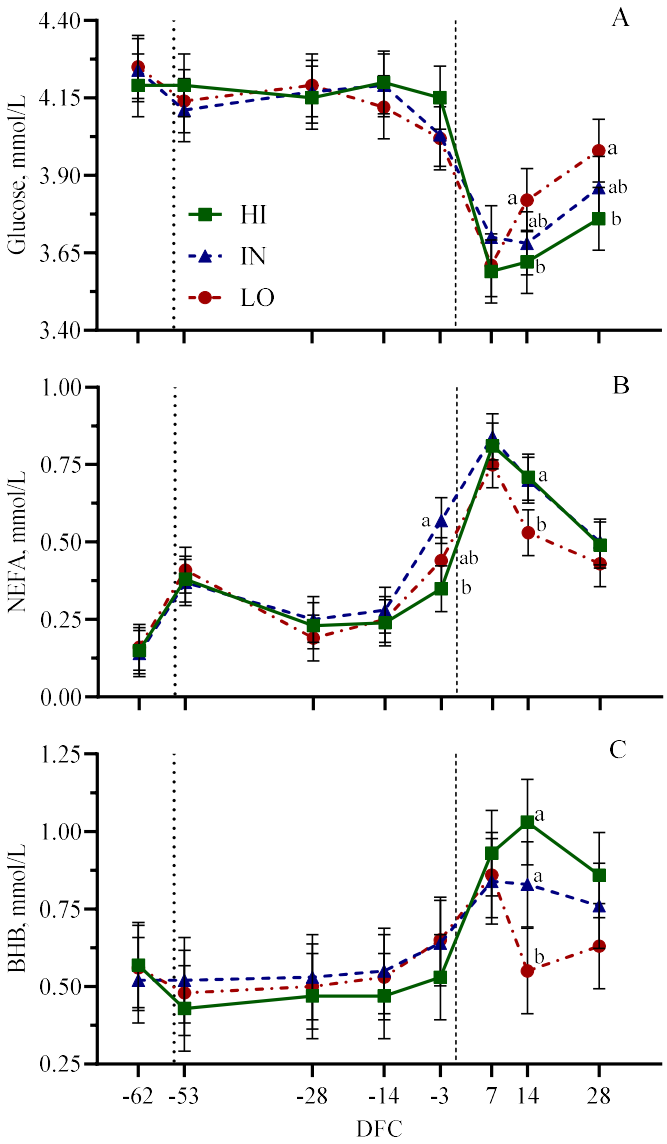

Figure 2.4. Time pattern of plasma energy metabolism biomarkers in Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n =25) albumin-to-globulin ratio (AG) before dry-off, from –62 to 28 days relative to calving (DFC). Values presented as least squares means ± standard error
[a–b] Differences ($P \leq 0.05$) between HI, IN, and LO groups within each time point relative to calving.

Among minerals, calcium was different among groups (G; $P = 0.03$; Table 2.3). In fact, especially around calving, HI showed greater calcemia than IN (–3 DFC; $P < 0.01$; Fig. 2.3A) and LO groups (7 and 14 DFC; $P = 0.01$ and $P = 0.04$, respectively). Magnesium revealed a different pattern over time among groups (G*T; $P = 0.05$; Fig. 2.3B), with marked differences around dry-off,

when LO had the lowest concentrations (–62 and –53 DFC; $P = 0.05$), and after calving, when IN peaked negatively (7 DFC; $P < 0.01$). Finally, zinc concentrations were lower in LO than in HI during almost the entire study period (G; $P < 0.01$; Table 2.3).

Overall, biomarkers of energy and protein metabolism (glucose, urea, NEFA, BHB, and creatinine; Fig. 2.4) did not differ between groups, and the same was observed of liver aminotransferase enzymes GOT and GGT. However, at 14 DFC, the LO group showed higher glucose than the HI group and lower NEFA and BHB than other groups ($P < 0.05$).

LFI was lower in LO than in HI (–1.58 vs 1.14; $P < 0.01$) and IN (–1.58 vs 0.13; $P = 0.04$).

2.4.3 Milk Yield, BCS, health status, and fertility

Before dry-off, milk yield did not differ among groups, whereas HI had lower SCS than other groups ($P < 0.01$). The milk yield trend from 4 to 30 DFC is shown in Fig. 2.5. Overall, there was a tendency towards greater milk production in the HI group than the LO group (G; $P = 0.08$). In addition, the interaction G*T demonstrated HI had a greater milk production than LO cows 10 to 14 DFC and 20 to 26 DFC (G*T; $P < 0.01$). Milk composition after calving (fat, protein, and lactose content) did not differ among groups (Table 2.4), but LO had the highest SCS ($P = 0.1$).

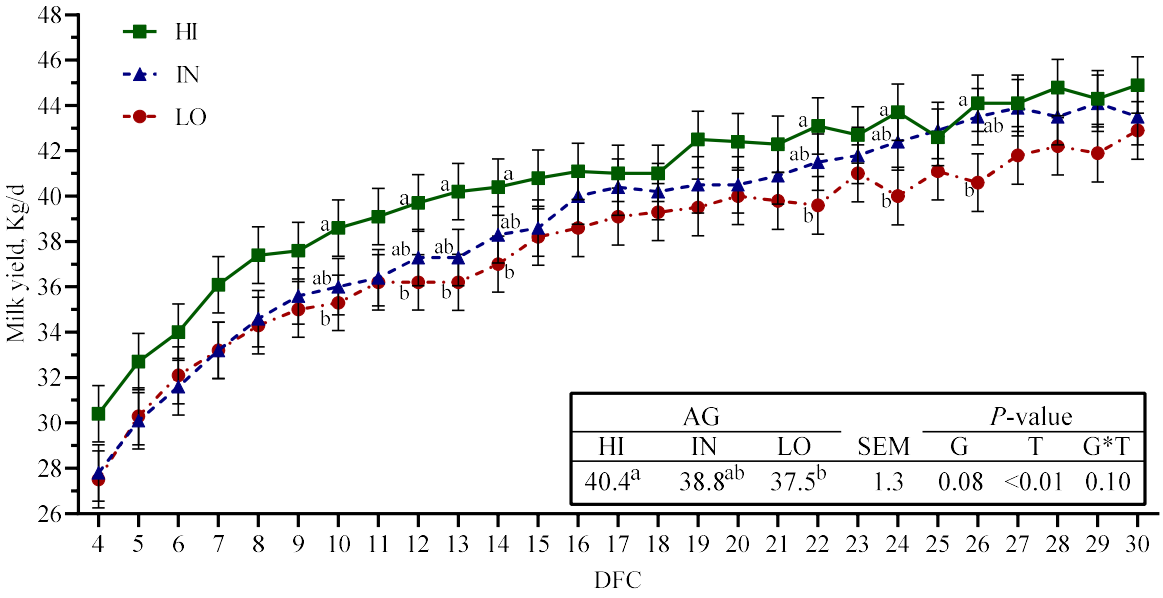

Figure 2.5. Time pattern of milk yield (Kg/d) in Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n =25) albumin-to-globulin ratio (AG) before dry-off, from 4 to 30 days from calving (DFC). Values presented as least squares means ± standard error

[a–b] Differences ($P \leq 0.05$) between HI, IN, and LO groups within each time point relative to calving.

Table 2.4. Milk composition in the subsequent lactation in 75 Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n = 25) albumin-to-globulin ratio (AG) before dry-off. Values presented as least squares means and greatest standard error

	AG				*P*-value
Item	HI	IN	LO	SEM[1]	G[2]
SCS[3]	2.12	2.07	3.24	0.63	0.11
Fat, %	3.86	3.81	3.78	0.24	0.94
Protein, %	3.08	3.13	3.17	0.08	0.52
Lactose, %	4.82	4.84	4.79	0.06	0.68

[a-b] Values in the column with different superscript differ (P < 0.05) for the albumin-to-globulin ratio effect
[1] Greatest standard error of the mean (SEM).
[2] Overall effect of albumin-to-globulin ratio
[3] Somatic cell score (Wiggans and Shook, 1987)

The incidence of diseases within groups is shown in Table 2.5. The number of cows diagnosed without any clinical disease did not differ among groups. Before calving, only lameness cases were diagnosed. Only one case of lameness was recorded in HI, whereas 7 cases were noted both in IN and LO ($P = 0.02$). Reasons for lameness included sole ulcer, digital dermatitis, white line disease, foot rot, and injuries. After calving, fewer cases of retained placenta were diagnosed in HI than LO (1 vs. 5; $P < 0.1$). The occurrence of mastitis, metritis, and ketosis did not differ among groups.

Table 2.5. Incidence of diseases diagnosed from –62 to 28 days relative to calving in 75 Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n = 25) albumin-to-globulin ratio (AG) before dry-off. Values presented as least squares means and greatest standard error

Item	HI	IN	LO
Pre-calving			
Lameness	1[b]	7[a]	7[a]
Post-calving			
Mastitis	2	2	4
Retained placenta[1]	1[b]	3[ab]	5[a]
Sub-clinical ketosis[2]	6	6	3
Metritis	6	5	6
Others	0[b]	3[a]	1[ab]

[1] Defined as fetal membranes retained ≥ 24 h after calving.
[2] Defined as cows having beta-hydroxybutyrate concentration greater than 1.4 in the first 2 wk after calving.
[a-b] Values in the column with different superscript differ (P < 0.1) for the albumin-to-globulin ratio effect

Reproductive performances are shown in Table 2.6. Superior reproductive performances were observed in the HI group than in other groups, as summarized by FSI (25.8, –4.9, and –34.1 for HI, IN, and LO, respectively). Moreover, the components of this synthetic index (pregnancy risk, pregnancy risk to first service, days open, and culling risk) revealed numerically better performance in HI cows. Cows culled before the first insemination were lower in HI than in the LO group (0 vs. 5; $P = 0.02$).

Table 2.6. Reproductive performance in 75 Holstein dairy cows with high (HI, n = 25), intermediate (IN, n = 25), and low (LO, n = 25) albumin-to-globulin ratio (AG) before dry-off

Item	HI	IN	LO
AI cows	25	24	20
Cows culled before first service[1]	0^{b}	1^{ab}	5^{a}
Pregnancy risk	80%	75%	70%
Pregnancy risk to first service	40%	22%	36%
AI/pregnancy	2.6	2.9	3.6
Days open[2]	119.6 ± 14.3	137.4 ± 14	164.1 ± 22.9
Culling risk[1]	20%	25%	30%
FSI[3]	25.8	–4.9	–34.1

[1] Forced culling: low fertility (HI, n = 4; IN, n = 3; LO, n= 5), severe mastitis (HI, n = 1; IN, n = 2; LO, n= 2), prototheca (LO, n= 2), severe injury (IN, n = 1), other (IN, n = 1; LO, n= 2)
[2] Mean ± standard error
[3] Fertility status index (Esslemont and Eddy, 1977)
[a-b] Values in the column with different superscript differ ($P < 0.05$) for the albumin-to-globulin ratio effect

2.5 Discussion

Identifying a simple proxy to evaluate the metabolic and inflammatory condition at dry-off in dairy cows is critically needed. The proxy should highlight physiological imbalances even in the absence of clinical signs of illness since the failure may result in disorders and impair the physiological response after calving. For instance, it is well known that changes in behavior and immune system occur before the onset of some diseases (Calamari et al., 2014; Mezzetti et al., 2019; Cattaneo et al., 2020). Moreover, cows housed in the same farming conditions can show different innate immune response and consequently different inflammatory condition as a result of stressors. Therefore, there would seem to be a different susceptibility to stressors at the individual cow level that can affect metabolism and performance, as observed in mammals (Tsyglakova et al., 2019). Thus, we aimed to evaluate the relationship between albumin-to-globulin ratios before dry-off, associated with systemic inflammation, and the metabolism and performance in Holstein dairy cows in both the dry period and next early lactation.

In the current study, inflammation was assessed through AG, which summarizes the protein profile in plasma in connection with body responses to stressors. AG is less sensitive to dehydration and fluid losses than the single values of albumin or globulin. It is widely used in human medicine as a marker of long-term survival in severe illness (e.g., cancer) and veterinary medicine as a diagnostic value. Traditionally, the blood protein profile is evaluated in serum (Russell and Roussel, 2007). However, blood protein profile measurement is common even in plasma samples (Allison, 2012). The presence of fibrinogen and clotting factors distinguishes plasma from serum. Fibrinogen, which is a +APP in ruminants, ranges from 3 to 7 g/L (Jones and Allison, 2007) and is included in the plasma globulin fraction. Moreover, simply subtract fibrinogen from plasma globulin to compare plasma and serum would not be possible due to differences in methodology. Therefore, our blood protein values might not be fully comparable with those in the literature, based mainly on serum samples. Considering the many variables measured and analyzed, in some case power of statistical analysis might be low. Therefore, data should be interpreted carefully.

2.5.1 Plasma protein profile

Before dry-off, the average AG value observed in the IN group (0.88 ± SEM) was within the reference values (0.84–0.94) proposed for serum by Kaneko et al. (2008), whereas LO and HI values (0.72 and 1.06, respectively) were outside the reference limits. Differences from reference values were observed both in albumin and globulin. A mild hyperalbuminemia was noted in IN and HI groups (> 35.5 g/L), whereas hyperglobulinemia was diagnosed in LO and IN groups (> 34.8 g/L). However, variations in albumin concentrations were smaller than those of globulin. Therefore, in LO cows, the ratio was lower mainly due to the increased globulin concentrations, suggesting greater inflammation. Such an interpretation is also supported by the higher concentrations of two important +APPs before dry-off (–62 DFC), haptoglobin and ceruloplasmin. However, globulin concentrations were lower in HI cows, resulting in higher AG. Nevertheless, high AG should not be a concern for cow's health in normal conditions. Albumin increases only during fluid loss and dehydration and decreased globulin occurs mainly in fetuses and neonatal animals (Eckersall, 2008). Meanwhile, low AG could be more common. In mature cows, selective hypoalbuminemia (without concurrent hypoglobulinemia) can occur in case of severe hepatic failure, malnutrition, gastrointestinal parasitism, severe intestinal failure, and glomerular diseases, whereas selective hyperglobulinemia can occur in case of inflammation, liver diseases, and diseases such as lymphoma, leukemia, myeloma, and extramedullary plasmacytoma (Allison, 2012).

Research available in literature proposed reference values for cows of different age or parity. However, Alberghina et al. (2011), although they reported numerically different limits, did not highlight significant differences among multiparous cows from the second to the sixth lactation. In

contrast, Bobbo et al. (2017) reported a constant decrease in AG over lactations, as a result of unchanged albumin and increasing globulin. Similar effect of parity on total protein and globulin was previously reported by other authors (Shaffer et al., 1981; Cozzi et al., 2011; Brscic et al., 2015). The higher globulin in older cows could be related to a more developed immune system and, consequently, increased antibodies (Eckersall, 2008) or to increased inflammation and APP. In our study, parity reflects AG stratification at dry-off, with the highest parity observed in LO.

Another factor potentially involved in the differences in AG could be genetics. Some authors suggested indeed a small genetic influence on plasma proteins, even though with a low to moderate heritability (Peterson et al., 1982; Cecchinato et al., 2018; Johnston et al., 2020). In our study, cows in the three groups had similar breeding values, but we cannot exclude that some genetic variant could be implied. Further research would be needed to investigate in depth the relationship between genome and plasma proteins.

The AG time pattern was the same for all the groups, and the differences observed when groups were defined were maintained throughout the monitoring period. Therefore, cows with a specific inflammatory status before dry-off maintained this condition through the dry period and even after calving. Cows with low AG had consistently higher globulin values, probably denoting a chronic innate immune deficiency associated with inflammation. Piccione et al. (2011) reported a similar pattern during dry and early lactation periods, and their values were similar to those of LO group in our study.

We could not determine which globulins fraction caused these greater values. Fractions mainly involved in the inflammatory response are α and γ-globulins. Acute inflammation leads to an increase in α-globulins, whereas chronic inflammation results in a higher γ fraction (Vavricka et al., 2009). Mammary gland inflammation might be a factor linked to increased globulins, as suggested also by (Bobbo et al., 2017b). Somatic cell count, both before dry-off and also after calving, reflects the inflammatory status as indicated by AG. Bobbo et al. (2017a) found an inverse relationship between SCC and AG in dairy cows from multi-breed herds, resulting from a linear relationship with globulin and an inverse with albumin. This relationship might be of practical interest. Indeed, SCC is the most commonly used proxy of udder health and is an easily obtainable value. According to our results, AG and SCC are two indexes of cow's health and inflammatory status that might be interchangeable as tools to evaluate cows' conditions before dry-off. However, AG could provide information not only about the udder but also about general health status.

Regardless of grouping, AG peaked immediately before calving, the result of the different trends of the two components of the ratio. While albumin and globulin had a similar pattern, near calving, globulin decreased before albumin, as previously reported by Rowlands et al. (1980).

Albumin reached its lowest concentrations after calving (7 DFC), whereas globulin was lowest immediately before calving (–3 DFC). After calving, the decrease in albumin concentration was related to the inflammatory-like conditions typical of the postpartum period (Bertoni et al., 2008). Globulin decreased before calving, probably due to the transfer of γ-globulins from blood to colostrum (Weaver et al., 2000). This shift caused a peak in AG values. Tóthová et al. (2018) and Lopreiato et al. (2019) observed a similar pattern around calving.

2.5.2 Blood biomarkers

APPs are a family of proteins that increase or decrease in concentration during inflammation. In our study, +APP concentrations allowed the groups to be divided into tertiles based on AG. Overall, LO appeared to have a worse inflammatory condition than HI, and IN was intermediate between the other two groups. Notably, the differences among groups observed before dry-off were maintained through the transition period. Haptoglobin, the main +APP in cows, highlighted important differences between groups. In fact, LO had constantly higher haptoglobin concentration than other groups until the 2nd week after calving, reaching values greater than 0.15–0.20 g/L, indicating inflammatory status (Eckersall and Bell, 2010; Bertoni and Trevisi, 2013). Although starting from higher baseline values, LO had a more pronounced increase after dry-off. The exacerbation of inflammation after dry-off in LO could be related to the drastic changes associated with switching from the lactating phase to the nonlactating phase at the rumen and mammary gland level (Mezzetti et al., 2020). Cows with low AG might have been more sensitive to these alterations. However, high haptoglobin concentration during this period might not be detrimental. Mammary gland involution and remodeling involve inflammation, and haptoglobin is implicated in tissue remodeling (Arslan et al., 2013). Similar pattern and differences in haptoglobin among groups were observed also after calving, suggesting a persistency of the inflammatory degree in animals with a different AG ratio. This result was confirmed by the plasma levels of ceruloplasmin, another important +APP in ruminants. Ceruloplasmin concentrations were fairly stable during the dry period due to the slow change of this protein in response to inflammatory events (Bertoni and Trevisi, 2013). Ceruloplasmin concentrations were greater in LO cows than in HI cows during the entire period. However, during the dry period, IN ceruloplasmin concentrations were between LO and HI, suggesting greater inflammation than in HI. This result is consistent with other plasma biomarkers and the superior performance of HI cows, such as greater milk production and fertility.

Most APP results also support the hypothesis of enhanced inflammation at dry-off in the LO group. Albumin concentrations maintained the initial differences thorough the period. Albumin has a slow turnover rate (Eckersall, 2008) and demonstrates a long-term response to inflammatory conditions (Nicholson et al., 2000). Cholesterol (as an indirect measure of lipoproteins; Bruss, 2008)

was lower in LO than the other groups before dry-off and in the early dry period. Decreases in plasma cholesterol are related to inflammatory conditions (Bionaz et al., 2007; Bertoni et al., 2008). Cholesterol and paraoxonase are strictly related because they are both within lipoproteins, and paraoxonase has an antioxidant role. Also, paraoxonase maintained the stratification throughout the period. Indeed, the greatest differences were observed in the first two weeks after calving. Bionaz et al. (2007) observed a decrease in plasma paraoxonase around calving due to reduced liver synthesis of its carrier HDL. However, in cows with higher paraoxonase concentration during the dry period, the reduction they observed was modest, as was the case of HI cows in our study. These results suggest that specific metabolic and inflammatory phenomena alter the concentration of paraoxonase in lipoproteins (Trevisi et al., 2012b). Notably, bilirubin concentration increased after dry-off, probably due to the stress caused by abrupt milking cessation, feeding, and group changes (Bertulat et al., 2013). Bilirubin is not a "true" –APP, but its concentration increases when liver synthesis of the enzymes responsible for its clearance is reduced (Tennant and Center, 2008). However, the bilirubin response to dry-off was somewhat reduced in HI compared to other groups. The same trend was observed after calving when the magnitude of the differences was greater. Retinol concentrations (considered an indicator of retinol-binding protein; Bertoni et al., 2008) were steadily higher in HI than in other groups. Inflammation can affect retinol-binding protein synthesis in the liver (Bertoni and Trevisi, 2013), leading to a reduction of retinol concentration in plasma.

Reactive oxygen metabolites, considered an oxidative stress biomarker, was lower in HI than other groups thorough the period, suggesting reduced oxidative stress in cows with higher AG despite the higher milk production. This result agreed with biomarkers of inflammation, as suggested by Sordillo and Aitken (2009). These authors highlighted the relationship between oxidative stress, inflammation, and susceptibility to diseases during the transition period.

Variation in inflammation between groups was confirmed by plasma concentration of calcium and zinc. Calcium concentrations were lower in LO than in the other groups. In the first days after dry-off, calcemia is expected to increase due to the milk cessation. Milk calcium demand decreases, and paracellular transport of calcium into the blood increases because of the weakening of tight junctions between mammary epithelial cells caused by milk accumulation in the udder (Putman et al., 2018). However, in the LO group, calcemia decreased after dry-off relative to the lactation's level. Calcium concentration usually decreases during acute inflammation (Bertoni and Trevisi, 2013). A decrease in blood calcium level was reported by Zebeli et al. (2010) as a result of rumen acidosis and by Waldron et al. (2003) following lipopolysaccharide infusion. Another possible explanation could be related to vitamin D, which regulates calcium homeostasis, and its deficiency correlates with inflammation (Guillot et al., 2010). Nonnecke et al. (2014) observed a strong decrease in serum

vitamin D during the calves' acute-phase response. Calcium and magnesium are closely related. During the dry period, magnesium concentrations steadily declined in LO. There was a subsequent sudden drop in IN magnesemia the first week after calving. However, in our study, differences were unlikely to be related to dietary intake since diets were the same for all the cows. Zinc levels were lower in LO, especially around calving. During the acute-phase response, zinc is sequestered into hepatocytes by metallothionein, the synthesis of which is enhanced by proinflammatory cytokines (Rink and Kirchner, 2000).

Greater BHB concentrations were observed in HI than LO fourteen days after calving, but at levels under the subclinical ketosis threshold. There are two possible reasons behind this increase in BHB. The first one could be the greater milk production of HI cows. The post-calving slightly lower glucose concentration in HI than LO cows also supported this hypothesis. Increased milk yield also led to increased lactose demand and, consequently, reduced blood glucose concentration (Aschenbach et al., 2010). The other possible explanation for the difference in BHB in early lactation could be related to liver function. From previous research, high BHB concentrations are usually related to liver dysfunction (Rodriguez-Jimenez et al., 2018; Mezzetti et al., 2019). However, as observed previously, in HI cows the liver was in overall better condition and BHB levels were only slightly higher. Therefore, this difference can be traced back to the greater milk production.

The LFI, a composite index of liver function in early postpartum, is consistent with previous observations of metabolic and inflammatory biomarkers (Trevisi et al., 2012a). Notably, the LFI differences reflected the AG stratification performed approximately 60 days before. Therefore, inflammatory condition before dry-off likely affected also postpartum condition. It is noteworthy that LFI incorporates changes in –APP (albumin, cholesterol, and bilirubin) in the 1st month of lactation to evaluate immune, inflammatory, and metabolic status in periparturient dairy cows (Bertoni and Trevisi, 2013). In contrast, AG is simpler to determine and, being calculated before dry-off, could provide key information about management during the transition period.

2.5.3 Performance

HI cows produced more milk during the first month of lactation than LO cows, whereas IN cows' milk yield was intermediate between the two other groups. Since BCS did not reveal any difference among groups, we hypothesized that there was no difference in the mobilization of body reserves to support this increased milk yield. Greater feed intake could explain the difference in milk production. Additional production may also be related to the improved inflammatory status observed in HI. Furthermore, the relationship between inflammation and milk yield in the transition period is well known. Bertoni et al. (2008) reported lower milk yield in early lactating cows with lower liver activity index (LAI) based on plasma –APP. Similarly, Huzzey et al. (2015) observed lower milk

yield in cows with higher plasma haptoglobin concentrations around calving. Furthermore, we grouped cows in this study according to the inflammatory status before dry-off, and the trend of inflammation biomarkers observed at dry-off reflected those around calving.

The FSI was used to summarize overall fertility condition because has the advantage of considering simultaneously days open, pregnancy risk, and culling risk. FSI scores in our study are far from the optimal (86 points), due to long calving to conception intervals and low pregnancy risk to first service. However, similar to milk production, fertility performance was better in HI cows. Castro et al. (2012) showed the influence of metabolic status during the dry period on fertility, highlighting the relationship between first ovulation and energy status during the dry period. Results of our study are also consistent with the observation that inflammatory status affects fertility outcomes and confirms the inverse relationship between inflammation and fertility (Bertoni et al., 2008; Ribeiro et al., 2016; Roche et al., 2018). Higher culling rate, both before and after the first insemination, observed in LO group could be related to higher parity, since culling risk usually increases with parity (De Vries et al., 2010; Pinedo et al., 2010).

2.5.4 Health status

The total number of cows diagnosed with at least one disease did not differ among groups. However, distinguishing the disorders in those noted before and after calving, we observed a marked difference in lameness diagnosed before calving. This observation might represent one of the reasons for the different inflammatory status observed before dry-off. Lameness is related to acute-phase response and increased haptoglobin blood concentrations (Smith et al., 2010). However, the cows in this study suffered from minor lameness, mostly identified after routine hoof trimming performed before dry-off. The trimming operation probably resolved the issue, evident after calving. In fact, during the first month of lactation, no case of lameness was recorded. After calving, a higher prevalence of retained placenta in LO than HI was noted. An increase in +APP (mainly haptoglobin) is related to infectious diseases (Skinner et al., 1991). However, most of the studies have usually focused on early postpartum haptoglobin concentrations (Huzzey et al., 2009; Pohl et al., 2015). To the best of our knowledge, no clear relationship was observed between inflammation before dry-off and the risk of developing uterine diseases.

2.6 Conclusions

The inflammatory status before dry-off influences a cow's condition during pregnancy and in the following lactation. Cows living under the same conditions experienced different inflammatory conditions in late lactation. These conditions resulted in different metabolic and inflammatory responses after dry-off, which were maintained in the following early lactation. Plasma protein profile

analysis, summarized by AG, highlighted its potential utility in predicting the outcome of the subsequent lactation. High AG before dry-off is related to superior adaptation to stresses in the lactation cycle (e.g., dry-off, calving), increased milk yield, reduced SCC, and better fertility. Further studies are needed to investigate the relationship between AG and the cow's status and performance in different lactation stages.

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Chapter 3: Drying-off Cows With Low Somatic Cell Count With or Without Antibiotic Therapy: A Pilot Study Addressing the Effects on Immunometabolism and Performance in the Subsequent Lactation

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3.1 Abstract

Pressure aimed at reducing the use of antibiotics in livestock is steadily increasing. In 2022, the prophylactic use of antibiotics for preventive purposes will be banned in the European Union (EU), including blanket therapy at dry-off. The objective of this study was to evaluate the short- and long-term effects of selectively treating cows with low somatic cell count (SCC) at dry-off using internal teat sealant with or without antibiotic therapy on udder health, milk production, metabolic, and inflammatory conditions through the next lactation. Fifteen Holstein dairy cows without intramammary infections and with SCC less than 200 x10^3 cells/mL at dry-off were enrolled in the study. Cows were abruptly dried off and treated either with antibiotic plus teat sealant (AB) or with teat sealant only (TS). Milk and blood samples were collected on scheduled days from 10 days before dry-off to 28 days after calving. Milk composition and both inflammatory and metabolic profiles were assessed. Fertility and milk production were recorded during the previous and subsequent lactation. Rumination time was monitored from three weeks before dry-off to four weeks after calving. Data were analyzed with PROC MIXED and PROC GLM of SAS software.

Total milk production, reproductive performance, rumination time, and mastitis incidence did not differ between groups. Milk yield during the first 120 days after calving was not affected by treatment. Milk composition and SCC were not affected during the first month. Compared with AB, TS had lower plasma calcium at –47 days from calving, gamma-glutamyl transferase at –50, –47, and –42 DFC, tocopherol at –65, –50, and –47 DFC, and alkaline phosphatase at 3 days from calving. Overall, both metabolic and inflammatory statuses were similar between AB and TS cows with only small transient differences. With the perspective of reducing antibiotic usage in dairy farms, internal teat sealant could be used alone in healthy cows with low SCC with no relevant effects on udder health and immunometabolic profile in the subsequent lactation.

***Keywords*:** Selective dry-cow therapy; Mastitis; Somatic cell count; Antimicrobial reduction

3.2 Introduction

Antimicrobial resistance represents a relevant concern for environment, the animal, and human health. An increasing amount of social and political pressure is being applied with respect to decreasing the use of antibiotics in animal husbandry in view of the One Health approach (Trevisi et al., 2014; ECDC/EFSA/EMA, 2017; Hernando-Amado et al., 2019). In dairy farms, one of the main sources of antibiotic use is for the control of intramammary infections (IMI; Kuipers et al., 2016). In particular, the dry-off contributes significantly to antibiotic use in this sector (Krömker and Leimbach, 2017). In fact, this routine practice consists in milking cessation approximately 60 days before the expected calving supported, in several cases, by antibiotic therapy.

Blanket dry cow therapy with the infusion in each quarter of intramammary antibiotic was traditionally performed to face udder infections and reduce the risk of developing mastitis in the early stages of subsequent lactation (Neave et al., 1969; Bradley and Green, 2004; Dufour et al., 2011). However, according to European Union (EU) Regulation 2019/6, the prophylactic use of antibiotics for preventive purposes in livestock will be banned in Europe starting in 2022.

In modern herds, the average bulk tank somatic cell count (SCC) is decreasing, and a remarkable number of animals do not suffer from IMI before dry-off. Therefore, avoiding antibiotic therapy in those cows is possible by adopting selective dry-cow therapy (DCT). The latter method consists in treating only cows with IMI and/or those with high milk SCC (Berry and Hillerton, 2002). Moreover, the application of internal teat sealant (TS) improves the protective effect of the keratin plug during the dry period. The use of TS was proven to be effective against new IMI during the dry period and early lactation both alone and in association with antibiotic treatment (Rabiee and Lean, 2013; Golder et al., 2016). Nevertheless, immunometabolic parameters have not been monitored in these animals to verify their adaptive mechanisms in the subsequent lactation period.

Several protocols for selective DCT have been proposed, and observed outcomes were shown to be different. Selective DCT could cause an increase in the incidence of IMI in the next lactation (Berry and Hillerton, 2002; Halasa et al., 2009b) and a reduction in the recovery rates of existing IMI (Halasa et al., 2009a). However, the reduction in antibiotic usage achieved at dry-off is not compensated by the amount of antibiotic used to treat a new IMI (Scherpenzeel et al., 2014; Vanhoudt et al., 2018). In the Netherlands, in which preventive use of antimicrobials was banned in 2013, intramammary antibiotic use (both at dry-off and at any point in time) decreased without producing any major negative effects on udder health (Santman-Berends et al., 2021). Furthermore, selective DCT should not result in additional costs and losses for the farmers if implemented appropriately (Scherpenzeel et al., 2018; Rowe et al., 2021). Thus, optimizing selective DCT protocols at the cow-level with a long-term perspective is required.

The main concerns in the selective DCT approach are related to the correct identification of the presence of IMI before dry-off and the evaluation of the long-term effects of the selective DCT on untreated cows. Criteria usually applied are based mainly on SCC at dry-off and mastitis history (Rajala-Schultz et al., 2011), results of milk culture (Cameron et al., 2013; Vasquez et al., 2018), and/or the California Mastitis Test (Swinkels et al., 2021). Usually, a threshold of 200 $x10^3$ cells/mL is set to distinguish healthy cows from those at risk of mastitis (Dohoo and Leslie, 1991). Additionally, many studies focused only on early lactation effects regardless of long-term effects on cow udder health, productive and reproductive performances, and metabolic and inflammatory status.

We hypothesized that avoiding antibiotic therapy at dry-off in favor of using TS alone in low-SCC cows could contribute to a reduction in antibiotic usage in dairy farms without impairing animal health and welfare. The aim of the present study was to compare udder health, milk yield and composition, rumination behavior, plasma metabolic traits, and inflammatory profile before and after dry-off in AB and TS cows free of IMI that had low milk SCC.

3.3 Materials and Methods

3.3.1 Experimental design and animal management

The trial was performed on the experimental dairy farm of Università Cattolica del Sacro Cuore (San Bonico, Piacenza, Italy) according to the Italian laws on animal experimentation (Italian Health Ministry authorization N 444/2019-PR in agreement with D. Lgs. n. 26, 04/03/2014). Fifteen Holstein dairy cows (parity 2.9 ± 1.2; average milk production in previous lactation 10,865 ± 2419 kg; 365.8 ± 80.2 days in milk; mean ± SD) were regularly monitored from the week before dry-off thorough the subsequent lactation.

Table 3.1. Ingredients and chemical composition of diets served as TMR during the study

	Dry cows	Lactating cows
Ingredient, % of DM		
Corn silage	11.6	32.6
Alfalfa hay	-	24.9
Corn ground	-	13.8
Soybean meal	4.8	11.4
Barley ground	-	9.2
Wheat silage	47.5	3.3
Sunflower meal	5.1	2.0
Mineral and vitamin	0.9	1.9
Hydrogenated fat	-	0.9
Straw	17.7	-
Grass hay	12.4	-
Chemical		
NE_L, Mcal/Kg	1.28	1.65
CP, % of DM	12.7	16.6
NSC, % of DM	9.7	30.0
NDF, % of DM	57.0	33.4
Calcium, % of DM	0.45	0.79
Phosphorus, % of DM	0.34	0.42

During lactation, cows were milked twice a day, i.e., at 05.00 and 17.00, and received lactation diet. After the last milking, cows were abruptly dried off 55.4 ± 3.6 days before expected calving, approximately around –55 days from calving (DFC). After dry-off, all animals received only grass

hay for 10 days. Afterward, dry period total mixed ration was provided. After calving, cows received a lactation diet and were milked again twice a day. Feeds and diet composition are shown in Table 3.1.

About 15 days before dry-off (–70 DFC), milk samples from each quarter were aseptically collected for bacteriological analysis. At the same timepoint, composite milk samples were collected for SCC determination. Cows with at least one-quarter positive for bacterial culture before dry-off or SCC over 200 x10^3 cells/mL were excluded from the trial.

Cows with SCC < 200 x10^3 cells/mL and with all quarters negative to bacteriological culture were randomly dried-off either with a single infusion in each quarter of intramammary antibiotic (Mamyzin A; Boehringer Ingelheim Animal Health Italia S.p.A) coupled with internal teat sealant (Noroseal, Norbrook Laboratories Limited; AB, n = 7) or with teat sealant only (TS, n = 8). The main characteristics of animals involved in the trial are shown in Table 3.2. Allocation to the different DCT was performed assigning randomly one cow to a treatment and the following one to the opposite. Only one of the authors, who performed the allocation and the DCT, was aware of the treatment assigned.

Table 3.2. Main characteristics recorded in the previous lactation of dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8).

	Treatment			
	AB	TS	SEM[1]	*P*-value[2]
Parity, n	1.70	2.00	0.46	0.66
Mature equivalent milk yield, kg	12419	11386	526	0.18
Services/conception, n	2.70	3.30	0.70	0.59
Days open, d	117	141	24.8	0.49
Lactation length, d	342	366	23.8	0.48
Clinical mastitis cases, n	1	2	-	0.43
SCC, x10^3 cells/mL	92.4	107.3	17.5	0.79
Dry period length, d	51.1	53.3	1.9	0.41
FSI[4]	53.2	16.3	-	-

[1] Highest standard error of the mean (SEM)

[2] *P*-value of grouping effect

[3] Fertility status index (Esslemont and Eddy, 1977)

3.3.2 Blood samples and immunometabolic profile assessment

Blood samples were collected in two different moments, i.e. around the day of dry-off (–15, 0, 3, and 10 days) and the calving date (–3, 3, and 28 DFC). Before the morning feeding, blood was collected via jugular venipuncture into heparinized collection tubes and immediately cooled in a water bath containing ice. An aliquot of whole blood was centrifuged to determine packed cell volume,

while the remaining was centrifuged at 3500×g for 15 min at 4 °C. Afterward, plasma was stored at –20 °C for subsequent tests.

According to Calamari et al. (2016), several parameters were analyzed using a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA): glucose, cholesterol, urea, calcium, phosphorus, magnesium, sodium, zinc, ceruloplasmin, total protein, albumin, globulin, aspartate amino transferase-glutamate oxaloacetate transaminase (AST-GOT), gamma-glutamyl transferase (GGT), bilirubin, alkaline phosphatase, haptoglobin, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), creatinine, paraoxonase, myeloperoxidase, ferric reducing antioxidant power (FRAP), thiol groups, reactive oxygen metabolites (ROM), triglycerides, retinol, tocopherol, and β-carotene. Albumin-to-globulin ratio (A:G) was calculated. To evaluate the consequences of inflammation occurring at or around calving time, plasma concentrations of albumin, bilirubin, and cholesterol at 3 and 28 DFC were used to calculate the Liver Functionality Index (LFI) according to Bertoni and Trevisi (2013). The LFI varies between –12 and +5 points, and values above zero were considered favorable.

3.3.3 BCS, rectal temperature, health status, fertility, and rumination time

Consistent with blood sampling time points, body condition score (BCS) and body temperature were measured. BCS was evaluated by the same operator with a 1- to 4- point scale (ADAS, 1986), rectal temperature was measured with a digital thermometer, and health status was monitored by the personnel and veterinarian staff operating on the farm facilities. Diseases and detection dates were registered, and mastitis, particularly, was diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis of suspicious cases. Results of pregnancy diagnosis, number of artificial inseminations (AI), and days open were recorded, and fertility status index (FSI) was calculated according to Esslemont and Eddy (1977). Rumination time was recorded daily for three weeks before and after dry-off and before and after calving using the Hr-LD tags (SCR by Allflex, Netanya, Israel).

3.3.4 Milk yield and composition

From 4 to 120 DFC, milk yield was automatically recorded daily in the milking parlor and expressed as weekly average. Representative milk samples were collected at –70, 7, 14, 21, and 28 DFC for milk composition assessment and SCC determination. Milk fat, protein, lactose, casein, urea, milk solids, solids-non-fat were assessed using a Milkoscan FT120 analyzer (Foss Analytics, Hillerød, Denmark), and SCC was analyzed by Fossomatic 180 (Foss Analytics).

At –70 DFC, milk samples were aseptically collected from each quarter into 5-mL sterile tubes. Samples were immediately refrigerated and transferred at “Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna” in which they were analyzed within 24 h from

the time of collection. For bacterial culture, 10 μL of milk was spread on plates of blood agar, Tallium Kristalviolette Tossin, and Gassner Agars (selective media for *Streptococci* and *Enterobacteriaceae*, respectively). Plates were incubated at 37 °C for 72 h and examined daily (Adkins et al., 2017). If at least a quarter was positive for bacteria, the cow was considered to have an IMI and therefore excluded from the study.

3.3.5 Statistical analysis

Data are presented as least squares means and standard error. All statistical analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC). SCC was presented as geometric means. Data were tested for normality using the Shapiro–Wilk test. Variables that did not satisfy these assumptions (milk fat and solids, rectal temperature, plasma haptoglobin, bilirubin, and NEFA) were log-transformed and reported as back-transformed data. Data for plasma biomarkers, rumination time, milk production, and composition were analyzed with analysis of variance (ANOVA) using a mixed model (MIXED procedure in SAS). The model included the fixed effect of treatment (DCT; AB and TS), the effect of time (T), and the interaction treatment × time effect (DCTxT); cows nested within the groups were included as the random effect. The covariance structure (compound symmetry, autoregressive order, or spatial power) with the lowest AICC (Littell et al., 1998) was included in the MIXED model. Data concerning general characteristics of cows in the previous and subsequent lactation were analyzed using a general linear model (GLM procedure), considering only DCT as a fixed effect. Mastitis incidence was compared with Fisher's exact test (FREQ procedure). The post-hoc pairwise comparison was performed using the least significant difference (LSD) test when the F-test of one of the factors was significant at $P < 0.10$. Statistical significance was declared as $P < 0.05$ and tendencies at $P < 0.10$.

3.4 Results

3.4.1 General characteristics

The main characteristics of the study groups during the lactation preceding the dry-off are reported in Table 3.2. Overall, the two groups were balanced with no significant differences in terms of parity, milk production, SCC, and clinical mastitis incidence during the previous lactation. Moreover, reproductive performance and average dry period length were similar between groups. At dry-off, milk yield, SCC, and rectal temperature were similar between groups (Table 3.3).

The effects of different DCT on main reproductive and udder health traits in the subsequent lactation are presented in Table 3.4, and no significant differences were observed between treatments. Nevertheless, cows treated with AB had better FSI (63.5 and 4.0 for AB and TS, respectively).

Table 3.3. Milk yield, somatic cell count, and rectal temperature at dry-off in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8).

	Treatment			
	AB	TS	SEM[1]	*P*-value[2]
Milk yield, Kg/d	19.6	15.1	2.6	0.23
SCC, x10^3 cells/mL	41.4	87.3	11.2	0.11
Rectal temperature, °C	38.7	38.7	0.13	0.99

[1] Highest standard error of the mean (SEM)
[2] *P*-value of grouping effect

Table 3.4. Main characteristics of dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8) during the subsequent lactation

	Treatment			
	AB	TS	SEM[1]	*P*-value[2]
Services/conception, n	2.71	2.88	0.79	0.88
Days open, d	102.4	158.1	27.9	0.17
Lactation length, d	328.3	379.3	27.8	0.20
Mastitis, n	2	4	-	0.30
SCC, x10^3 cells/mL	53.1	101.0	15.0	0.17
LFI[3]	0.91	1.03	0.47	0.86
FSI[4]	63.5	4.0	-	-

[1] Highest standard error of the mean (SEM)
[2] *P*-value of grouping effect
[3] Liver functionality index (Bertoni and Trevisi, 2013)
[4] Fertility status index (Esslemont and Eddy, 1977)

3.4.2 Milk production and rumination time

AB and TS cows produced a similar amount of milk during the first 120 days of lactation (Fig. 3.1). Milk composition from 7 to 28 DFC was affected by time (T; $P < 0.05$; Table 3.5), except for urea, but was not affected by treatment.

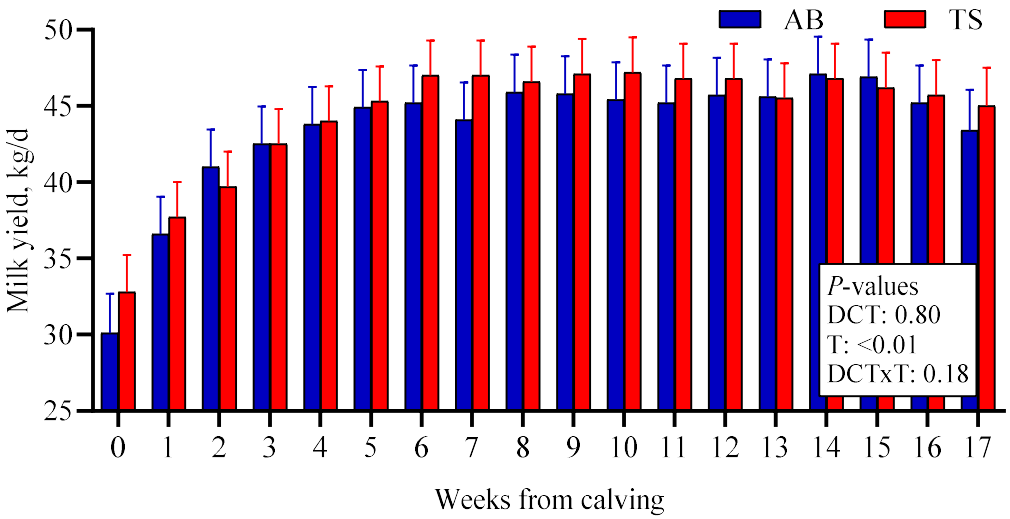

Figure 3.1. Milk yield (Kg/d) in the first 17 weeks of lactation in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8). Data presented as least squares means ± standard error. DCT is the effect of treatment, T is the effect of sampling day, and DCTxT is the effect of interaction between treatment and sampling day (*; $P \leq 0.05$).

Table 3.5. Mean milk composition in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8) in the first 28 days of the next lactation.

	Treatment			*P*-value[2]		
	AB	TS	SEM[1]	DCT	T	DCTxT
SCC, x10^3 cells/mL	26.6	30.6	4.02	0.76	<0.01	0.59
Fat, mg/100 mL	4.10	4.06	0.13	0.82	<0.01	0.96
Protein, mg/100 mL	3.57	3.50	0.05	0.31	<0.01	0.92
Lactose, mg/100 mL	5.08	5.14	0.03	0.27	<0.01	0.53
Casein, mg/100 mL	2.66	2.64	0.04	0.66	<0.01	0.77
Urea-N, mg/dL	16.2	17.3	3.88	0.85	0.96	0.22
Milk solids, %	12.9	13.0	0.23	0.71	0.01	0.72
Solids-non-fat, %	9.14	9.11	0.07	0.72	<0.01	0.68

[1] Highest standard error of the mean (SEM).
[2] *P*-value of treatment effects: DCT = effect of treatment; T = effect of sampling day; DCTxT = effect of interaction between treatment and sampling day.
* Variable log-transformed and presented as back-transformed.

Similar trends in rumination time were observed between groups both around dry-off and calving, but AB cows had greater rumination time than TS cows 16 days before calving ($P = 0.02$; Fig. 3.2).

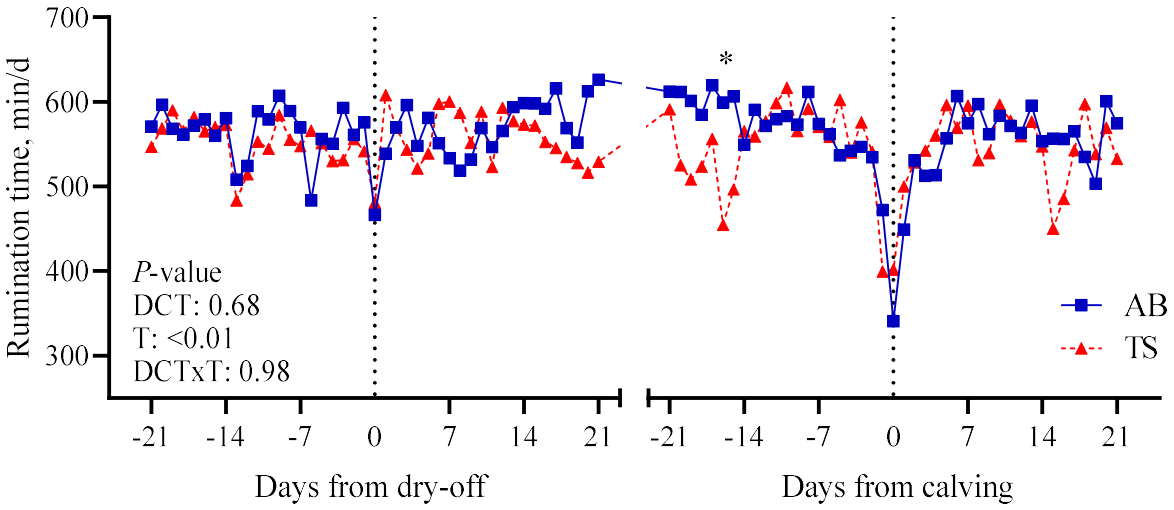

Figure 3.2. Daily rumination time (min/d) for 3 weeks before and after dry-off and before and after calving in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8). DCT is the effect of treatment, T is the effect of sampling day, and DCTxT is the effect of interaction between treatment and sampling day (*; $P \leq 0.05$).

3.4.3 BCS, rectal temperature, and immunometabolic profile

Least-squares means of BCS, rectal temperature, and blood biomarkers are reported in Table 3.6. All analyzed variables were affected by time (T; $P < 0.05$) and BCS, rectal temperature, and PCV did not differ between groups. Among minerals, calcium had a greater increase after dry-off in AB than TS (DCTxT; $P = 0.07$; Fig. 3.3A) with the greatest difference observed at –52 DFC (DCTxT; $P < 0.01$). No differences were observed in the plasma concentrations of other minerals.

Among liver function biomarkers, alkaline phosphatase revealed an interaction DCTxT due to the greater increase of its concentration observed around calving in AB when compared with TS ($P = 0.02$; Fig. 3.3B). GGT was higher in AB than TS (DCT; $P = 0.05$; Fig. 3.3C), particularly after dry-off (–52 and –45 DFC), whereas GOT was not different between treatments. Tocopherol concentrations tended to be higher in AB than TS around the drying-off period (DCT; $P = 0.09$, Fig. 3.3D), whereas retinol and β-carotene did not differ between groups. In addition, inflammatory, oxidative stress, and energy metabolism biomarkers were not affected by either treatment or the interaction with time.

Table 3.6. Body condition score, rectal temperature, packed cell volume, and plasma concentrations of immunometabolic biomarkers in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8) from 15 days before dry-off to 28 days after calving

	Before dry-off		Dry period		Lactation			*P*-value[3]		
Item[1]	AB	TS	AB	TS	AB	TS	SEM[2]	DCT	T	DCTxT
BCS	2.61	2.73	2.66	2.72	2.49	2.59	0.07	0.38	<0.01	0.31
* Temperature, °C	38.6	38.7	38.6	38.7	38.7	38.8	0.07	0.29	0.03	0.63
Packed cell volume	0.32	0.33	0.33	0.34	0.32	0.32	0.01	0.43	0.02	0.80
Calcium, mmol/L	2.62	2.58	2.70	2.64	2.44	2.48	0.02	0.28	<0.01	0.07
Chlorine, mmol/L	104.6	104.4	105.7	105.7	103.5	102.4	0.40	0.54	<0.01	0.84
Phosphorus, mmol/L	1.85	1.76	2.25	2.16	1.78	1.63	0.07	0.29	<0.01	0.92
Potassium, mmol/L	1.05	1.03	0.91	0.89	1.03	0.94	0.11	0.99	<0.01	0.28
Magnesium, mmol/L	145.1	145.2	146.6	147.2	145.5	145.6	0.02	0.17	<0.01	0.52
Sodium, mmol/L	4.20	4.23	4.23	4.30	4.10	3.95	0.50	0.63	<0.01	0.84
Zinc, µmol/L	15.4	15.7	17.0	15.8	13.9	14.9	0.55	0.89	<0.01	0.44
GOT, U/L	94.3	87.6	84.8	79.3	99.7	98.8	3.30	0.32	<0.01	0.99
GGT, U/L	30.5	25.5	28.9	24.1	24.1	22.3	1.40	0.05	<0.01	0.67
Alkaline phosphatase, U/L	50.5	52.3	59.8	52.4	56.3	44.3	5.00	0.39	<0.01	0.02
Total protein, g/L	78.2	77.2	77.5	77.0	77.3	77.2	1.70	0.82	<0.01	0.65
Albumin, g/L	38.9	37.8	37.9	36.6	36.9	36.5	0.40	0.13	<0.01	0.47
Globulin, g/L	39.3	39.4	39.6	40.4	40.4	40.7	1.50	0.84	<0.01	0.80
A:G	1.00	0.97	0.97	0.92	0.94	0.92	0.04	0.54	<0.01	0.76
* Haptoglobin, g/L	0.14	0.15	0.18	0.32	0.47	0.34	0.03	0.71	<0.01	0.51
Ceruloplasmin, µmol/L	2.10	2.00	2.35	2.26	2.62	2.49	0.06	0.22	<0.01	0.79
* Bilirubin, µmol/L	1.69	1.71	2.46	2.22	3.85	3.69	0.20	0.83	<0.01	0.86
Myeloperoxidase, U/L	318.7	315.3	371.7	380.5	443.4	445.5	16.1	0.88	<0.01	0.89
Cholesterol, mmol/L	5.00	4.40	3.72	3.41	2.96	3.00	0.18	0.25	<0.01	0.33
Paraoxonase, U/mL	100.8	97.0	98.1	92.6	90.6	85.9	5.40	0.53	<0.01	0.98
ROM, mgH_2O_2/100mL	11.6	11.6	13.5	13.3	14.9	14.3	0.41	0.62	<0.01	0.85
FRAP, µmol/L TE	145.6	134.7	119.4	117.0	128.5	137.6	3.80	0.77	<0.01	0.47
SHp, µmol/L	379.6	382.3	364.7	370.7	368.5	373.4	8.40	0.69	<0.01	0.99
Glucose, mmol/L	4.22	4.39	4.36	4.34	3.98	4.05	0.07	0.57	<0.01	0.72
* NEFA, mmol/L	0.16	0.14	0.27	0.21	0.41	0.42	0.03	0.44	<0.01	0.89
BHB, mmol/L	0.57	0.47	0.42	0.41	0.64	0.58	0.03	0.24	<0.01	0.16
Urea, mmol/L	5.04	4.88	3.81	3.66	4.18	4.33	0.15	0.73	<0.01	0.42
Creatinine, µmol/L	94.4	92.6	96.3	94.8	88.5	93.4	1.90	0.93	0.02	0.25
Triglycerides, mmol/L	0.13	0.13	0.24	0.23	0.09	0.10	0.01	0.78	<0.01	0.46
Retinol, µg/100 mL	46.9	48.1	35.5	35.9	33.1	36.5	2.00	0.60	<0.01	0.42
Tocopherol, µg/mL	6.93	5.64	4.06	3.36	2.79	2.71	0.28	0.09	<0.01	0.51
β-carotene, µg/100 mL	0.67	0.59	0.49	0.39	0.24	0.26	0.07	0.50	<0.01	0.29

[1] GOT = glutamic oxaloacetic transaminase; GGT = γ-glutamyltransferase; A:G = albumin-to-globulin ratio; ROM = reactive oxygen metabolites; FRAP = ferric-reducing ability of plasma; NEFA = non esterified fatty acids; BHB = β-hydroxybutyrate.

[2] Highest standard error of the mean (SEM).

[3] *P*-value of treatment effects: DCT = effect of treatment; T = effect of sampling day; DCTxT = effect of interaction between treatment and sampling day.

* Variable log-transformed and presented as back-transformed.

[#] 95% confidence interval: before dry-off (0.90-1.10 for AB and 0.88-1.07 for TS); dry period (0.88-1.07 for AB and 0.83-1.01 for TS), lactation (0.84-1.04 for AB and 0.82-1.01 for TS).

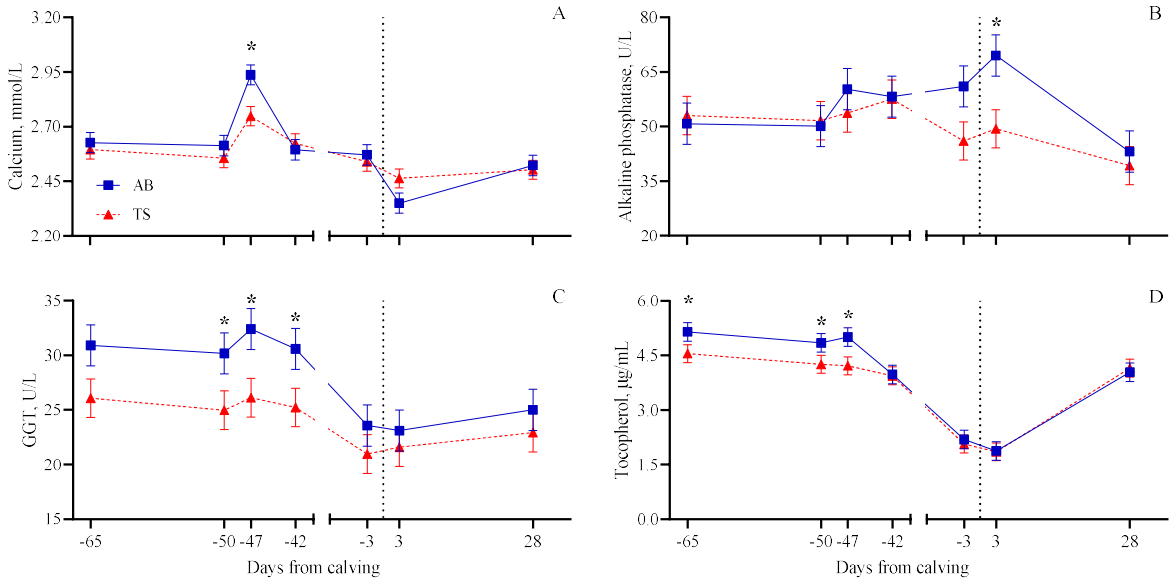

Figure 3.3. Plasma concentrations of calcium (A), alkaline phosphatase (B), γ-glutamyltransferase (GGT; C), and tocopherol (D) in dairy cows with low somatic cell count at dry-off (< 200 x10^3 cells/mL) treated either with antibiotic dry-cow therapy (AB, n = 7) or with internal teat sealant alone (TS, n = 8). Differences ($P \leq 0.05$) between treatments at each time point are denoted with an asterisk (*).

3.5 Discussion

In this preliminary study, we evaluated the effects of selective dry-cow therapy on milk production, udder health, metabolism, and inflammatory status during the dry period and the subsequent lactation with the final goal of understanding whether reduction antibiotic use is feasible in the dairy sector. Cows enrolled were clinically healthy, negative for bacteriological culture (all four quarters), and had low SCC (< 200 x10^3 cells/mL). The threshold of 200 x10^3 cells/mL is commonly used in similar studies (Vasquez et al., 2018; Lipkens et al., 2019) to distinguish healthy cows from those having IMI (Dohoo and Leslie, 1991). Although they might be clinically healthy, cows with SCC over 200 x10^3 cells/mL are at greater risk of developing IMI during the dry and early lactation periods. Therefore, we focused on low-SCC cows and compared the use of TS alone versus the combination of TS and AB to evaluate the effects of avoiding AB at dry-off in cows with healthy udders. Previous studies have shown promising results, and no difference in the likelihood of contracting mastitis was detected during the first 100 (Bradley et al., 2010) and 120 days in milk (Cameron et al., 2014) between uninfected quarters treated with antibiotic plus teat sealant and teat sealant alone, respectively. Additionally, no detrimental effects on milk microbiome and bacterial

load were observed in cows treated with teat sealant only or antibiotic therapy at dry-off (Bonsaglia et al., 2017; Biscarini et al., 2020). According to Winder et al. (2019), the risk of IMI at calving in selectively treated cows was found to be higher than blanket DCT cows, but when internal TS is used, this difference vanishes.

The number of involved subjects was limited; therefore, the observation of some significant differences might be affected by a certain lack of power. Moreover, other factors not in our control could influence the outcomes of DCT. Variables, such as parity or milk yield (average during lactation or at dry-off) were related to SCC and mastitis trends after calving (Niemi et al., 2021). To limit this influence in the present study, groups were balanced for those factors. In fact, cows' performances were similar in the two groups, both in the previous and subsequent lactation. At dry-off, SCC was similar between groups and far below the threshold of 200 $x10^3$ cells/mL, and during the first month of lactation, SCC was extremely low in both treatments. Interestingly, the geometric mean of SCC during the subsequent lactation was numerically higher in TS (53 versus 101 $x10^3$ cells/mL for AB and TS, respectively) but lower than the safety threshold of 200 $x10^3$ cells/mL. In a larger study, McParland et al. (2019) found similar results. These authors found that low-SCC cows treated with TS without AB had consistently a slightly higher SCC throughout lactation than cows treated with both TS and AB. In the current study, despite not being significant, the numerically higher clinical mastitis incidence (4 versus 2 for AB and TS, respectively) paired with the slightly higher SCC across the whole lactation could represent one negative outcome of the selective dry-cow therapy even though during the first month after calving, SCC was similar between treatments. An increase in SCC or IMI incidence after calving in selectively treated cows might have been expected (Berry and Hillerton, 2002; Scherpenzeel et al., 2014; Niemi et al., 2021). Differences in SCC before dry-off could have affected the results since SCC seems to be a factor associated with the odds of having high SCC after calving and of mastitis treatment during early lactation (Niemi et al., 2021). Although in the present study the numerical difference was apparent, groups were well-balanced on SCC during the previous lactation. Nevertheless, we did not repeat the microbiological culture in the next lactation period; therefore, we could not determine whether mastitis resulted from new IMI developed during the dry period or had its origin later. Moreover, increased treatments for new cases of mastitis in the TS group were compensated by the lack of antibiotic treatment before dry-off in accordance also with previous studies (Scherpenzeel et al., 2014; Vanhoudt et al., 2018). However, results of this study need to be carefully interpreted. Although cows involved in the study had low SCC at dry-off, and all tested negative on bacteriological cultures, some minor adverse effects on udder health in the long-term might have occurred in untreated cows. The limited number of enrolled animals and the large variability observed in many of the considered variables suggest a heterogenous condition of the

cows, which might be due to several individual factors and could have prevented the achievement of statistical significance between groups. On commercial dairy farms, quarter-level bacteriological analysis of milk of all the cows approaching dry-off to identify microbial infections was not feasible, and cows having an IMI despite low SCC would likely benefit from antibiotic DCT (Berry and Hillerton, 2002). However, proper selective DCT protocols that identify infected cows or those at risk of developing IMI during the dry period could reduce the use of antibiotics at dry-off without impairing cow health and performance (Vanhoudt et al., 2018; Rowe et al., 2020; Santman-Berends et al., 2021).

As expected, milk production and composition varied with time (Silvestre et al., 2009; Amalfitano et al., 2021) due to the changes in energy balance taking place at the onset of lactation (de Vries and Veerkamp, 2000; Xu et al., 2018) and to the physiological trend of the lactation curve. DCT had minimal effects on milk production during the first 120 days of lactation and on milk components during the first 28 days. The lack of differences in milk composition between AB and TS agreed with the results of Kok et al. (2021) in cows with a 60-days dry period. Despite the slightly higher SCC in TS cows after considering the whole lactation and the negative relationship between SCC and milk yield (Deluyker et al., 1993; Miller et al., 1993; Green et al., 2006), it was shown that cows in the two groups produced approximately the same amount of milk. Effects of selective DCT on milk yield are generally unclear and probably depend on criteria used to allocate cows to different therapies and also on possible IMI. In fact, McParland et al. (2019) observed lower milk production in cows treated with antibiotics, as we noted in the first week of lactation, whereas Wittek et al. (2018) reported opposite results. Other studies comparing blanket and selective DCT did not report significant differences in milk yield (Cameron et al., 2015; Rowe et al., 2020). However, the milk yield at dry-off in both treatments (particularly AB) was high and greater than the safety threshold of 15 kg/d (19.6 versus 15.1 kg/d, for AB and TS, respectively) proposed by Mezzetti et al. (2020) and Vilar and Rajala-Schultz (2020). High milk yield at dry-off is related to a prolonged involution process, increased inflammation, and altered antioxidant system (Rajala-Schultz et al., 2005; Silanikove et al., 2013; Mezzetti et al., 2020). Therefore, it would have been useful to implement strategies to reduce milk yield before dry-off in cows still producing a relevant amount of milk to produce a smooth transition from the lactating to the non-lactating phase (Zobel et al., 2015; Vilar and Rajala-Schultz, 2020).

Rumination time was similar between groups, except for the late dry period. Throughout the study period, two relevant drops were observed. The plunge observed at dry-off, most likely linked to the stresses of re-grouping and abrupt diet change and to the pain and inflammation associated with drying-off (Schirmann et al., 2011; Dancy et al., 2019; Abuelo et al., 2021), was smaller than that at

calving, and the drastic decrease in rumination time on the day of calving was consistent with previous studies (Soriani et al., 2012; Calamari et al., 2014). However, the return to pre-calving values occurred rapidly. Recorded ones were in accordance with those reported by Soriani et al. (2012) in cows with high LAI, an index that summarizes the inflammatory status during the transition period (Bertoni and Trevisi, 2013). Overall, the values recorded were similar (> 500 min/d) to those reported by other authors in healthy cows (Schirmann et al., 2016; Stangaferro et al., 2016), and do not represent a risk for cow's health and metabolism.

To the best of our knowledge, this is the first study concerning the effects of different DCT on plasma biomarkers of metabolism, inflammation, and immune function. The analysis of plasma metabolic and inflammatory profiles suggested an overall similar condition in the two groups over such a long time. Relevant changes over time were obtained for almost all markers. Samples were collected close to two critical events in the lactation cycle, dry-off and calving, both of which deeply affect metabolism and inflammation (Putman et al., 2018; Trevisi and Minuti, 2018; Mezzetti et al., 2020). The differences that were observed between treatments were minimal and limited to a single time point, and the lack of relevant differences in inflammatory and liver conditions in the transition period was supported by the LFI, which summarizes inflammatory condition and liver function after calving (Bertoni and Trevisi, 2013). The slightly greater increase in observed plasma calcium concentration in AB a few days after dry-off could be related to the greater milk production before dry-off in these cows. Blood calcium usually increases after dry-off due to the decreased calcium output in milk, and because the accumulation of milk in the mammary gland causes a weakening of the tight junctions between mammary epithelial cells, thus increasing the paracellular transport of calcium into the blood (Aslam and Tucker, 1998; Putman et al., 2018). Consistent increases in GGT and alkaline phosphatase concentrations are usually related to liver damage or fatty liver (Fernandez and Kidney, 2007; Russell and Roussel, 2007; Lopreiato et al., 2019). Therefore, the lower values in TS in comparison with AB suggest a better liver function during the critical phase of transition from lactation to dry period in these cows. However, this hypothesis was not supported by other biomarkers of liver function, such as GOT, albumin, and bilirubin. Moreover, the A:G ratio, an indicator of an inflammatory condition (Cattaneo et al., 2021), revealed overall good conditions in both groups during the dry and the postpartum periods as A:G values were fairly high (~ 1). Therefore, the small effects that avoiding AB at dry-off were likely limited to the udder and did not determine systemic metabolic and inflammatory involvement. Furthermore, although our results showed negligible differences in terms of innate immune response, oxidative stress, and metabolism between the two groups that were selected for a good udder functionality at the end of lactation, it would be advisable to monitor the overall health and inflammatory status of cows before this critical phase in larger

experiments. This assessment may allow the discovery of subjects with subclinical disorders and help adopt proper strategies that are not limited to only the intramammary antibiotic treatment to reduce the metabolic dysfunction and possible impairment of the immune defenses in the following transition period.

3.6 Conclusions

Selectively treating cows at dry-off according to SCC could represent a way to reduce antibiotic consumption in the dairy sector and comply with future EU legislation concerning veterinary medicinal products. From the results of this preliminary study, antibiotic dry-cow therapy could be avoided in cows with low SCC (< 200 $x10^3$ cells/mL) if mammary infection status is monitored, and internal teat sealant confirmed its potential to be used alone in those cows. The analysis of the immunometabolic profile highlighted a similar response to the different dry-cow therapies performed, and milk composition did not change. Nevertheless, further research enrolling a large number of cows and carefully analyzing IMI in the following lactation, SCC dynamic, and incidence of clinical mastitis is needed.

3.7 Acknowledgments

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Chapter 4: Drying-off Dairy Cows Without Antibiotic Therapy and Orally Supplemented With Lyophilized *Aloe Arborescens*: Effects on Rumen Activity, Immunometabolic Profile, and Milk Yield

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4.1 Abstract

The drying-off is a stressful stage of the lactation cycle of dairy cows, that deeply affects cows' metabolism, inflammatory status, and immune system. The promising effects observed during the transition period resulting from supplementation with *Aloe arborescens* Mill. suggest its potential utility during this phase. A group of 23 Holstein dairy cows with somatic cell count (SCC) less than 200 x10^3 cells/mL and without intramammary infections were enrolled in the study. Cows were divided into two groups: one orally receiving 10 g/day of *A. arborescens* Mill. lyophilized powder (AL; 11 cows) between –7 and 7 days from dry-off (DFD), and a control group (CTR; 12 cows). From –14 and 7 DFD and 7 and 28 days from calving (DFC), the body condition score and rectal temperature were determined, and rumen fluid, feces, milk, and blood samples were collected. Daily rumination times and milk yield were recorded. Data were analyzed through repeated measures mixed models. Compared to the CTR group, AL cows tended to show reduced production of volatile fatty acids in the rumen with acetate proportion that tended to be higher and valerate proportion that was lower. Moreover, *Aloe* supplementation caused a reduction in fecal dry matter. At the end of drying-off, AL cows presented better liver function, as suggested by higher paraoxonase plasma concentrations at 7 DFD, higher glucose, and lower urea, but showed increased reactive oxygen metabolites (ROM). *Aloe* supplementation at dry-off ameliorated inflammatory status after calving (lower haptoglobin and ceruloplasmin levels), and improved milk yield in the first weeks of subsequent lactation, without influencing milk composition, SCC, and incidence of intramammary infections. These results confirmed the positive effects of *Aloe* administration on liver function in dairy cows but indicate the need for further studies investigating the effects of *Aloe* on rumen fermentation profile and oxidative status.

Keywords: Rumen fermentation; Nutraceutical; Teat sealant; Selective dry-cow therapy.

4.2 Introduction

The dry-off is a challenging event of the lactation cycle in dairy cows. The transition from lactation to the dry period together with abrupt milking cessation, regrouping, and dietary changes, leads to a significant physiological imbalance (von Keyserlingk et al., 2008; Zobel et al., 2015). In fact, dry-off is a stressful event that causes impairment of metabolism, liver function, and antioxidant system and also triggers inflammatory responses (Bertulat et al., 2013; Mezzetti et al., 2020b; Cattaneo et al., 2021b). In particular, after dry-off, the NEFA and BHB increase due to the lower energy content of the diet (Putman et al., 2018) can harm immune system function (Lacetera et al., 2004). Moreover, mammary gland starts a period of intense remodeling, known as active involution (Hurley, 1989). During this remodeling phase, which begins two days after dry-off and lasts

approximately 21 days, susceptibility to new intramammary infections is high (Eberhart, 1986; Bradley and Green, 2004). In addition, social pressure to decrease the use of antibiotics in livestock with the aim of limiting antibiotic resistance is increasing (Trevisi et al., 2014; ECDC/EFSA/EMA, 2017). In light of this decrease, the use of selective dry-cow therapy is increasing, but cows that are not treated with antibiotic therapy at dry-off could be at a higher risk of developing new infections during the mammary remodeling phase (Scherpenzeel et al., 2014; Winder et al., 2019). However, recent evidence showed that selective dry-cow therapy can effectively reduce the use of antimicrobials at dry-off without jeopardizing udder health or milk production at the onset of lactation (Kabera et al., 2021; Weber et al., 2021).

Additionally, in cows with still high milk yield at dry-off, all these processes are exacerbated (Rajala-Schultz et al., 2005; Silanikove et al., 2013; Mezzetti et al., 2020b), and many strategies to face these issues have been proposed (Vilar and Rajala-Schultz, 2020). Most interventions that have been commonly studied and applied are related to dietary changes (Odensten et al., 2007; Ollier et al., 2014), reduction in milking frequency (Gott et al., 2017; Rajala-Schultz et al., 2018; Martin et al., 2020), or a combination of both (Tucker et al., 2009; Dancy et al., 2019). An alternative option could be represented by the administration of nutraceuticals (Bertoni et al., 2015, 2016). These products have been shown to produce positive effects on regulating immune responses and metabolism during the transition period (Lopreiato et al., 2020), but literature addressing the application of these products at dry-off is scarce.

Aloe spp. plants have been used for ages in traditional medicine for their therapeutic properties, such as wound healing, anti-inflammatory, antioxidant, antitumor, antimicrobial, and immunomodulatory effects (Singab et al., 2015). The most relevant species are *A. barbadensis* Mill. (also known as *A. vera*) and *A. arborescens* Mill. (Liao et al., 2006). *Aloe* leaves are rich in anthraquinones and their glycosides, found in the green rind, whereas the parenchyma is abundant in complex carbohydrates (Hamman, 2008). Although the effects of *Aloe* are likely due to a synergic activity of several compounds (e.g. phenolics, polysaccharides, and vitamins), its main active compounds are the anthraquinones aloin A and B (also known as barbaloin) and acemannans (Pellizzoni et al., 2012). Anthraquinones have antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Hamman, 2008), and acemannans might have an indirect antimicrobial activity because they stimulate phagocytic leukocytes (Pugh et al., 2001).

Previous research demonstrated that aloin A is detectable in the blood of dairy cows as early as 2 h after oral administration of 200 g/d of whole leaves *A. arborescens* homogenate (obtained from 3 years plants) frozen preserved and thawed just before the use (Bani et al., 2016). In the same study, the authors did not observe any adverse effects resulting from *A. arborescens* administration on feed

intake, digestibility, and rumen fermentation. In a subsequent experiment, Mezzetti et al. (2020a) fed in the same way 200 g/d of *A. arborescens* homogenate to dairy cows during the transition period. In this study, positive effects of *Aloe* on lipid mobilization, hepatic and renal function, and on mitigating the inflammatory responses typical of the calving event were detected. However, the storage, preservation, and administration of homogenate are impractical in larger settings, and lyophilization of the homogenate can address these issues.

We hypothesized that *Aloe* could improve the transition from lactation to the dry period in dairy cows due to this plant's anti-hyperlipidemic and anti-inflammatory effects, particularly in subjects that did not receive antibiotic therapy during this phase. Thus, in cows treated with internal teat sealant alone at dry-off, we evaluated the effects of 10 g/d of lyophilized *A. arborescens* Mill. powder supplementation from –7 to 7 days from dry-off (DFD) on rumen function, milk production, SCC, and hematological biomarkers in dairy cows at dry-off and during the subsequent lactation.

4.3 Materials and Methods

4.3.1 Animal management and experimental design

The research was conducted at Università Cattolica del Sacro Cuore dairy barn (Cerzoo, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation and ethics (Italian Health Ministry Authorization N 444/2019-PR in agreement with D. Lgs. n. 26, 04/03/2014). Fourteen days before dry-off (–14 DFD), sterile milk from each quarter and composite milk-were collected for bacteriological and SCC analysis, respectively. Cows with SCC less than 200 $x10^3$ cells/mL and without intramammary infections due to major pathogens were included in the study. A group of 23 Holstein dairy cows (parity expressed as mean ± standard deviation [SD] 2.43 ± 1.34), was enrolled in the study. During lactation, cows were housed in a free-stall pen, received lactation diet, and were milked twice daily (5:00 and 17:00 h). About 55 days before expected calving, cows were abruptly dried off with the infusion of internal teat sealant (Noroseal, Norbrook Laboratories Limited, Newry, UK) after the last milking. The average milk yield during the week leading to dry-off was 22.7 ± 5.7 kg/d (mean ± SD). Afterwards, they were moved to a dry pen and received only grass hay for seven days after which a diet for dry cows was administered. Rations were formulated according to the National Research Council (NRC, 2001) guidelines, and chemical composition of the rations is reported in Table 4.1. Regular checks were performed during the the study period and several samples were collected according to the schedule shown in Figure 4.1 and described below.

Table 4.1. Ingredients and chemical composition of diets served as TMR during the study

	Dry cows	Lactating cows
Ingredient, % of DM		
Corn silage	11.6	32.6
Alfalfa hay	-	24.9
Corn ground	-	13.8
Soybean meal	4.8	11.4
Barley ground	-	9.2
Wheat silage	47.5	3.3
Sunflower meal	5.1	2.0
Mineral and vitamin	0.9	1.9
Hydrogenated fat	-	0.9
Straw	17.7	-
Grass hay	12.4	-
Chemical		
NE_L, Mcal/Kg	1.28	1.65
CP, % of DM	12.7	16.6
NSC, % of DM	9.7	30.0
NDF, % of DM	57.0	33.4
Calcium, % of DM	0.45	0.79
Phosphorus, % of DM	0.34	0.42

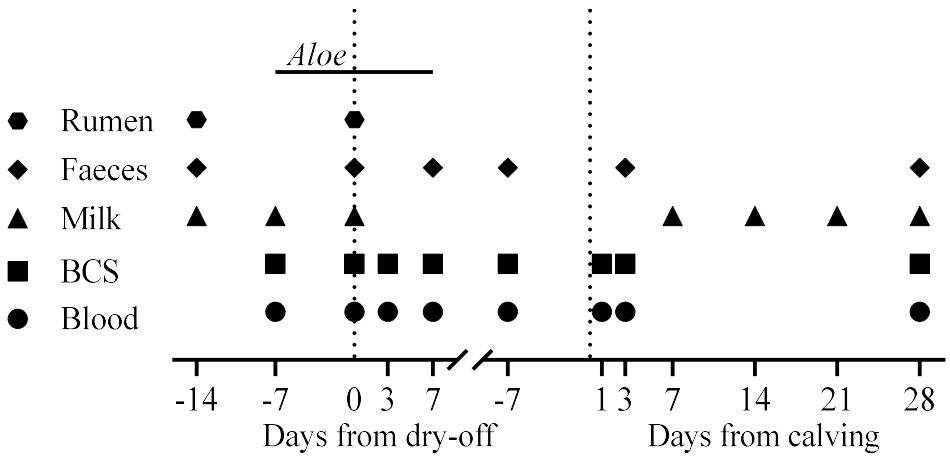

Figure 4.1. Study design reporting the scheduled time points of the different sampling procedures carried out. *Aloe arborescens* Mill. lyophilized powder was supplemented from –7 to 7 days from dry-off (DFD). Blood samples (together with BCS evaluation) were collected at –7, 0, 3, 7 DFD and –7, 1, 3, 28 days from calving (DFC); milk samples were collected at –14, –7, 0 DFD and 7, 14, 21, 28 DFC; fecal samples at –14, 0, 7 DFD and –7, 3, 28 DFC; rumen samples at –14 and 0 DFD.

Cows were selected for supplementation with 10 g/d of lyophilized *A. arborescens* Mill. whole leaves homogenate from –7 to 7 DFD (AL; n = 11) or none for the control group (CTR; n =

12). Groups were balanced for parity, previous lactation length, and SCC history. The dose of *Aloe* was calculated to provide similar DM amount as in the study of Mezzetti et al. (2020a). Since 200 g/d of homogenate *Aloe* with a DM content of about 7% were used (Bani et al., 2016), to provide a similar amount of DM we chose to use 10 g/d. Before total mixed ration (TMR) distribution, feed bunk was cleaned, all cows were restrained in the headlocks at distance to avoid cross-feeding, and each dose of lyophilized *Aloe* was mixed with 1 kg of lactation TMR and fed to AL cows, whereas CTR cows received only 1 kg of lactation TMR. During the whole trial, an operator checked that the cows ate all the supplemented TMR, and no leftovers were ever found so the treatment had to be administered in another way.

4.3.2 Aloe processing and aloin determination

A. arborescens Mill. whole leaves (Dester Gardens, Crociale di Manerba del Garda, Italy) were cut and homogenized as reported by Bani et al. (2016). Briefly, whole leaves were cut and homogenized by a vegetable cutter (model R6, Robot Coupe, Vincennes Cedex, France). A sample was collected, and the homogenate was immediately frozen in plastic bags, with no additives. Afterward, all the homogenate was lyophilized at the same time, through evaporation of water content at –5 mbar at an increasing temperature from –40 to 25° C (Biostarters S.r.l., Polesine Parmense, Italy). Lyophilizate was aliquoted and stored in the dark until use. Aloin content was determined using liquid chromatography coupled to triple quadrupole mass spectrometry via an electrospray ionization source (LC-ESI/MS/MS), as previously described (Lucini et al., 2013, 2015).

4.3.3 Rumen fluid and fecal samples

At –14 and 0 DFD, rumen fluid samples were collected before the morning feeding using a ruminal probe specially designed for cattle (Ruminator; profs-products.com, Wittibreut, Germany), as described by Wallace et al. (2019). To avoid salivary contamination, the first liter of collected rumen fluid was discarded, and the next 0.5 L was retained. For all samples, the pH of the rumen fluid was recorded immediately with a pH meter (GLP 21; Crison Instruments SA, Alella, Spain). Afterward, samples were gently mixed, and five aliquots of 1 mL each were pipetted into 2 mL tubes and immediately stored at –20 °C for volatile fatty acid (VFA) measurements. Concentrations of VFA and D- and L-lactic acid were determined as described by Minuti et al. (2014). At –14, 0, and 7 DFD and –7, 3, and 28 days from calving (DFC), fecal samples were collected manually in plastic bags from the rectal ampulla immediately after rumen sampling. For all samples, pH was determined on fresh material after mixing, as the mean of six readings in different points of the. Fecal dry matter was determined from 200g of fresh material after 96 h in a ventilated oven at 65°C.

4.3.4 Blood samples and immunometabolic profile

Blood samples were collected from the jugular vein into heparinized tubes before the morning feeding on –7, 0, 3, and 7 DFD and –7, 1, 3, and 28 DFC. Tubes were immediately cooled in a water bath containing ice. An aliquot of whole blood was centrifuged to determine packed cell volume, while the remaining sample was centrifuged at 3500×g for 15 min at 4 °C. Afterwards, plasma was stored at –20 °C for use in subsequent assays. Several parameters were analyzed using a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA) as reported by Calamari et al. (2016): calcium, phosphorus, magnesium, zinc, glucose, cholesterol, urea, ceruloplasmin, total protein, albumin, globulin, aspartate-aminotransferase (AST-GOT), γ-glutamyl transferase (GGT), alkaline phosphatase, bilirubin, haptoglobin, non-esterified fatty acids (NEFA), β-hydroxybutyric acid (BHB), creatinine, paraoxonase, myeloperoxidase, total reactive oxygen metabolites (ROM), thiol groups, advanced oxidation protein products (AOPP), ferric reducing antioxidant power (FRAP), retinol, tocopherol, and β-carotene. Albumin and globulin concentrations were used to calculate the albumin-to-globulin ratio, the liver functionality index (LFI) was calculated according to Bertoni and Trevisi (2013), and the Oxidative Status Index (OSI) was calculated as the ratio between ROM and FRAP.

4.3.5 BCS, rectal temperature, health status, and rumination time

At the time of blood sampling, the BCS was evaluated by the same operator using a 1- to 4-point scale (ADAS, 1986), and body temperature was measured with a digital thermometer. Health status was monitored, and diseases and their corresponding detection dates were registered. Mastitis was diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases. Rumination time was recorded daily with the Hr-LD tags (SCR by Allflex, Netanya, Israel) from –7 to 14 DFD, and from –14 to 28 DFC.

4.3.6 Milk yield and composition

Milk yields were automatically recorded at each milking session in the parlor and expressed as a weekly average. At –14, –7, and 0 DFD and 7, 14, 21, and 28 DFC, approximately 100 mL of milk were collected for the determination of milk composition and SCC. Milk composition (fat, protein, lactose, casein, urea) was using a Milkoscan FT120 analyzer (Foss Analytics, Hillerød, Denmark), and SCC by Fossomatic 180 (Foss Analytics). SCC was expressed as linear score (SCS; Wiggans and Shook, 1987).

At –14, –7, and 0 DFD and 7, 21, and 28 DFC, milk samples were aseptically collected from each quarter and analyzed for bacterial contamination within 24 h of the collection as described in Cattaneo et al. (2021c). Samples at -14 DFD were used to enroll the cows in the trial, to avoid the presence of pathogens that made compulsory antibiotic treatment. In subsequent samples, carried out

to provide information about intramammary infection status thorough the experiment, those tested positive for *Staphylococcus aureus*, *Streptococcus uberis*, *Enterobacter* spp., *Escherichia coli*, *Trueperella pyogenes*, and *Serratia marcescens* were considered infected by major pathogens, whereas samples tested positive for coagulase-negative staphylococci and *Corynebacterium bovis* were considered infected by minor ones. Samples positive to more than three different bacterial species were considered contaminated.

4.3.7 Statistical analysis

Sample size was calculated based on the expected anti-hyperlipidemic and anti-inflammatory effect of Aloe supplementation, considering NEFA and haptoglobin as the primary outcomes. From previous investigations in this phase (Cattaneo et al., 2021a), the required sample size in a repeated measures design to achieve a statistical power of 0.8 with $\alpha = 0.05$ and an expected effect size of 0.4 in NEFA levels was 11 subjects per group (G*Power package; Faul et al., 2007). A similar sample size was required according to haptoglobin.

Data were analyzed with SAS software, version 9.4 (SAS Inst. Inc., Cary, NC, USA). Rumination time, milk, feces, and plasma parameter data were subject to analysis of variance (ANOVA) testing using a mixed model for repeated measures (GLIMMIX Procedure, SAS Inst. Inc., Cary, NC, USA). The statistical model included fixed effects of the treatment (TRT) for CTR and AL groups, time (T), and interactions of the two factors (TRT×T), and cows as random effects. In the milk yield model, mature equivalent milk yield in the previous lactation was included in the model as a covariate. Covariance structures (compound symmetry, autoregressive order, and spatial power) were included in the model according to the Akaike information criterion with the one having the lowest criterion being chosen (Littell et al., 1998). Plasma biomarkers and rumination times around dry-off and calving were analyzed separately to evaluate the effects in these two distinct phases. Rumen fluid analysis performed at 0 DFD was subject to analysis of covariance (ANCOVA) testing (GLM Procedure, SAS Inst. Inc., Cary, NC, USA) with the use of baseline (–14 DFD) as a covariate (Bland and Altman, 2015) after considering only the fixed effect of treatment. The pairwise comparison was done using the Tukey test. Post-hoc comparisons were discussed when $P \leq 0.05$. The main effects at $P \leq 0.10$ were discussed as tendencies.

4.4 Results

The main characteristics of the two groups of cows at the enrollment in the experiment are reported in Table 4.2. Overall, groups were balanced with respect to the recruitment of cows with no relevant differences between them, except for milk yield in the previous lactation resulting numerically higher in AL cows.

Table 4.2. Main characteristics at the enrollment of dairy cows in the control group (CTR) or receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off.

	Treatment			
Item, unit	AL	CTR	SEM†	*P*-value‡
Parity, n	2.5	2.3	0.4	0.71
Mature equivalent milk production, kg	12317	11539	373	0.15
Lactation length, d	332	352	15.0	0.35
Average somatic cell count, SCS§	2.08	1.96	0.29	0.77
Dry period length, d	50.3	51.9	2.5	0.64
Milk yield at dry-off, kg/d	24.2	21.4	1.7	0.26
Somatic cell count at dry-off, SCS§	2.61	2.27	0.29	0.41

† SEM = greatest standard error of the mean

‡ *P*-value of the treatment effect

§ SCS = Somatic Cell Score (Wiggans and Shook, 1987)

Aloe homogenate's aloin content was 4.6 ± 0.5 % (mean ± SD; on a DM basis), whereas lyophilizate had 1.3 ± 0.4 % DM of aloin.

4.4.1 Rumen fluid, feces, and rumination time

Table 4.3. Rumen fluid pH and concentration of ammonia, D-lactate, L-lactate, total volatile fatty acids (VFA), and VFA proportions (%, mmol/100 mol of VFA) on the day of dry-off in dairy cows in the control group (CTR) or receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off

	Treatment			
Item, unit	AL	CTR	SEM†	*P*-value‡
pH	6.88	6.76	0.08	0.30
Ammonia, mmol/L	18.0	19.0	1.8	0.72
D-lactate, mmol/L	217	191	49	0.71
L-lactate, mmol/L	217	193	48	0.72
Total VFA production, mmol/L	89.2	101.3	4.8	0.08
VFA proportion, %				
Acetate	65.7	64.0	0.6	0.06
Propionate	18.5	20.0	0.7	0.13
Butyrate	11.0	11.3	0.4	0.55
Isobutyrate	1.19	1.11	0.06	0.40
Valerate	1.14	1.28	0.04	0.03
Isovalerate acid	1.86	1.81	0.08	0.68

† SEM = greatest standard error of the mean

‡ *P*-value of the treatment effect adjusted for baseline (–14 days from dry-off)

Rumen fluid pH was not affected by *Aloe* treatment (Table 4.3). Otherwise, as shown in Table 4.3, the treatment affected the VFA production at 0 DFD. Compared with the CTR group, the AL group tended to have greater proportion of acetate ($P = 0.06$) and a lower valerate proportion ($P = 0.03$) was detected. Concentrations of ammonia, D- and L-lactate, and other VFA proportions were not affected by *Aloe* treatment. Daily rumination time did not differ between groups but varied with time both at drying-off and at calving (T; $P < 0.01$; Fig. 4.2).

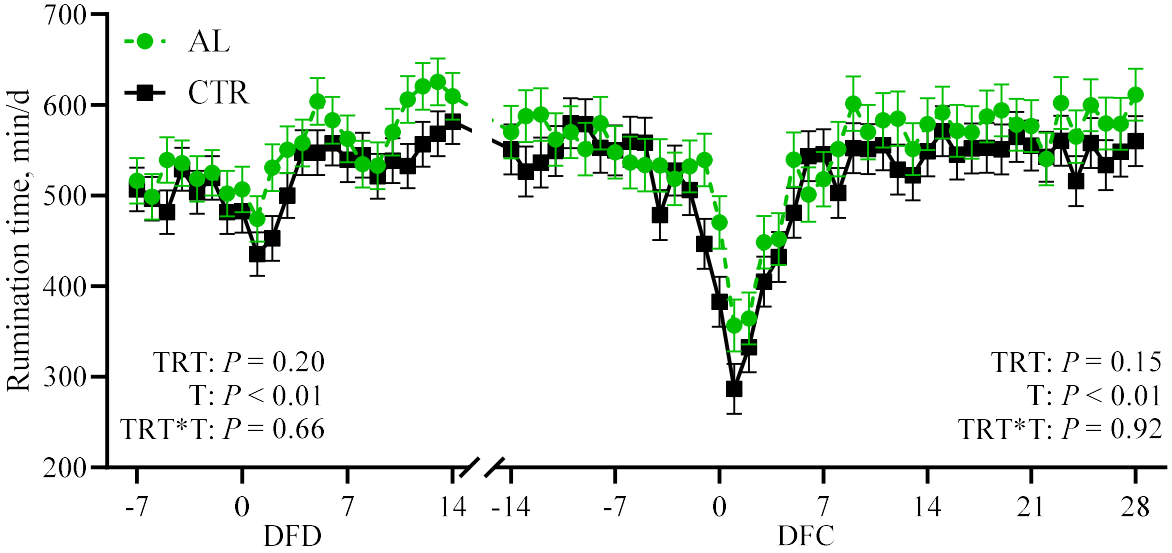

Figure 4.2. Daily rumination time around dry-off (from –7 to 14 days from dry-off [DFD]) and calving (from –14 to 28 days from calving [DFC]) in dairy cows receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off or in the control group (CTR). Values presented as least squares means ± standard error. In the text box, *P*-values for the main effect of treatment (TRT), time (T), and interaction of treatment and time (TRT×T) are shown.

Table 4.4. Dry matter and pH of feces collected around dry-off (–14, 0, and 7 days relative to dry-off) and calving (–7, 3, and 28 days relative to calving) in dairy cows in the control group (CTR) or receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off

	Treatment			*P*-value‡		
Item, unit	AL	CTR	SEM†	TRT	T	TRT×T
Around dry-off						
Fecal pH	6.51	6.49	0.05	0.79	<0.01	0.63
Fecal dry matter, %	12.80	13.45	0.26	0.08	0.05	0.32
Around calving						
Fecal pH	6.44	6.47	0.05	0.61	<0.01	0.43
Fecal dry matter, %	12.69	13.53	0.32	0.07	0.14	0.06

† Greatest standard error of the mean

‡ *P*-value of main effects: treatment (TRT), time (T), and interaction between treatment and time (TRT×T)

Fecal pH did not differ between groups both at dry-off and calving (Table 4.4), whereas a tendency toward an effect of treatment was observed in fecal dry matter with lower values observed in AL versus CTR cows, both at dry-off and calving ($P = 0.08$ and 0.07, respectively).

4.4.2 Udder health

The presence of pathogens in the udder before dry-off and in the first month of lactation is shown in Table 4.5. Incidence of mastitis in the subsequent lactation did not differ between groups (36% versus 25% for AL and CTR, respectively; $P = 0.55$).

Table 4.5. Distribution of quarters at dry-off and calving in dairy cows in the control group (CTR) or receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off

	Before dry-off		After calving	
	AL (n = 125)	CTR (n = 112)	AL (n = 94)	CTR (n = 107)
Uninfected	94%	96%	94%	92%
Infected with major pathogens†	1%	1%	2%	3%
Infected with minor pathogens‡	5%	3%	4%	6%

† *Staphylococcus aureus*, *Streptococcus uberis*, *Enterobacter* spp., *Escherichia coli*, *Trueperella pyogenes*, *Serratia marcescens*

‡ Coagulase-negative staphylococci and *Corynebacterium bovis*

Before dry-off and after calving, SCS did not differ between groups (Table 4.6). On average, during the whole lactation period, no differences were detected between groups (2.78 ± 0.37 versus 2.12 ± 0.35 for AL and TS, respectively; $P = 0.21$).

4.4.3 Milk yield and composition

Milk yield in the first 30 weeks of lactation is shown in Fig. 4.3. Cows receiving AL at dry-off tended to have higher milk yield, particularly during the second month of lactation (weeks 4 to 6 after calving; $P < 0.1$), resulting in an interaction effect (TRT×T; $P < 0.01$). In Table 4.6, milk composition during the last week of lactation and in the first month after calving is reported. Except for urea, all analyzed variables varied with time after calving ($P < 0.01$), but not before dry-off. *Aloe* treatment did not lead to any difference between groups in the periods investigated.

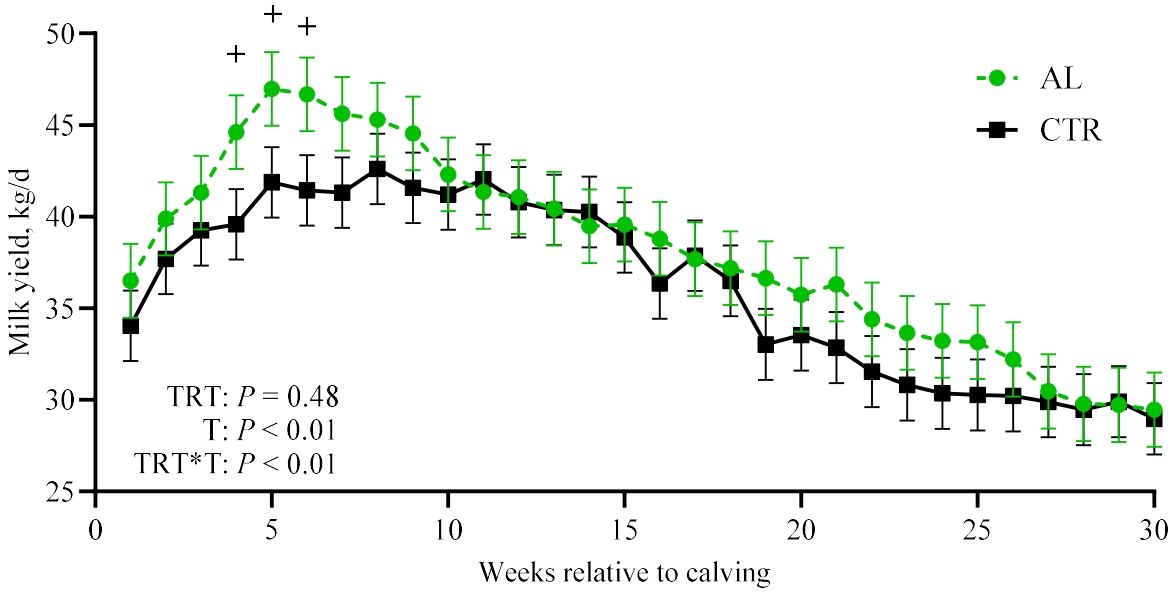

Figure 4.3. Milk yield in the first 25 weeks after calving in dairy cows receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off or in the control group (CTR). Values presented as least squares means ± standard error. Differences between groups within each time point are denoted with * ($P \leq 0.05$) and tendencies with + ($P \leq 0.1$). In the text box, *P*-values for the main effect of treatment (TRT), time (T), and interaction of treatment and time (TRT×T) are shown.

Table 4.6. Average milk composition before dry-off (–7 and 0 days relative to dry-off) and after caving (7, 14, 21, and 28 days relative to calving) in dairy cows in the control group (CTR) or receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off

	Treatment			*P*-value‡		
	AL	CTR	SEM†	TRT	T	TRT×T
Before dry-off						
Fat, mg/100 mL	5.02	4.72	0.27	0.42	0.23	0.75
Protein, mg/100 mL	3.87	3.95	0.11	0.60	0.11	0.24
Casein, mg/100 mL	2.91	2.98	0.09	0.60	0.07	0.17
Lactose, mg/100 mL	4.96	4.91	0.08	0.66	0.41	0.78
Somatic cell count, SCS§	2.51	2.35	0.27	0.67	0.98	0.31
After calving						
Fat, mg/100 mL	4.28	4.45	0.21	0.58	<0.01	0.99
Protein, mg/100 mL	3.57	3.55	0.08	0.83	<0.01	0.52
Casein, mg/100 mL	2.63	2.62	0.06	0.83	<0.01	0.52
Lactose, mg/100 mL	5.03	5.01	0.05	0.76	<0.01	0.41
Somatic cell count, SCS§	2.27	2.49	0.42	0.71	<0.01	0.51

† Greatest standard error of the mean

‡ *P*-value of main effects: treatment (TRT), time (T), and interaction between treatment and time (TRT×T)

§ SCS = Somatic Cell Score (Wiggans and Shook, 1987)

4.4.4 BCS, rectal temperature, and immunometabolic profile

Despite significant variations during the study period, BCS and rectal temperature did not present any difference between groups.

Dry-off caused significant alterations in most of the plasma biomarkers ($P \leq 0.05$), except for AOPP, haptoglobin, thiol groups, and albumin (Supplementary Table S1; https://doi.org/10.1111/jpn.13777). *Aloe* administration at dry-off had a significant effect on plasma concentrations of glucose, urea, paraoxonase, and ROM around the drying-off period (Fig. 4.4). Glucose increased more in AL than in the CTR group starting from 0 DFD (TRT×T; $P = 0.03$), whereas urea concentration tended to be lower in AL after dry-off (TRT; $P = 0.07$). Paraoxonase and ROM concentration increased more in AL group starting from fry-off (TRT×T; $P = 0.01$ and 0.04, respectively). Other plasma biomarkers and OSI did not show any differences between groups around dry-off.

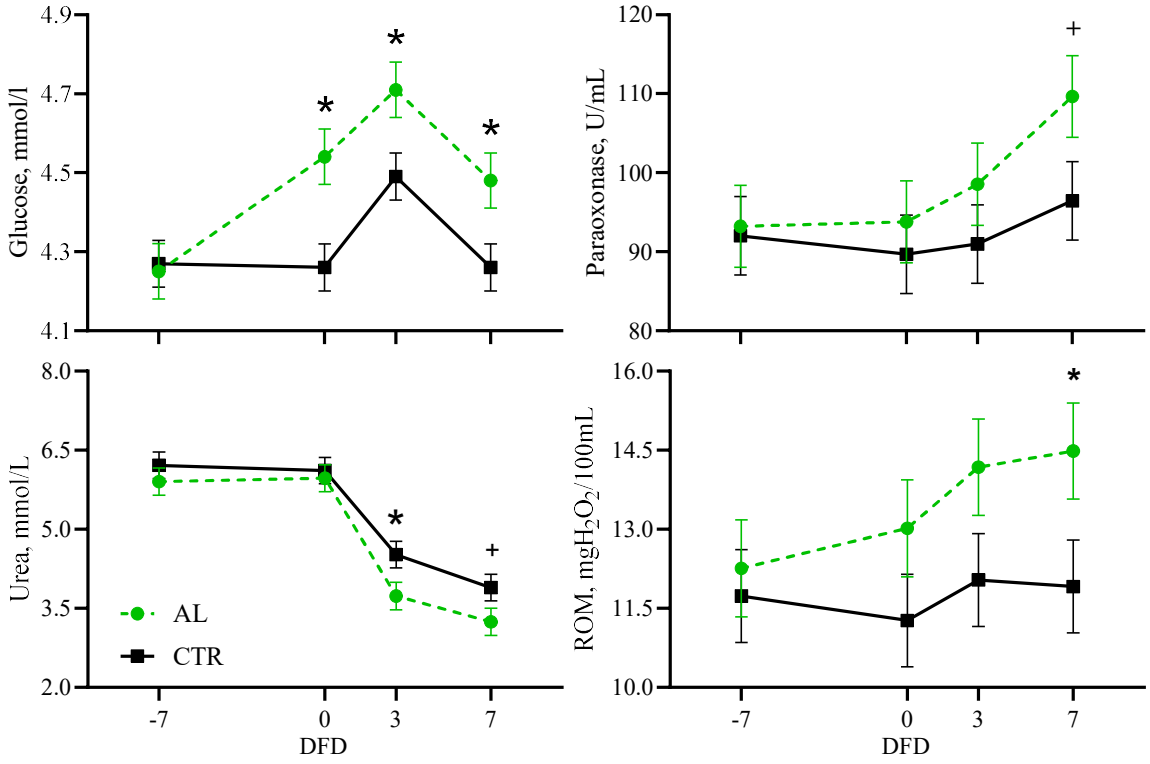

Figure 4.4. Plasma concentration of glucose, paraoxonase, urea, and reactive oxygen metabolites (ROM) from –7 to 7 days from dry-off (DFD) in dairy cows receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off or in the control group (CTR). Values presented as least squares means ± standard error. Differences between groups within each time point are denoted with * ($P \leq 0.05$) and tendencies with + ($P \leq 0.1$).

Around calving, all tested plasma biomarkers varied with time ($P < 0.01$; Supplementary Table S2; https://doi.org/10.1111/jpn.13777). *Aloe* treatment at dry-off tended to influence biomarkers of inflammation levels (Fig. 4.5). The peak in haptoglobin concentration after calving was lower in the AL than in the CTR group (3 DFC; $P = 0.04$), resulting in a tendency towards an interaction effect (TRT×T; $P = 0.08$). Ceruloplasmin levels tended to be higher in the CTR group around calving (TRT; $P = 0.09$). Other plasma biomarkers, OSI, and LFI (1.28 ± 0.76 and 0.63 ± 0.73 points for AL and TS, respectively; $P = 0.54$) did not differ between groups around calving.

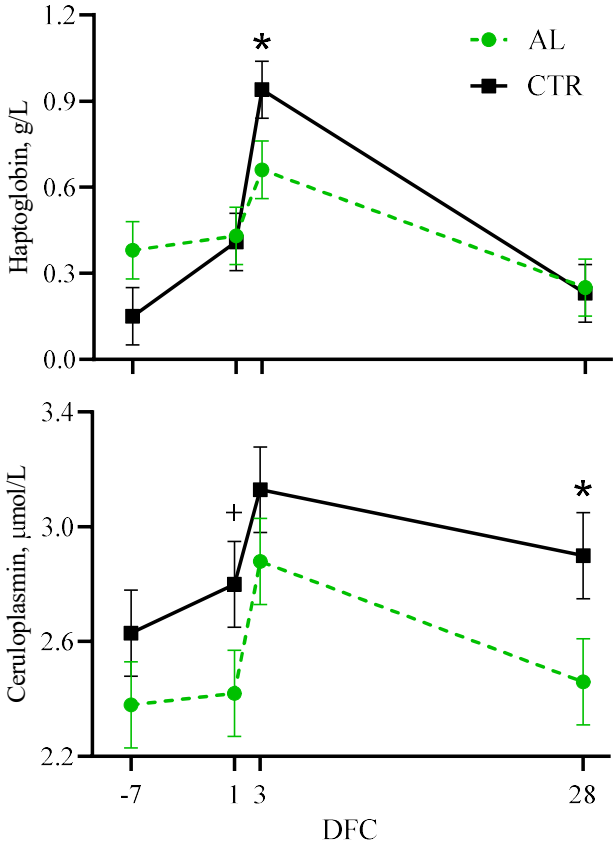

Figure 4.5. Plasma concentration of haptoglobin and ceruloplasmin from –7 to 28 days from calving (DFC) in dairy cows receiving 10 g/d of lyophilized *Aloe arborescens* Mill. (AL) from –7 to 7 days relative to dry-off or in the control group (CTR). Values presented as least squares means ± standard error. Differences between groups within each time point are denoted with * ($P \leq 0.05$) and tendencies with + ($P \leq 0.1$).

4.5 Discussion

Aloe is a plant known worldwide for its therapeutic use. In particular, *A. arborescens* leaves are rich in aloin, and aloenin, contain a good amount of polyphenols and flavonoids and showed antioxidant activity (Lucini et al., 2015; Cardarelli et al., 2017). Remarkably, among *Aloe* species, *A. arborescens* has the highest total phenolic concentration and total antioxidant activity (Zapata et al.,

2013). In a previous paper, Bani et al. (2016) described the use of *A. arborescens* Mill. homogenate as a nutraceutical in dairy cattle. In fact, the authors demonstrated that aloin, one of the main *Aloe* active compounds, is successfully absorbed into the blood. Moreover, no side effects of the *Aloe* on feeding behavior and health were reported. Afterward, Mezzetti et al. (2020a) tested the supplementation of 200 g/d of *Aloe* homogenate in transition dairy cows and obtained promising results. They added supplemental *Aloe* for two weeks before and after calving and observed positive effects on liver and kidney functions. Those effects could have been related to the anti-hyperlipidemic and anti-inflammatory effects of *Aloe*. Moreover, an improvement in antioxidant status in early lactation was also found, which likely mitigated the liver dysfunction typical of this physiological phase (Bertoni et al., 2008). Therefore, since the dry-off represents another critical and potentially stressful transition for dairy cows (Zobel et al., 2015; Mezzetti et al., 2020b; Cattaneo et al., 2021b), we evaluated whether *Aloe* supplementation for seven days before and after dry-off could have positive outcomes also in this phase and if this could affect the subsequent early lactation period. However, the processing and delivery of *Aloe* homogenate are time-consuming and not easily implantable on a larger scale. Lyophilization has shown the best results in preserving the chemical quality of Aloe (Saravanan et al., 2015; Pawłowicz et al., 2021). Therefore, to facilitate easier administration of this plant, in this trial we lyophilized its homogenate, allowing also a more efficient storing and the possibility of feeding it together with the ration. The dry matter of *Aloe* provided in the present study was comparable to that used in previous studies (Bani et al., 2016; Mezzetti et al., 2020), but aloin content was slightly lower (1.3 vs 2.4 % of DM). The lyophilization process or differences in the raw material might account for this reduction. *Aloe* properties are dependent on the species, growing conditions, extraction, and preservation methods. Similar aloin concentration was obtained in A. arborescens lyophilized leaf extract by other authors (2.0%; Froldi et al., 2019). Moreover, many properties of Aloe are linked also to its phenolic components, which are preserved with this processing procedure (Lucini et al., 2015; Froldi et al., 2019).

In the current study, supplementation with lyophilized *Aloe* whole leaves did not alter rumen fluid pH but affected VFA proportions. Although it is known that nutraceuticals through their essential oils can modify rumen fermentation (Calsamiglia et al., 2007), Bani et al. (2016) reported a lack of effects of *Aloe* homogenate on *in vitro* fermentation parameters even though *Aloe* tended to cause an increase in total VFA production. With regards to molar proportions, acetate tended to increase after one week of lyophilized *Aloe* supplementation when compared with the control, while valerate tended to decrease. Effects on rumen VFA due to *Aloe* administration that are reported in literature are inconsistent. Total VFA production increased in all previously reported trials, but effects on individual VFA varied. Calabrò et al. (2013) recorded an increase in acetate and a tendency for

propionate and butyrate to decrease after testing *in vitro* dried leaves of *A. arborescens*. Singh et al. (2021) reported an increase in acetate and propionate when *A. barbadensis* waste was fermented *in vitro*, whereas Bani et al. (2016) did not observe any effect on these VFA after adding *A. arborescens* homogenate to rumen juice *in vitro*. These differences compared with the current study can be due to diets, different species, doses tested, time of sampling, and techniques used to manipulate the plant. Moreover, all of the previous studies analyzed VFA production *in vitro*, whereas ours is indeed the first study evaluating rumen fermentations during *Aloe* spp. supplementation *in vivo.* Characterization of ruminal microbial populations during aloe supplementation would help to explain the fermentation dynamic. Active compounds contained in *Aloe* might have caused a slowing of dry matter ruminal degradability, resulting in lower VFA production. However, our samples were collected once a day before the morning feeding when fermentation patterns reached their nadir. Therefore, differences in absorption or fermentation dynamic could have led to a discrepancy between actual VFA production and our measurements. Rumination time decreased immediately after dry-off resulting from the many different stressors occurring at this time (Abuelo et al., 2021), and increased one week after despite the reduced dry matter intake, probably due to the adaptation to the diet of the dry period, higher in fiber than the lactation one. However, lyophilized *Aloe* supplementation did not significantly influence rumination time during the periods investigated. Despite the lack of differences at calving, the values observed in both groups were higher than those recorded by Calamari et al. (2014), suggesting a good metabolic condition in all enrolled cows.

Cows supplemented with lyophilized *Aloe* had lower feces dry matter at dry-off and also at calving. Even though it is difficult to ascribe the lower dry fecal matter at calving to *Aloe* supplementation, the difference in this parameter at dry-off is noteworthy. *Aloe* spp. is also widely used by humans as a remedy for constipation (Ramkumar and Rao, 2005; Cirillo and Capasso, 2015), and its laxative effect has been confirmed in rats (Wintola et al., 2010). Aloin was indeed shown to cause an increase in water content and stimulation of peristalsis in rat intestines (Ishii et al., 1994). However, the reduction in dry matter did not result in issues, such as diarrhea, at the dosage of *Aloe* used in this study. Previous research has shown the lack of toxicity of *Aloe* whole leaf extract in mice and rats up to a dose of around 100 mg/kg/d (Matsuda et al., 2008; Guo and Mei, 2016). Therefore, the dose used in the present study, equivalent to approximately 15 mg/kg/d, was safe and should have not caused any toxic or adverse effects. Moreover, the metabolization of *Aloe* bioactive compounds that likely takes place in the bovine forestomaches further reduces the availability for gut absorption (Bani et al., 2016).

Aloe spp. is also known for its antimicrobial properties (Maan et al., 2018; Forno-Bell et al., 2019), and it has been used as an intramammary remedy to treat mastitis in organic systems (Pol and

Ruegg, 2007). In the present study, we did not observe any effect of *Aloe* on SCC and mastitis incidence. *Aloe* was fed to dairy cows rather than applying it directly to the udder, and its main effects likely occurred in the digestive tract as previously described. Moreover, despite having not undergone antibiotic dry-cow therapy, cows were selected for good udder health at dry-off, and this selection might have limited the potential effects of *Aloe.* Furthermore, the presence of intramammary pathogens was similar between groups, both before dry-off and after calving. However, considering the limited number of subjects involved, the aim of these samplings was not to find differences in intramammary infection risk due to the oral supplement treatment but to identify potential factors that could have affected other results. Regardless of treatment, the lack of differences in mammary pathogen infections and SCC between dry-off and early lactation confirm the protective effects of internal teat sealant during the dry period when used alone without the antibiotic dry-cow therapy in healthy cows (Winder et al., 2019; Niemi et al., 2021), even when milk yield at dry-off is higher than the safety threshold proposed (Vilar and Rajala-Schultz, 2020).

Dramatic changes in plasma biomarkers happened around dry-off, as previously reported (Putman et al., 2018; Mezzetti et al., 2020b). In this scenario, lyophilized *Aloe* administration caused a likely amelioration of liver function. These effects are similar to those reported by Mezzetti et al. (2020a) during the transition period. Aloin content was indeed lower in the lyophilized form we supplemented in the present study compared with the homogenate previously used. Nevertheless, *Aloe* contains a variety of bioactive compounds (e.g. phenols, flavonoids, vitamins), which have shown to have beneficial effects on human health and, together with aloin, could have been involved in the observed responses. Paraoxonase is an index of liver function (Bionaz et al., 2007). The higher concentration of paraoxonase suggested better liver function at the beginning of the dry period in the cows that received *Aloe*. Besides, the higher glycemia and the lower urea concentrations that resulted from *Aloe* supplementation might also be related to liver conditions. Glucose supply heavily relies on liver endogenous production, and impairment in liver function can harm glucose metabolism (Drackley et al., 2001; Hammon et al., 2009). Moreover, lower glucose levels can suggest an immune system activation at dry-off in the control group (Kvidera et al., 2017). Urea blood content is yielded by liver synthesis, starting from ammonia absorbed from the rumen (exogenous), or derived from deamination of amino acids (endogenous). In our experiment, since rumen ammonia was similar, the tendency towards lower urea in AL cows could suggest a less recourse to deamination of amino acids in the liver, which, despite the slightly higher milk yield in AL and a similar rumination time between groups (i.e., a similar dry matte intake) could indicate a lower energy demand in AL cows, likely for a lower immunity system activity. In contrast, reactive oxygen metabolite (ROM) concentrations increased in AL cows when compared with the CTR group for a few days starting at dry-off. *Aloe* is

known to improve antioxidant availability (Ozsoy et al., 2009), even in cattle (Mezzetti et al., 2020a). During mammary gland involution, the production of oxidant species increases (Silanikove et al., 2005; Mezzetti et al., 2020b) in addition to the expression of several antioxidant genes (Singh et al., 2008). Excessive ROM concentration is suggestive of oxidative stress and inflammation (Celi and Gabai, 2015) although values recorded in AL were similar to those reported after dry-off in healthy cows in other studies (Putman et al., 2018; Mezzetti et al., 2020b; Cattaneo et al., 2021c). Moreover, the increase in ROM was paired with a general increase in the antioxidant capacity, as supported by the lack of differences in the Oxidative Status Index. Besides, ROM are markers of intense cell activity and are necessary for immune and inflammatory responses (Halliwell, B.; Gutteridge, 2007; Kvietys and Granger, 2012; Abuelo et al., 2015). At the same time, paraoxonase, which is inversely related to oxidative stress (Turk et al., 2005), was slightly higher in AL cows. The slightly higher milk yield at dry-off that was observed in the AL group can hardly account for this difference. Another possible reason for this difference could be related to immune system activation. Among its many applications, *Aloe* spp. is used as drug absorption enhancing agent. *A. barbadensis* leaves have the potential to enhance drug permeation across the intestinal epithelial barrier thus causing an increase in tight junction permeability (Haasbroek et al., 2019). The possible increase in gastrointestinal permeability might have resulted in the translocation of lipopolysaccharides to the bloodstream (Gozho et al., 2005; Khafipour et al., 2009), causing the activation of the immune system and the consequent production of ROM (Bogdan et al., 2000). However, lipopolysaccharide translocation is known to induce a systemic inflammatory response (Minuti et al., 2014; Ghosh et al., 2020), and we did not observe that type of phenomenon in AL cows. Although the degree of this immune response can vary significantly (Plaizier et al., 2012), further research investigating the underlying mechanism of the ROM increase is needed.

The main result of *Aloe* administration on metabolites as reported by Mezzetti et al. (2020a) was an anti-hyperlipidemic effect. In that experience, periparturient cows supplemented with *Aloe* showed an improvement in lipid metabolism, increase in BHB metabolization, and NEFA removal from the bloodstream, thus affecting milk fat output. In the current study, no effect was detected, and body fat mobilization was not affected. However, the different phase during which *Aloe* was supplemented or the lower aloin content measured in the lyophilized form could account for this difference. The transition period is indeed a period of intense lipid mobilization, whereas, during the dry-off phase, this process is mild and the potential to reduce the NEFA concentration is limited.

Trying to connect changes in plasma biomarkers during the periparturient period to nutraceutical supplements given about two months earlier is difficult. However, as observed in previous studies, better conditions in the drying-off phase can influence the success of early lactation

(Cattaneo et al., 2021a) or the development of early-lactation diseases (Abuelo et al., 2021). In the present study, lyophilized *Aloe* administration during dry-off seems to have led to a reduction in the magnitude of the acute phase liver response typical of the calving event (Trevisi and Minuti, 2018) as demonstrated by lower plasma haptoglobin and ceruloplasmin in this phase. The effect of *Aloe* might not have been direct but having improved cows' condition at dry-off could have ameliorated inflammatory status and metabolism during the dry period, allowing them to better adapt to the transition period. Haptoglobin and ceruloplasmin are indeed positive acute-phase protein concentrations (Ceciliani et al., 2012), and their plasma concentration increase because of this acute-phase response. Therefore, the better general condition observed both at dry-off and calving in the present study in AL cows could account for the slightly higher milk yield in the subsequent early lactation period. Our results seem to confirm previous observations suggesting that cows experiencing a better drying-off phase will also have an improved condition at lactation onset and produce more milk (Cattaneo et al., 2021a). Therefore, particular attention during the dry-off period is needed to ensure that cows are in proper health, metabolic, and inflammatory condition. Those cows will cope better with transition period challenges and likely have better performance during the subsequent lactation period.

4.6 Conclusions

Our results indicate that supplementation of lyophilized *Aloe* in the dry-off period altered VFA proportions and lowered dry fecal content. Lyophilized *Aloe* also led to an improvement in liver function and increased the production of ROM. At the onset of lactation, cows that received *Aloe* at dry-off showed a decrease in inflammatory response after calving. Moreover, milk yield in the subsequent lactation was higher, whereas milk composition, SCC, and mastitis incidence were not affected by *Aloe* treatment. These results highlight the importance of improving cows' condition even before dry-off to obtain healthier cows during the transition period, particularly in cows not treated with antibiotics at milking cessation. Further research to elucidate *Aloe* spp. mode of action is needed.

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Chapter 5: Gene Network Expression of Whole Blood Leukocytes in Dairy Cows with Different Milk Yield at Dry-off

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5.1 Abstract

Dairy cows at dry-off undergo several management and physiological changes, resulting in alterations in plasma biomarkers of inflammation, oxidative stress, and immune system. High milk yield at the end of lactation exacerbates these responses. The underlying mechanism of these changes has yet to be elucidated. We hypothesized altered leukocyte gene expression after dry-off and different responses in cows with different milk yield. Thirteen Holstein dairy cows were sampled at the turn of dry-off to investigated whole blood leukocyte gene expression and were grouped according to the average milk yield during the last week of lactation: low (< 15 kg/d) and high milk yield (> 15 kg/d). Blood samples were collected in PAXgene tubes (Preanalytix, Hombrechtikon, Switzerland) at –7, 7, and 34 days from dry-off (DFD) to measure mRNA abundance of 37 genes. Normalized gene abundance data were subjected to MIXED model ANOVA (SAS Institute Inc., Cary, NC). Compared with –7 DFD, at 7 DFD RNA abundance of lipoxygenase genes (*ALOX5*, *ALOX15*) and myeloperoxidase (*MPO*) increased, and that of the antioxidant gene (*SOD2*) decreased. Meanwhile, genes related to recognition and immune mediation (*CD16*, *MYD88*, *TLR2*), migration and cell adhesion (*CX3CR1*, *ITGAL*, *ITGB2*, *TLN1*), and the antimicrobial gene *MMP9* were downregulated at 7 or 34 DFD, whereas the antimicrobial *IDO1* gene was upregulated. Compared with low-producing cows, cows with high milk yield at dry-off cows had upregulated expression of the pro-inflammatory cytokines *IL8* and *IL18* and a greater reduction in transcript abundance of the toll-like receptor (TLR) recognition-related gene *TLR2*. Overall, the dry-off confirmed to be a phase of intense changes, triggering an inflammatory response and somewhat suppressing leukocyte immune function. In cows with high milk yield during the week before dry-off, the inflammatory response was exacerbated.

Keywords: inflammation; oxidative stress; mammary gland; white blood cells

5.2 Introduction

At dry-off, dairy cows have to face the transition from a lactating to a non-lactating state. After halting of milk removal, active mammary gland involution begins (Oliver and Sordillo, 1988; Hurley, 1989) and many behavioral and physiological modifications happen (Zobel et al., 2015; Putman et al., 2018). Milk synthesis stops, the mammary epithelium is partially renewed, different proteases are activated, and the permeability of tight junctions between epithelial cells increases (Zhao et al., 2019). Moreover, diet is changed and rumen papillae need to adapt (Dieho et al., 2016). Altogether, these changes affect metabolism, inflammation, and oxidative stress, even though at a lower degree than in the periparturient period (Putman et al., 2018). In particular, blood NEFA increased immediately after dry-off, and concentrations of liver enzymes indicators, positive acute-

phase proteins, and nitrogen species increased after dry-off, whereas negative APPs and antioxidant species decreased (Putman et al., 2018; Mezzetti et al., 2020b). Moreover, blood total leukocytes count decreased, mainly due to the reduction in neutrophils and monocytes (Putman et al., 2018; Mezzetti et al., 2020b).

With the increase of genetic merit and the improvements in nutrition and management, cows that approach the scheduled dry-off day maintaining high milk yields are increasingly common (Stefanon et al., 2002). In these cows, the metabolic and inflammatory response at dry-off is exacerbated and mammary gland involution is impaired (Vilar and Rajala-Schultz, 2020). Therefore, high milk production before dry-off represents a threat to cow's udder health in the following lactation, and a safety threshold of 15 Kg/day has been proposed (Vilar and Rajala-Schultz, 2020) In a previous study, Mezzetti et al. (Mezzetti et al., 2020b) observed that cows with an average milk yield above 15 Kg/day during the week before dry-off had an increased inflammatory response compared with those having a milk yield below this threshold.

Altered gene expression has been reported around calving in neutrophils (Crookenden et al., 2016) and leukocytes (Lopreiato et al., 2020; Minuti et al., 2020), whereas the effects of dry-off on leukocytes gene expression have been poorly investigated. Nevertheless, insights on molecular changes of the immune cells at dry-off can provide information for a more accurate management of this fundamental physiological phase of the high-yielding dairy cow.

We hypothesized that leukocyte gene expression would differ around dry-off and between cows with high and low milk production before dry-off. Thus, we investigated the effect of dry off on genes involved in recognition, immune mediation, migration, cell adhesion, antimicrobial mechanisms, inflammatory cascade, oxidative stress, and the leukotriene pathway in leukocytes from cows with different milk yields during the week before dry-off.

5.3 Materials and Methods

5.3.1 Animal management and PAXgene tubes sampling

All procedures were approved by the Università Cattolica Animal Welfare Committee and carried out in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 1047/2015-PR). The trial was performed at the Università Cattolica del Sacro Cuore research dairy barn. The details about animal management and sampling procedure are described in previous work (Mezzetti et al., 2020b). Briefly, 13 Holstein dairy cows (parity 1.9 ± 1.1; mean ± SD) were housed in individual tied stalls with controlled environmental conditions and milked twice daily until dry-off. Cows were abruptly dried off 55 days before the expected calving day and treated with an intramammary antibiotic and an injection of internal teat

sealant (Mamyzin-A; Haupt Pharma Latina S.r.l, Italy). Before dry-off, cows were individually fed with the lactation diet. For 10 days after dry-off, cows were fed grass hay only. Afterward, dry period ration was administered. The diet composition was previously reported (Mezzetti et al., 2020b). According to the average milk yield during the week before dry-off, cows were retrospectively divided into two groups, with a threshold of 15 Kg/day: low milk yield (LM; n = 7; 10.6 ± 3.7 Kg/d) and high milk yield (HM; n = 6; 16.5 ± 5.3 Kg/d). At –7, 7, and 34 days from dry-off (DFD), blood samples were collected through jugular venipuncture into PAXgene Blood RNA System tubes (Preanalytix, Hombrechtikon, Switzerland) for RNA extraction.

5.3.2 RNA extraction, cDNA synthesis, and gene expression

RNA extraction from PAXgene tubes was performed according to the manufacturer's protocol (Blood RNA Kit Handbook, PreAnalitix GmbH, Qiagen, Hilden, Germany), as described previously (Lopreiato et al., 2020) and described in S1 Appendix. Afterward, RNA was quantified using the Qubit RNA BR Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and RNA quality was assessed with the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA). The average RNA quality was 9.5 ± 0.6 (mean ± SD). Samples were diluted to 100 ng RNA/µL using nuclease-free water, and synthesis of cDNA was carried out with a reverse transcription kit (RevertAid RT Reverse Transcription Kit; Thermo Fisher Scientific). Diluted cDNA (4 µL) was combined with 6 µL of a 5 µL 1 × SYBR Green Master Mix (Applied Biosystems, Woolston Warrington, UK) + 0.4 µL each of 10 µM forward and reverse primers + 0.2 µL of nuclease-free water mixture., qPCR was performed with an Optical 384-Well Reaction Plate (CFX384 Touch; BioRad, Hercules, CA, USA), running three replicates for each sample. The qPCR efficiency and quantification cycle values were obtained for each reaction using LinReg-PCR (Version 2017.1; Amsterdam UMC, Amsterdam, the Netherlands). Genes selected for transcript analysis were those related to leukotrienes and oxidative status (*ALOX5*, *ALOX15*, *SOD1*, *SOD2*), inflammatory cascade (*CASP1*, *IL1B*, *IL1R*, *IL4*, *IL6*, *IL6R*, *IL10*, *IL18*, *IRAK1*, *IRAK4*, *NLRP3*, *S100A8*, *TNFRSF1A*, *TNF*), migration and cell adhesion (*CCR2*, *CD44*, *CX3CR1*, *IL8*, *ITGAL*, *ITGB2*, *LGALS8*, *SELL*, *SELPLG*, *TLN1*), recognition and immune mediation (*CD14*, *CD16*, *MYD88*, *TLR2*), and antimicrobial strategies (*IDO1*, *LCN2*, *MMP9*, *MPO*, *TLN2*). The final data were normalized using the geometric mean of three internal control genes: *ACTB*, *YWHAZ*, and *SDHA*. Gene names and functions, primer information, and primer sequencing results are included in Supporting tables (https://doi.org/10.1371/journal.pone.0260745). The stability of the normalization factor of these three control genes was assessed using GeNorm software and no improvement in stability was obtained with the addition of a fourth endogenous control gene.

5.3.3 Statistical analysis

Normalized arbitrary mRNA abundance data were analyzed using the repeated measure mixed model, with the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). The fixed effects were milk yield at dry-off (MY; LM and HM), sampling day (DFD; –7, 7, and 34), and their interaction (MY*DFD), whereas cows were included as random effect. All means were compared using the PDIFF statement of SAS and Dunnett's adjustment was applied to compare sampling days (7 and 34 DFD) with the reference timepoint (–7 DFD). Significant differences were declared at $P \leq 0.05$.

5.4 Results and Discussion

At dry-off, milking is stopped and mammary involution begin (Putman et al., 2018; Zhao et al., 2019), and, at the same time, energy content of the diet is dramatically reduced and rumen need to adapt (Dingwell et al., 2001; Dieho et al., 2016). Therefore, the dry-off represents a potentially stressful event of the lactation cycle of dairy cows (Abuelo et al., 2021), with relevant effects on the subsequent lactation, which leads to huge alterations in plasma biomarkers of inflammation, metabolism, liver function, and oxidative stress (Putman et al., 2018; Mezzetti et al., 2020b). These responses are exacerbated in high-yielding dairy cows (Mezzetti et al., 2020b; Vilar and Rajala-Schultz, 2020). Mammary gland gene expression is altered at the turn of dry-off (Piantoni et al., 2010; Dado-Senn et al., 2018), but information about circulating leukocyte gene expression in this phase is lacking. Thus, we investigated the effect of dry off on peripheral blood leukocyte RNA abundance of genes involved in several pathways in cows with different average milk production during the last week before dry-off.

5.4.1 Effects of dry-off on gene expression

In the present study, we evaluated mRNA abundance in circulating leukocytes. Blood leukocyte profile is affected by mammary involution, due to the migration of white blood cells to the mammary gland (Atabai et al., 2007; Putman et al., 2018; Mezzetti et al., 2020a). Therefore, some changes in mRNA abundance might be related to differential expression of genes in a specific leukocyte population. Dry-off affected ($P < 0.05$) transcript abundance of genes involved in inflammatory cascade (*NLRP3*), leukotriene regulation (*ALOX5*, *ALOX15*), recognition and immune mediation (*CD16*, *MYD88*, *TLR2*), migration and cell adhesion (*CX3CR1*, *ITGAL*, *ITGB2*, *TLN1*), antimicrobial strategies (*IDO1*, *MMP9*, *MPO*), and oxidative stress (*SOD2*).

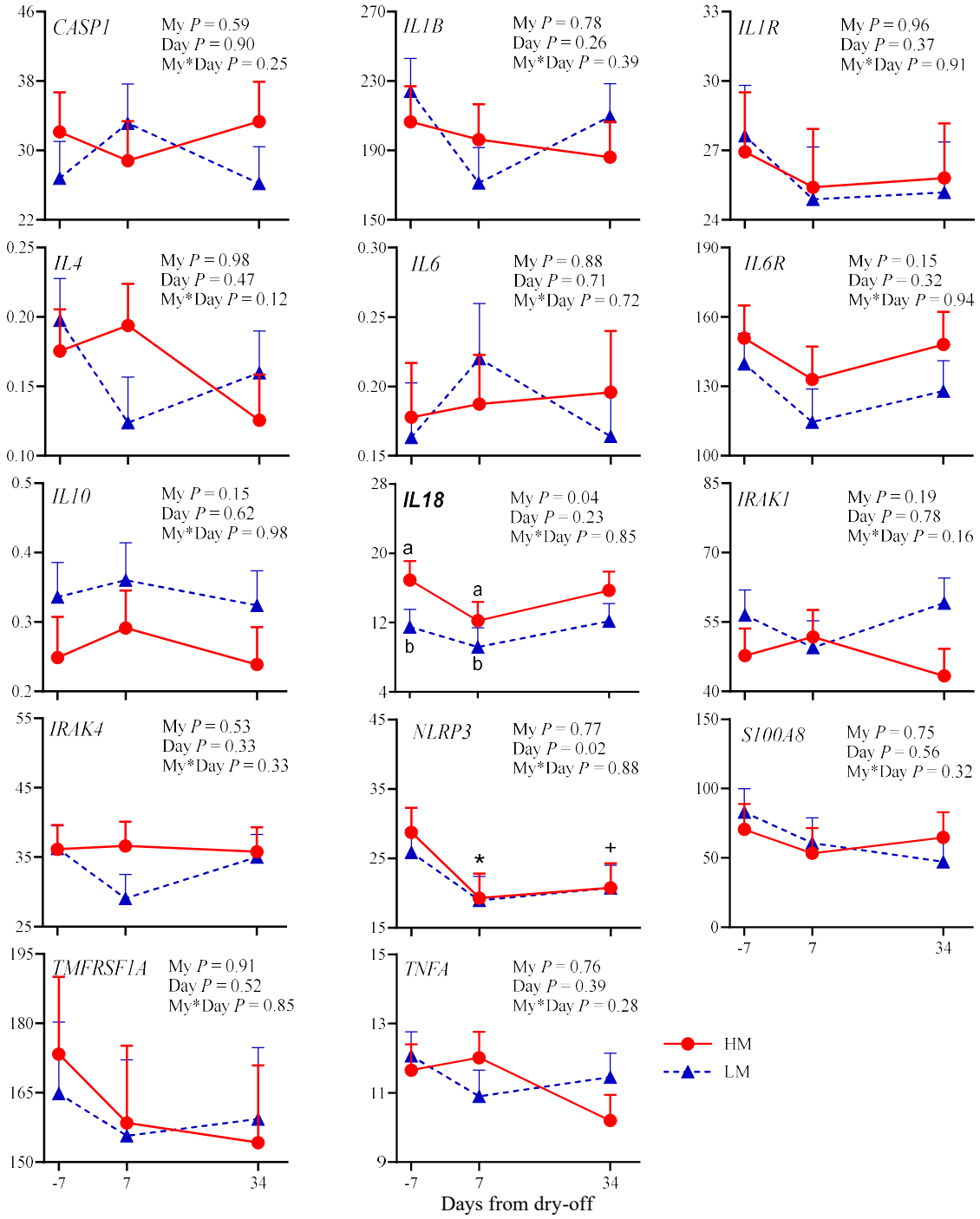

Figure 5.1. Changes from –7 days from dry-off (DFD) to 34 DFD in dairy cows with high (HM; red line) or low (LM; blue line) milk yield at dry-off in mRNA abundance (mean ± SEM) for gene expression of genes involved in the inflammatory cascade: CASP1 (Caspase 1), IL1B (Interleukin 1 Beta), IL1R (Interleukin 1 Receptor), IL4 (Interleukin 4), IL6 (Interleukin 6), IL6R (Interleukin 6 Receptor), IL10 (Interleukin 10), IL18 (Interleukin 18), IRAK1 (Interleukin 1 Receptor-Associated Kinase 1), IRAK4 (Interleukin 1 Receptor-Associated Kinase 4), NLRP3 (NOD-Like Receptor Protein 3), S100A8 (S100 Calcium Binding Protein A8), TMFRSF1A (TNF Receptor Superfamily Member 1A), TNFA (Tumor Necrosis Factor Alfa). P-values for main effect of milk yield at dry-off (My), day, and interaction of milk yield × day (My*Day) are shown. Significant differences (P ≤ 0.05) between groups on the same day are denoted with lowercase a and b, and

differences between –7 DFD and 7 or 34 DFD are denoted with an asterisk (*; $P \leq 0.05$) or a plus sign (+; $P \leq 0.1$).

The mRNA abundance of the gene encoding for NOD-like receptor protein 3 inflammasome (*NLRP3*) was reduced after dry-off (Fig. 5.1). *NLRP3* is mainly expressed in monocytes and macrophages (Awad et al., 2017). Its activation has been reported due to a variety of unrelated stimuli that induce cellular stress (Swanson et al., 2019), and also by reactive oxygen species (ROS) (Tschopp and Schroder, 2010), while nitric oxide inhibits the activation of the NLRP3 inflammasome (Chen and Sun, 2013). We observed a reduced monocytes percentage on total leukocytes count (Supporting Table S6; https://doi.org/10.1371/journal.pone.0260745.s007) and an increased plasma concentration of nitric oxide were found after dry-off, probably accounting for the reduced *NLRP3* expression found in the present study. Arachidonate 5-Lipoxygenase (*ALOX5*) and arachidonate 15-Lipoxygenase (*ALOX15*) are genes involved in the leukotriene pathway and the inflammatory process. The enzyme *ALOX5* catalyzes the oxidation of arachidonic acid into leukotriene A4. It is increased during inflammation and is also involved in homeostasis restoration (Serhan et al., 2008). Through its pathway, it also produces hydroxyl and hydroperoxyl derivatives that are often elevated during inflammation (Aitken et al., 2011). *ALOX5* exists in the cytoplasm and nucleoplasm of cells, and its upregulation may occur during the maturation of leukocytes (Anwar et al., 2014). *ALOX15* plays a pivotal role in the resolution of inflammation (Tian et al., 2017), through the formation of key lipid mediators (e.g., lipoxins and resolvins) but through arachidonic acid metabolism also produces eicosanoids that act as pro-inflammatory mediators (Olson et al., 1995; Sordillo et al., 2008) and are capable of generating ROS, metabolites strictly related to oxidative stress (Aitken et al., 2009). Therefore, their upregulation one week after dry-off (Fig. 5.2) might suggest the activation of the inflammatory cascade after dry-off, but also the need for a modulatory mechanism that allows a rapid termination of the inflammatory process linked to the drastic changes taking place after milking cessation.

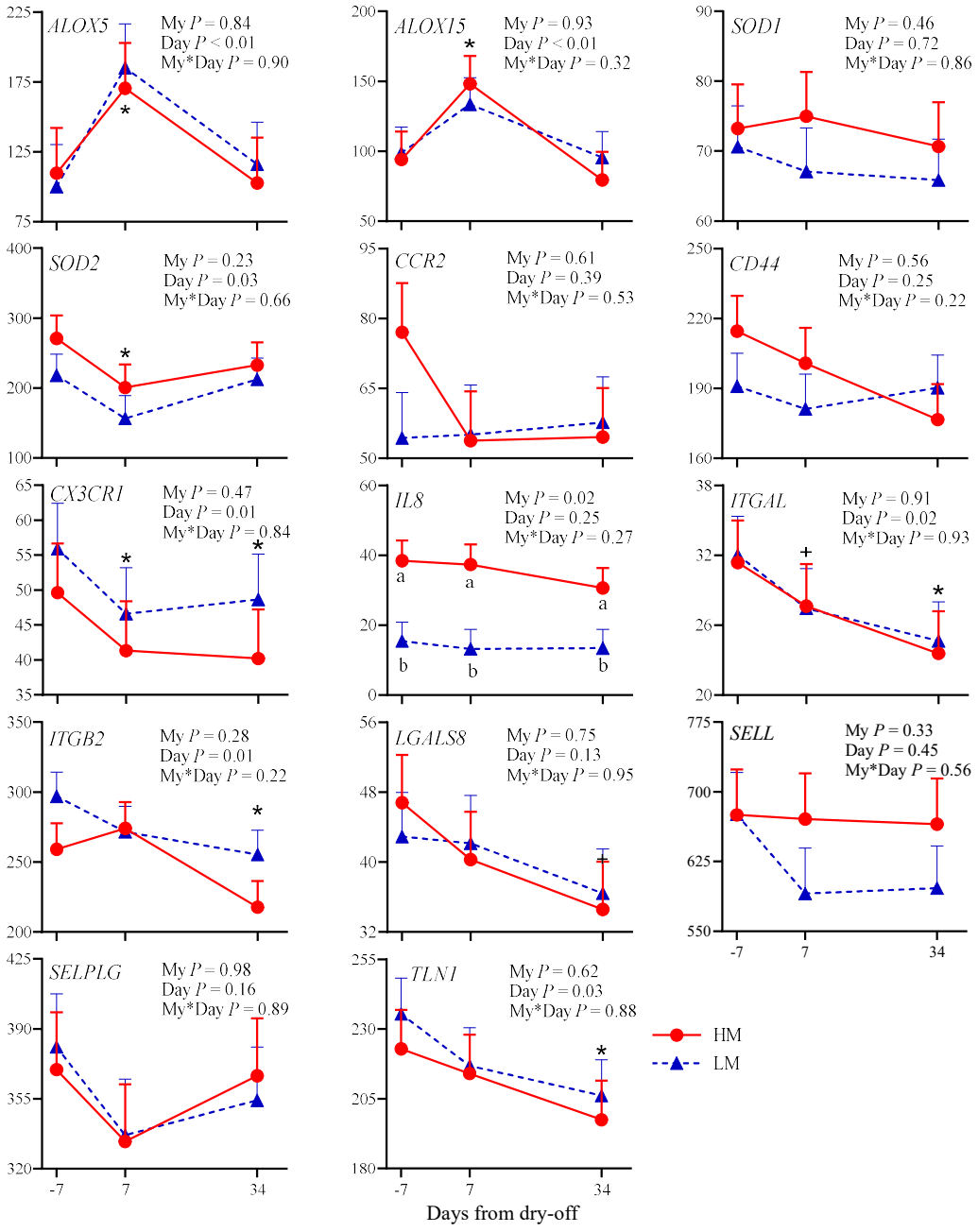

Figure 5.2. Changes from –7 days from dry-off (DFD) to 34 DFD in dairy cows with high (HM; red line) or low (LM; blue line) milk yield at dry-off in mRNA abundance (mean ± SEM) for gene expression of genes involved in leukotrienes and oxidative status pathways and migration and cell adhesion: *ALOX5* (Arachidonate 5-Lipoxygenase), *ALOX15* (Arachidonate 15-Lipoxygenase), *SOD1* (Superoxide Dismutase 1), *SOD2* (Superoxide Dismutase 2), *CCR2* (C-C Chemokine Receptor Type 2), *CD44* (Hematopoietic Cell E- and L-Selectin Ligand), *CX3CR1* (CX3C Chemokine Receptor 1), *IL8* (Interleukin 8), *ITGAL* (Integrin Subunit Alpha L), *ITGB2* (Integrin Subunit Beta 2), *LGALS8* (Lectin, Galactoside-Binding, Soluble 8), *SELL* (Selectin L), *SELPLG* (Selectin P Ligand), *TLN1* (Talin 1). *P*-values for main effect of milk yield at dry-off (My), day, and interaction of milk yield × day (My*Day) are shown. Significant differences ($P \leq 0.05$) between groups on the same day are denoted with lowercase a and b, and differences between –7 DFD and 7 or 34 DFD are denoted with an asterisk (*; $P \leq 0.05$) or a plus sign (+; $P \leq 0.1$).

The dry-off also affected the RNA abundance of Pathogen Associated Molecular Patterns - related genes (Fig. 5.3). *CD16*, which is involved in the removal of the antigen-antibody complex from the circulation, had a lower abundance after dry-off, which can lead to a lower innate immune system efficiency against pathogens. However, CD16 is a cluster of differentiation molecule found on the surface of natural killer cells, neutrophils, monocytes, and macrophages (Janeway et al., 2001). The reduced *CD16* expression could be partially explained by the decrease in the number of these cells observed after dry-off (Mezzetti et al., 2020b) Similar responses were observed in genes involved in the toll-like receptors (TLRs) signaling, such as the myeloid differentiation primary response gene 88 (*MYD88*) and *TLR2*. TLRs recognize foreign non-self molecular products, initiating an inflammatory response against invading pathogens, consisting of alerting the body to infection, neutralizing pathogens, and repairing damaged tissues (Foster and Medzhitov, 2009). In particular, *TLR2* has bacterial peptidoglycan and lipoproteins as ligands (Taraktsoglou et al., 2011). After pathogens invasion, microbial products signal through TLRs on tissue-resident mast cells and macrophages, activate these cells to produce proinflammatory cytokines, which coordinates the recruitment of leukocytes together with the antimicrobial function (Foster and Medzhitov, 2009). *MYD88* acts as a signaling transductor of TLRs (not only of *TLR2*), by which is recruited (Janssens and Beyaert, 2002). The lower abundance during the week after dry-off of *TLR2* and *MYD88* might be a proxy of the suppression of the immune system activity in this phase. However, the exact reason why dry-off depressed immune system remains unknown. First, we analyzed RNA abundance in circulating leukocytes and their transcriptome could be different from that of milk or mammary tissue. We could hypothesize that the mammary gland has priority over other tissues during this phase of involution and remodeling, as is the case of the metabolic priority of the mammary gland in early lactation (Gross and Bruckmaier, 2019). During involution, leukocyte concentration in the mammary gland increases (Sordillo and Nickerson, 1988), whereas decreased in the bloodstream (Mezzetti et al., 2020b), likely due to migration to the mammary gland. Moreover, blood phagocytic cells are more efficient than their milk counterparts (Sordillo et al., 1997; Rainard and Riollet, 2006). Therefore, even with a lower expression of related genes, they might be able to cope with systemic stimuli, at the same time prioritizing mammary gland immunity. Additionally, the switch from the high-energy diet of lactation to hay-feeding first and high-fiber dry period diet then might have played a role. Plasma NEFA concentration increased during the days following the dry-off (Mezzetti et al., 2020b) and they are known to have an immunosuppressive effect (Ster et al., 2012).

The suppression state of leukocyte immune function could be confirmed also by the genes related to migration and cell adhesion, that were downregulated after dry-off (Fig. 5.2). Abrupt cessation of milking and the beginning of active involution leads to the recruitment of immune cells

into the mammary gland (Hurley, 1989; Atabai et al., 2007). To avoid an excessive rate of migration, it would be possible to hypothesize that the migration capacity of circulating cells, not absorbed by the mammary gland, might be inhibited. A similar negative feedback mechanism was proposed for the control of neutrophils diapedesis and chemotaxis mediated by lysozyme in severe local inflammatory processes to prevent excessive tissue damage (Gordon et al., 1979). The protein CX3C chemokine receptor 1 (*CX3CR1*) is the receptor for CX3CL1, also known as fractalkine. It is expressed in immune and non-immune cells and their interaction mediates the chemotaxis of immune cells (Lee et al., 2018). The combination of integrin alpha L chain (*ITGAL)* and the beta 2 chain (*ITGB2*) forms the lymphocyte function-associated antigen-1 (LFA-1), which has a relevant role in the extravasation of immune cells from the bloodstream to tissues (Salmi and Jalkanen, 1997). In this case, the main target was probably the mammary gland, which faces huge challenges in the transition from milking to dry period. The cytoskeletal protein Talin-1 (*TLN1*) is also involved in neutrophil chemotaxis (Dixit et al., 2012). Together, the decrease in the mRNA abundance of these genes after dry-off might suggest a reduced leukocytes migration capacity, as observed in other studies after calving (Seo et al., 2013; Crookenden et al., 2016). Additionally, in the present study, the effect of these genes was maintained over one month after dry-off (34 DFD), likely indicating a persistency of this condition. Meanwhile, the antibiotic therapy at dry-off might have influenced these processes, even though, without a comparison with cows that did not receive the antibiotic therapy, we could not confirm this speculation. Alongside the immunosuppression noted after the dry-off, the antibiotic therapy may have reduced the bacterial load in the udder, causing the lack of immune system activation.

Moreover, dry-off, milk stasis, and mammary gland involution affected also genes involved in antimicrobial strategies (Fig. 5.3). Indolamine 2,3-dioxygenase (*IDO1*) encodes a protein that catalyzes the degradation of the essential amino acid tryptophan, reducing its availability for pathogens at the site of infection (Mellor, 2005). Myeloperoxidase (*MPO*) release stimulates neutrophils killing of pathogens by phagocytosis or by antimicrobials release (Teng et al., 2017), catalyzes the production of hypochlorous acid (Winterbourn and Kettle, 2000), and induces neutrophils activation (Lau et al., 2005). Interestingly, *MPO* was upregulated. The upregulation of both these genes shortly after dry-off might suggest a more active innate immune system in this phase, in order to cope with milk and pathogens stasis in the udder. These results are consistent with the increased lipoxygenase RNA abundance, suggesting the activation of inflammatory response after dry-off. Meanwhile, matrix metalloproteinase 9 (*MMP9*) abundance was lower after dry-off. *MMP9* is a collagenase of the gelatinase B group, which are zinc dependent proteinases degrading at least one component of the extracellular matrix or basement membrane (Hanthorn et al., 2014). In this

way, they assist neutrophils' migration from blood to the site of inflammation. Therefore, *MMP9* downregulation is consistent with that of genes involved in migration and cell adhesion (*CX3CR1*, *ITGAL*, *ITGB2*, and *TLN1*), and may be a signal of reduced immune cells migration capacity in the early dry period. The previously hypothesized negative feedback system, mediated by lysozyme or by another compound present in the mammary gland, might be implied. Opposite results have been reported in mammary dry secretions (Yu et al., 2012; Ollier et al., 2013), where *MMP9* dramatically increases during mammary involution, due to neutrophils infiltration and degranulation. Therefore, it seems that an increased immune system efficiency in the target site was paired with a reduced migration capacity into the bloodstream.

The dramatic changes at the turn of dry-off (i.e. diet change and abrupt milking cessation) resulted in oxidative stress, paired with an inflammatory response (Mezzetti et al., 2020b). Superoxide dismutase (SOD) enzymes are one of the most efficient antioxidant systems, catalyzing the reduction of ROS (Sordillo and Aitken, 2009). In our study, *SOD2* abundance was reduced during the week after dry-off (Fig. 5.2). A depletion of the antioxidant system was reported both after dry-off (Mezzetti et al., 2020b) and after calving (Sordillo and Mavangira, 2014). Moreover, being *SOD2* the mitochondrial superoxide dismutase directly involved with the respiratory chain, its downregulation after dry-off might be related to the intense cell metabolism (Bühler et al., 2018), typical of active mammary gland involution (Hurley, 1989; Zhao et al., 2019). Genes associated with oxidative stress, in particular in *SOD2*, were upregulated after dry-off (Singh et al., 2008). However, in that research, the expression of alveolar tissue was analyzed, which is directly involved in mammary gland involution. In the present study, we analyzed blood leukocytes expression, and the different responses observed could be related to this important difference in the studies.

5.4.1 Effects of milk yield at dry-off on gene expression

Milk yield before dry-off had a significant effect only on the abundance of genes involved in the inflammatory cascade. In fact, HM cows had increased peripheral leukocyte mRNA abundance of *IL18* and *IL8* related genes compared with LM ($P = 0.04$ and $P = 0.02$, respectively; Figs 5.1 and 5.2). *IL18* gene encodes a pro-inflammatory cytokine that enhances natural killer cell activity, the proliferation of activated T cells, and induces interferon-γ production from spleen cells, liver lymphocytes, and type-I T-helper cells (Takeda et al., 1998), whereas *IL8* mediates the chemotaxis of neutrophils and other inflammatory cells from the blood into the mammary gland (Lahouassa et al., 2008). RNA abundance of *TLR2* decreased more markedly in HM cows ($P = 0.10$; Fig. 5.3), likely suggesting a greater reduction in innate immune system activity in these cows. These results are consistent with previous findings of Mezzetti et al. (2020b), who observed increased inflammation after dry-off in higher producing cows, likely due to longer mammary tissue remodeling required.

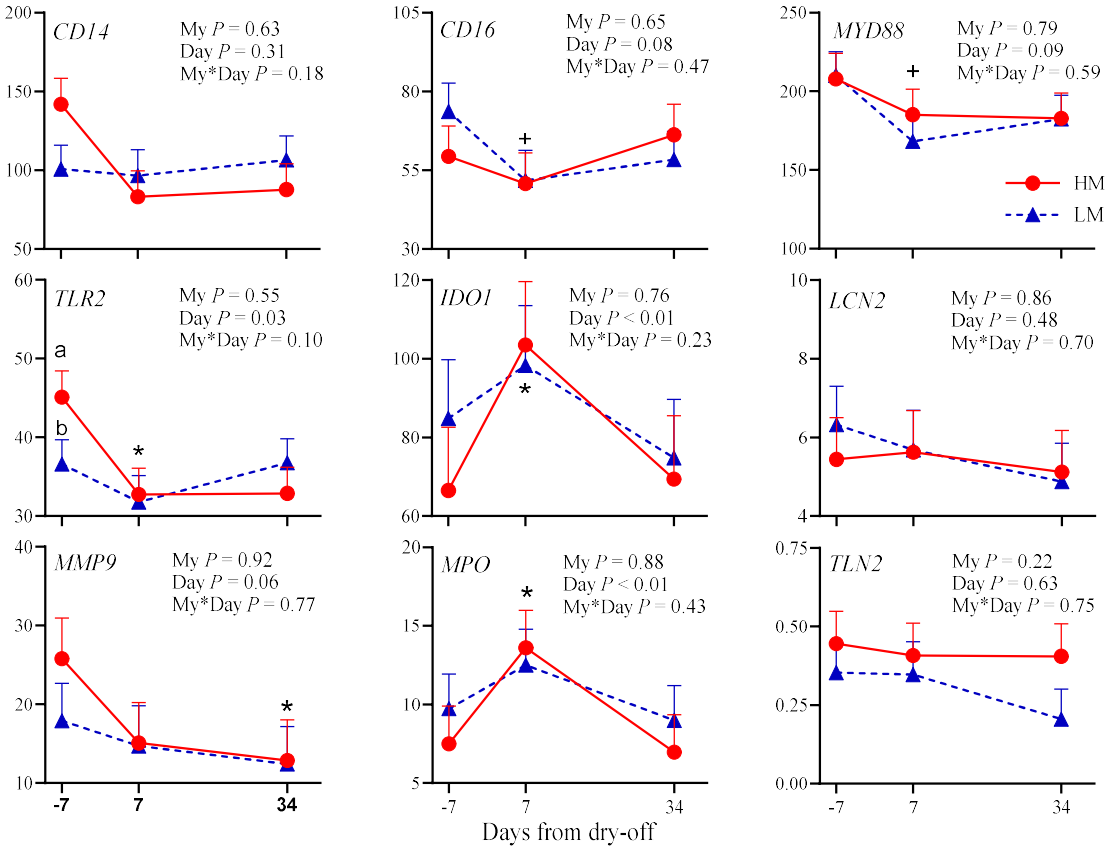

Figure 5.3. Changes from –7 days from dry-off (DFD) to 34 DFD in dairy cows with high (HM; red line) or low (LM; blue line) milk yield at dry-off in mRNA abundance (mean ± SEM) for gene expression of genes involved in recognition and immune mediation and antimicrobial strategies: *CD14* (Cluster of Differentiation 14), *CD16* (Cluster of Differentiation 16 or Fc Fragment of Igg Receptor IIIa), *MYD88* (Myeloid Differentiation Primary Response Gene 88), *TLR2* (Toll-Like Receptor 2), *IDO1* (Indoleamine 2,3-Dioxygenase 1), *LCN2* (Lipocalin 2), *MMP9* (Matrix Metallopeptidase 9), *MPO* (Myeloperoxidase), *TLN2* (Talin 2). *P*-values for main effect of milk yield at dry-off (My), day, and interaction of milk yield × day (My*Day) are shown. Significant differences ($P \leq 0.05$) between groups on the same day are denoted with lowercase a and b, and differences between –7 DFD and 7 or 34 DFD are denoted with an asterisk (*; $P \leq 0.05$) or a plus sign (+; $P \leq 0.1$).

5.5 Conclusions

The dry-off alters blood biomarkers of nutrient metabolism, inflammation, and oxidative stress. This study investigated peripheral blood leukocyte RNA abundance of genes involved in pathways of inflammation, immune system, and oxidative stress. The dry-off triggered an inflammatory response and increased oxidative stress. Peripheral leukocytes antimicrobial and antioxidant capacity were somewhat impaired, but the exact reasons were unclear. In cows that produced more than 15 kg/day during the week before dry-off, the inflammatory response after dry-off was exacerbated. Therefore, the transition from lactation to the dry period needs special attention, in particular in high-yielding cows. Further research on gene expression would be needed in substrates

closer to the mammary gland, such as the milk somatic cells, mammary epithelial cells, or mammary tissue.

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Chapter 6: Impact of Nutrient Restriction at Dry-off on Performance and Metabolism

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6.1 Abstract

Thanks to improvements in genetics, nutrition, and management, modern cows can still produce large amounts of milk at the end of lactation, with possible negative effects on health and welfare, particularly when milking is stopped abruptly. To limit yield at dry-off, strategies involving different degrees of feed restriction have been used worldwide. Thus, we aimed to investigate the effects of a reduced nutrient density at dry-off on milk production, metabolism, rumen fermentation pattern, and milk fatty acid profile around dry-off and in the ensuing transition period. During the last week before dry-off, 26 Holstein dairy cows were either fed *ad libitum* ryegrass hay (NR; n = 12) or continued to receive lactation diet (CTR, n = 12). After dry-off, both groups received only grass hay for 7 days, and free access to water was always provided. Blood, milk, and rumen samples were collected from 7 days before dry-off to 28 days in milk. Milk production, lying and rumination times were recorded daily. At dry-off, compared to CTR, NR decreased milk yield (– 65%) and milk lactose but had higher fat and protein contents. In the subsequent lactation, no differences were observed in milk composition, but yield was slightly lower in NR in the first 2 weeks of lactation. The BCS did not differ between groups. Before dry-off, NR had decreased glucose, urea, and insulin, but higher creatinine, β-hydroxybutyrate, and non-esterified fatty acids. The day after dry-off, non-esterified fatty acids were lower in FR, but they were higher 7 days after calving. At dry-off, NR had higher rumen pH, lower lactate, urea, and total VFA production. Considering VFA molar proportions, NR had increased acetate but decreased propionate and butyrate at dry-off. Rumination time dropped 6 days before dry-off in NR and after dry-off in CTR. No differences were observed in the periparturient period and, overall, in lying time. Milk FA profile revealed a remarkably lower proportion of short-chain fatty acids in NR at dry-off and a higher proportion of medium- and long-chain ones. These results confirmed that decreasing nutrient density can effectively reduce milk yield before dry-off, with minor effects on milk production in the following early lactation. However, metabolism around dry-off was significantly impacted, as suggested by plasma, rumen fluid, and milk analysis. Further research is required to investigate the impact of the metabolic effects on the inflammatory response, liver function, and immune system, particularly concerning the mammary gland.

Keywords: mammary gland involution; stress; milk cessation; transition period

6.2 Introduction

The dry-off represents an important phase of the lactation cycle in dairy cattle, with the transition to a nonlactating period. At this time, several changes happen, and cows need to adapt to them. Among those, the milking cessation promotes the beginning of the mammary involution process (Zhao et al., 2019), which allows the regeneration of the secretory tissue. The diet changes

as well, with the use of more fibrous rations to which the rumen papillae need to adapt (Dieho et al., 2016). Furthermore, cows are moved to a different pen, requiring the establishment of a new social hierarchy (von Keyserlingk et al., 2008). Together, these factors can develop a potentially stressful condition for the animals (Zobel et al., 2015), as pointed out by a drop in rumination time (Abuelo et al., 2021). The increase in stress levels after dry-off can be observed also in the blood (Putman et al., 2018; Mezzetti et al., 2020; Cattaneo et al., 2021c), with dramatic changes in terms of metabolism, inflammation, oxidative stress, and also at the gene level (Cattaneo et al., 2021b).

Besides, due to the progress in management, nutrition, and genetics, modern cows have increased persistency of lactation, resulting in still high milk yield in late gestation. Cows dried off with productions over 15 kg/d show a worse adaptation to the dry period, with carryover effects in the ensuing lactation (Vilar and Rajala-Schultz, 2020). In particular, in high-yielding cows there is a delayed involution process (Silanikove et al., 2013), an exacerbated and prolonged inflammatory response (Mezzetti et al., 2020; Cattaneo et al., 2021b), and also an increased milk leakage (Dingwell et al., 2004). To avoid these adverse reactions, many strategies have been proposed (Vilar and Rajala-Schultz, 2020). Most of them include a feed restriction to reduce production and promote the onset of the involution process.

Finally, the condition of the cows around dry-off appears to influence the subsequent adaptation to the transition period and the performance in the following lactation (Cattaneo et al., 2021a).

We hypothesized that reducing the diet's nutrient density could decrease yield before dry-off without impairing future performance. The aim of this study was to investigate the impact of nutrient restriction at dry-off on milk production, metabolism, rumen fermentations, and milk fatty acids (FA) composition around dry-off and in the subsequent transition period.

6.3 Materials and Methods

6.3.1 Animal management and experimental design

The research was carried out at Università Cattolica del Sacro Cuore dairy barn (Cerzoo, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation and ethics (Italian Health Ministry authorization N 139/2021-PR in agreement with D. Lgs. n. 26, 04/03/2014). A group of 26 Holstein dairy cows (parity 1.8 ± 1.6; mean ± SD) was enrolled in the study. Cows were selected based on milk yield and udder health 21 days before dry-off. The inclusion criteria were a milk yield of at least 15 kg/d, composite milk SCC below 400,000 cells/mL, and absence of major pathogens in any quarter.

During lactation, cows were housed in a freestall pen with rubber mattresses covered by shavings, received the lactation diet, and were milked twice daily (5:00 and 17:00h). Feed intake was measured daily with Roughage Intake Control (RIC) troughs (Insentec BV, Marknesse, The Netherlands), that records the visits to the feed bin (i.e., number and duration of visits and feed intake). Two cows were assigned to each bin and water was provided ad libitum.

Seven days before dry-off, cows were moved to a straw-bedded pen, where they remain for 14 days. Here, they either were fed ryegrass hay (CP = 7.1 ± 1.6% DM; NDF = 59.8 ± 4.3% DM) ad libitum (NR, n = 13) or continued to receive lactation diet (CTR, n = 13) until dry-off. In this pen, RIC troughs were not present. Fifty-five days before expected calving, cows were dried off with a single injection in each quarter of intramammary antibiotic (Mamyzin A; Boehringer Ingelheim Animal Health Italia S.p.A) and internal teat sealant (Noroseal; Norbrook Laboratories Limited). Afterward, both groups were fed ryegrass hay ad libitum for 7 days. At the end of the drying-off phase, cows were moved to the dry pen, which had the same design as the lactation pen, and received the dry period diet. Rations were formulated according to NRC (2001) guidelines and their chemical composition is reported in Table 6.1.

Table 6.1. Ingredients and chemical composition of diets served as TMR during the study

	Lactation	Dry period
Ingredient, % of DM		
Corn silage	32.6	11.6
Alfalfa hay	24.9	-
Corn ground	13.8	-
Soybean meal	11.4	4.8
Barley ground	9.2	-
Wheat silage	3.3	47.5
Sunflower meal	2	5.1
Mineral and vitamin	1.9	0.9
Hydrogenated fat	0.9	-
Straw	-	17.7
Grass hay	-	12.4
Chemical		
NE_L, Mcal/Kg	1.65	1.28
CP, % of DM	16.6	12.7
NSC, % of DM	30	9.7
NDF, % of DM	33.4	57
Calcium, % of DM	0.79	0.45
Phosphorus, % of DM	0.42	0.34

Groups were balanced for parity, milk production, days open in the previous lactation, and milk production at –21 DFD. One cow per group was removed from the final dataset due to illness unrelated to treatment. A descriptive analysis of animals involved in the trial is shown in Table 6.2.

Table 6.2. General characteristics of the cows enrolled in the study

	Mean	SD
Parity at enrollment	1.79	1.14
DIM at enrollment, d	350	71.2
Days carrying calf at enrollment, d	218	3.26
Previous lactation length, d	357	70.8
Milk yield at -21 DFD, kg/d	24.0	4.59
SCC at -21 DFD, n×10^3/mL	83.0	71.1
SCC at -21 DFD, logSCC	1.76	0.40
Mature cow equivalent milk yield, kg/d	11301	1065
Average milk yield previous lactation, kg/d	29.4	3.15
Average SCC previous lactation, logSCC	2.07	1.05

6.3.2 Blood sampling and analysis

Blood samples were collected from the jugular vein into heparinized tubes before the morning feeding at –7, –3, 0, 1, 4, 7, 14, and 28 DFD and at –14, –3, 3, 7, 14, and 28 DFC. Samples were immediately placed into an ice-water bath and processed as described by Calamari et al. (2016) for the determination of the markers of energy and protein metabolism: glucose, β-hydroxybutyric acid (BHB), non-esterified fatty acids (NEFA), urea, and creatinine. Concentrations of insulin were analyzed using an ELISA kit (Mercodia Bovine Insulin ELISA, Mercodia Inc., Uppsala, Sweden). Intra- and interassay CV were 2.3 and 5.2 %, respectively.

6.3.3 Milk yield, lying, and rumination time

Before dry-off and from 4 to 120 DFC, milk yield was automatically recorded daily in the milking parlor. From 0 to 28 DFD and from –28 to 120 DFC, lying time and lying bouts were recorded daily with the AfiTag II pedometer (SAE Afikim, Israel), previously validated in dairy cows (Henriksen and Munksgaard, 2019), and rumination time was recorded using the Hr-LD tags (SCR by Allflex, Netanya, Israel). Data were expressed as weekly average.

6.3.4 Body weight, body condition score, rectal temperature, health status

Body weight was recorded at each milking with a single walking-in scale (SAE Afikim, Israel). With the same schedule of blood sampling, body condition score (BCS) was assessed by the same person (ADAS, 1986) and rectal temperature was measured with a digital thermometer. Health status was monitored by the personnel and veterinarian staff operating in the farm facilities, and any sign of disorder was noted. Mastitis was diagnosed by visual evaluation of abnormal milk from each

quarter and SCC analysis on suspicious cases. Outcomes of pregnancy diagnosis, number of artificial inseminations (AI), and days open were recorded.

6.3.5 Milk samples

At –21, –7, –3, and 0 DFD, and at 7, 14, and 28 DFC, composite milk samples were collected in the morning milking determine milk composition and milk FA profile. Fat, protein, casein, lactose, and urea were determined with Milkoscan FT120 analyzer (Foss Analytics, Hillerød, Denmark). Extraction and methylation of milk fat FA were performed following the method of O'Fallon et al., (2007). Briefly, 1 ml of milk samples were placed into a 16 × 125 mm screw-cap Pyrex culture tube, then 0.7 mL of 10 *N* KOH in water and 5.3 mL of MeOH were added. Tubes were incubated in a water bath (1.5 h at 55°C) and vigorously hand-shaken for 5 s every 20 min. After cooling in a cold tap water bath, 0.58 mL of 24 *N* H_2SO_4 in water was added. After mixing and precipitation of K_2SO_4, tubes were incubated again (1.5 h at 55°C), hand-shaken for 5 s every 20 min, and, after FA methyl ester synthesis, cooled in a cold tap water bath. The FA methyl esters were extracted by addition of 3 mL of hexane followed by mixing and centrifugation (5 min at 3000g). Hexane layer containing FA methyl esters was put into gas chromatography vial and used for the gas chromatographic analysis. The assay was performed using equipment and conditions previously reported (Mezzetti et al., 2022).

At –21 DFD, 7, and 28 DFC, a milk sample was aseptically collected from each quarter. Briefly, after pre-dipping application with foaming wipes, each teat was accurately cleaned with a cotton swab soaked in ethyl alcohol, and the first 5 streams of milk were discarded. Then, samples were collected into 5-mL sterile tubes, immediately refrigerated, and transferred at "Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna", where they were analyzed within 24 h from the collection, as described in Cattaneo et al. (2021).

6.3.6 Rumen fluid samples

Rumen fluid samples were collected at –7, 0, and 7 DFD and –14, 7, and 28 DFC before the morning feeding with a ruminal probe specially designed for cattle (Ruminator; profs-products.com, Germany), as previously described (Wallace et al., 2019). Samples were immediately placed in an ice-water bath. Within 30 min, the pH was measured with a pH meter (GLP 21; Crison Instruments SA, Spain). Then, samples were gently mixed, and five aliquots of 1 mL each were pipetted into 2 mL tubes and immediately stored at -20°C for volatile fatty acids (VFA) measurement. Concentrations of total and individual VFA were determined as described by Minuti et al. (2014) and expressed as molar proportions. Ammonia was measured using a urea nitrogen kit (Instrumentation laboratory, Milano, Italy) with a clinical auto-analyzer (ILAB-650), whereas D- and L-lactic acid

with a D-/L-Lactate assay Kit (Megazyme International Ltd., Wicklow, Ireland), following manufacturer's instruction.

6.3.7 Statistical analysis

Data were analyzed with SAS software (version 9.4; SAS Institute Inc., Cary, NC). Sample size was based on the availability of cows in the herd and supported by a power analysis (POWER procedure of SAS). From previous data collected in the same farm (Cattaneo et al., 2022b), considering high-producing cows in late lactation, a sample size of 12 subjects/group would be sufficient to detect a significant difference of 6 kg/d (with mean = 24 kg/d and SD = 5 kg/d) with α = 0.05 and β = 0.20. Before analysis, the normality of distributions was checked (UNIVARIATE procedure of SAS), and non-normally distributed variables were log-transformed. Data with a single measure were subjected to ANOVA (GLM procedure of SAS). Data about multiple observations were analyzed with repeated measures mixed models (GLIMMIX procedure of SAS) with the compound symmetry covariance structure. The periods around dry-off and calving were analyzed separately. Besides, one NR cow left the herd before the end of the trial and was excluded from the analysis of the periparturient period. The models included the fixed effects of treatment (Trt; CTR vs NR), time (T), their interaction (Trt×T), parity as a covariate (primiparous vs multiparous), and the random effect of the cow nested within treatment. Baseline value was included as a covariate when appropriate. Pairwise comparisons were carried out with the Tukey adjustment. Moreover, the milk fatty acid profile was evaluated by a principal component (PC) analysis (PRINCOMP procedure of SAS). Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

6.4 Results

6.4.1 Milk production

During the week before dry-off, milk yield dropped in the NR group, while was constant in the CTR group (22.6 ± 1.01 kg/d; $P < 0.01$; Fig. 6.1). Particularly, NR cows produced 12.6 ± 1.37 kg/d after 2 days of treatment, and 7.75 ± 0.98 kg/d the day before dry-off (–65% compared with CTR). In the subsequent early lactation, groups had a different trend ($P = 0.02$; Fig. 6.2), with numerically higher production in the CTR group compared with NR in the first 2 weeks of lactation (39.4 and 42.5 ± 1.23 kg/d for CTR and 37.4 and 40.0 ± 1.28 kg/d for NR).

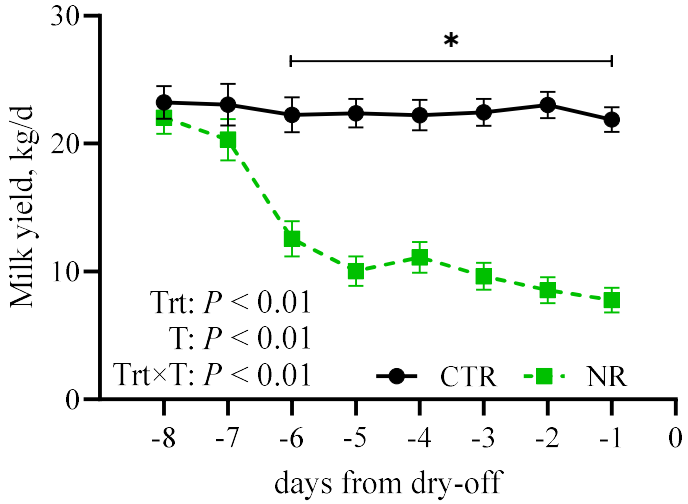

Figure 6.1. Milk yield in the week preceding dry-off in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported.

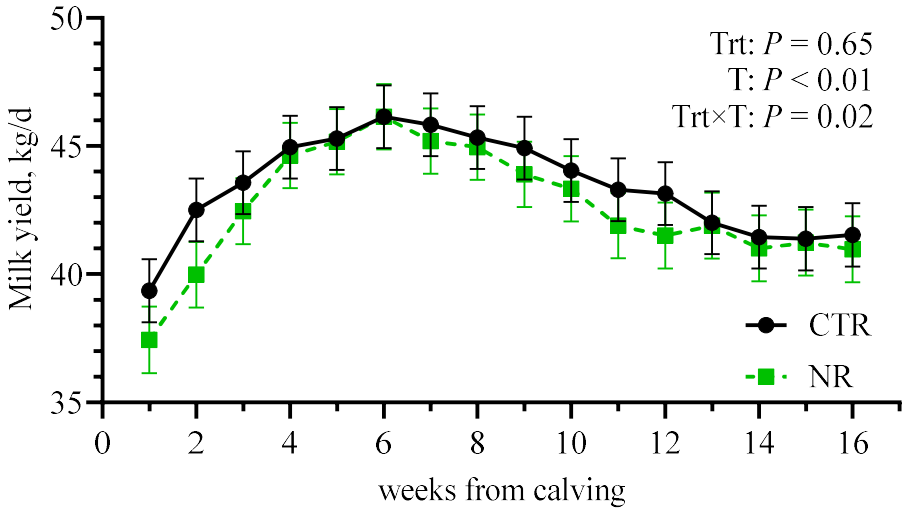

Figure 6.2. Milk yield in the subsequent lactation in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported.

The week before dry-off, milk composition was pretty stable in the CTR group (Fig. 6.3), whereas in the NR group milk fat ($P < 0.01$), protein ($P = 0.04$), and fat:protein ratio ($P < 0.01$) increased, and lactose decreased ($P < 0.01$). In the first month of lactation, no difference was observed between groups (data not shown).

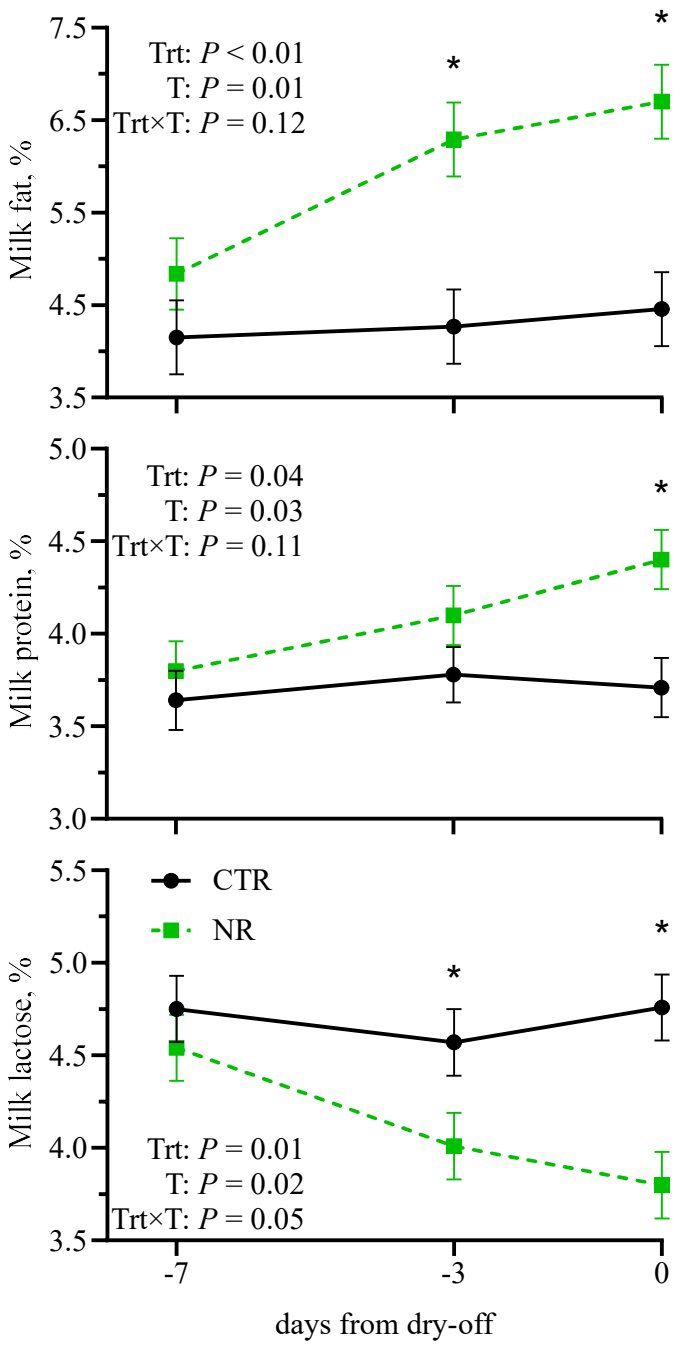

Figure 6.3. Milk composition in the week preceding dry-off in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported.

6.4.2 Health and fertility

No differences were observed in disease incidence between groups (data not shown). Rectal temperature was overall similar between groups, but at –3 DFD was lower in NR compared with CTR

(38.25 vs 38.63 ± 0.09°C; $P < 0.01$; Table 6.3). No difference was observed during the transition period.

Table 6.3. Milk yield, SCC, and gestation length in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off

	Treatment			
Item	CTR	FR	SEM	*P*-value[1]
Milk yield at -7 DFD, kg/d	22.6	21.8	1.38	0.68
SCC at -7 DFD, logSCC	1.76	1.76	0.11	1.00
Gestation length, d	278	277	1.08	0.31
Difference from expected calving, d	-1.67	-3.25	1.08	0.31
Dry period, d	52.5	53.2	1.52	0.76

[1] Overall effect of the treatment (Trt), time (T), and their interaction (Trt×T)

Body weight decreased by around 20 kg in NR during the last week of lactation (668 vs 652 ± 4.5 kg at –8 and –1, respectively, while was unchanged in CTR (668 vs 675 ± 4.5 kg at –8 and –1, respectively; $P < 0.01$; Table 6.3). Despite similar body weight at calving, it was lower in CTR compared with NR in early lactation (Trt×T; $P < 0.01$). The BCS was not different between groups both at dry-off and calving (Table 6.3).

Days open (121.7 vs 119.4 ± 13.76 d; $P = 0.90$) and services per conception (2.58 vs 2.00 ± 0.42; $P = 0.33$) in the subsequent lactation did not differ between groups.

6.4.3 Feeding and lying behavior

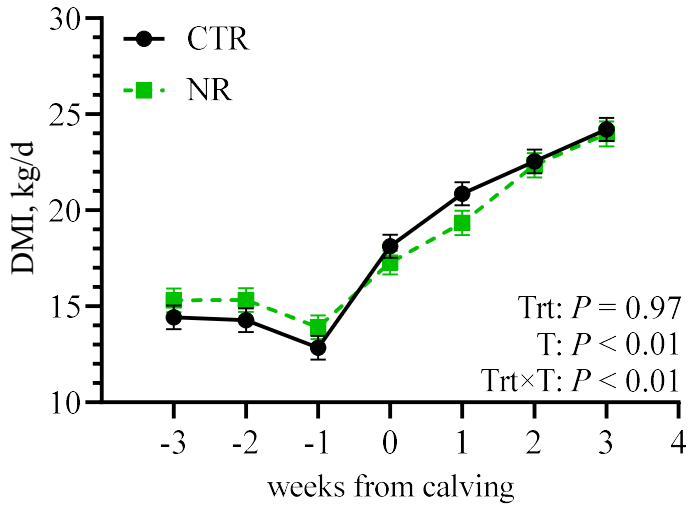

Figure 6.4. Dry matter intake from –3 to 3 weeks from calving in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported.

Dry matter intake had a different trend between groups during the transition period ($P < 0.01$; Fig. 6.4). Compared with CTR, NR cows ate slightly more before calving, but less during the second week of lactation (20.9 vs 19.3 ± 0.63 kg/d for CTR and FR, respectively; $P = 0.09$).

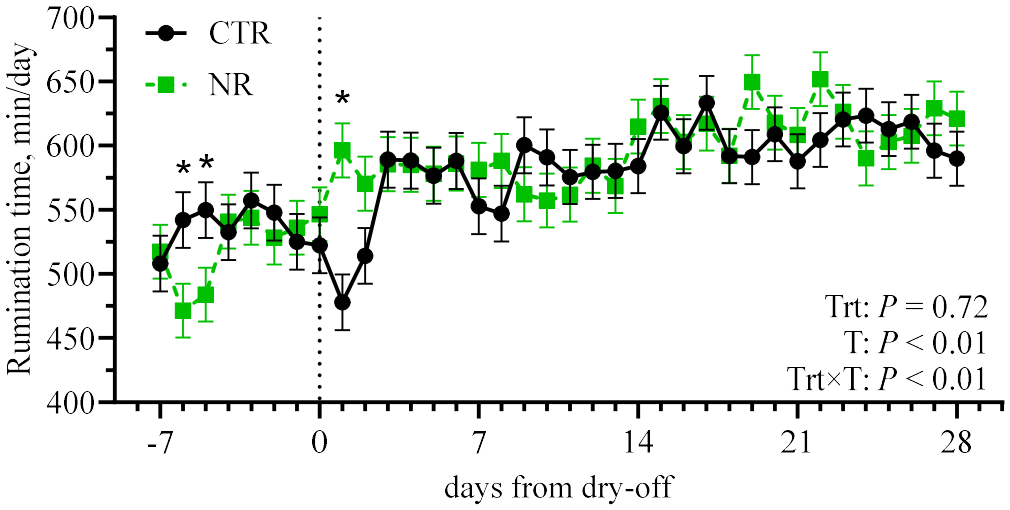

Figure 6.5. Daily rumination time from 7 days before dry-off to 28 days after in high producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the model are reported.

Rumination time had a different trend between groups ($P < 0.01$; Fig. 6.5). It dropped at –6 and –5 DFD in NR (471 and 484 ± 21.1 min/d, respectively in FR, compared with 542 and 550 ± 21.8 min/d in CTR), and then increased again to similar values to CTR until dry-off. Conversely, values were stable in CTR before dry-off, dropped at 1 and 2 DFD (478 and 514 ± 21.8 vs 596 570 ± 21.1 min/d), and rose again afterward. No difference was observed around calving (544 vs 557 ± 14.4 min/d for CTR and FR, respectively; $P = 0.51$). Lying time after dry-off (817 vs 796 ± 20.7 min/d for CTR and FR, respectively; $P = 0.48$) and around calving (670 vs 693 ± 25 min/d; $P = 0.54$) did not differ between groups.

6.4.4 Rumen fermentation

Rumen fluid composition is reported in Table 6.4. Rumen pH was not different between groups at –7 and 7 DFD but at 0 DFD was higher in NR than in CTR (6.89 vs 7.36 ± 0.06 in CTR and FR, respectively; $P < 0.01$; Fig. 6.6). At calving, compared with CTR, pH tended to be higher in NR at 7 DFC and lower at 28 DFC (Trt×T; $P = 0.05$). D-lactate (49.2vs 12.5 ± 3.9 mmol/L at 0 DFD; $P < 0.01$), L-lactate (41.4 vs 12.9 ± 3.30 mmol/L at 0 DFD; $P < 0.01$), and ammonia (6.17 vs 2.25 ± 0.48 mmol/L at 0 DFD; $P < 0.01$) had similar trends, and they decreased in both groups at 7 compared with –7 DFD. No differences in D- and L-lactate, and ammonia were observed around calving.

Table 6.4. Rumen fluid composition around dry-off (from –7 to 28 days from dry-off) and calving (from –14 to 28 days from calving) in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off

	Treatment			*P*-value[1]		
Item	CTR	FR	SEM	TRT	T	Trt×T
Around dry-off						
pH	7.05	7.21	0.03	<0.01	0.01	<0.01
L-Lactate, mmol/L	31.9	21.6	1.84	<0.01	<0.01	<0.01
D-lactate, mmol/L	36.0	22.7	2.11	<0.01	<0.01	<0.01
Ammonia, mg/L	4.76	3.19	0.28	<0.01	<0.01	<0.01
Total VFA, mmol/L	80.2	67.9	2.30	<0.01	0.03	<0.01
Acetate, %	66.0	69.9	0.57	<0.01	<0.01	<0.01
Propionate, %	19.5	17.7	0.53	0.02	<0.01	<0.01
Butyrate, %	10.36	8.92	0.14	<0.01	<0.01	<0.01
Isobutyrate, %	1.01	0.98	0.04	0.65	<0.01	0.11
Valerate, %	1.09	0.86	0.04	<0.01	<0.01	<0.01
Isovalerate, %	1.45	1.25	0.06	0.03	<0.01	<0.01
Hexanoate, %	0.53	0.37	0.04	0.01	<0.01	<0.01
Heptanoate, %	0.041	0.027	0.004	0.03	<0.01	<0.01
Around calving						
pH	6.96	6.95	0.05	0.93	<0.01	0.05
L-Lactate, mmol/L	89.3	94.2	13.3	0.80	0.07	0.79
D-lactate, mmol/L	91.6	100.4	15.8	0.70	0.11	0.77
Ammonia, mg/L	7.68	8.30	0.49	0.38	<0.01	0.19
Total VFA, mmol/L	79.4	79.0	2.19	0.89	<0.01	0.15
Acetate, %	65.2	65.6	0.57	0.64	<0.01	0.08
Propionate, %	21.0	20.6	0.64	0.67	<0.01	0.04
Butyrate, %	9.82	9.58	0.15	0.30	<0.01	0.96
Isobutyrate, %	0.94	1.01	0.05	0.34	<0.01	0.07
Valerate, %	1.15	1.18	0.04	0.56	<0.01	0.07
Isovalerate, %	1.40	1.53	0.07	0.16	0.02	0.06
Hexanoate, %	0.42	0.43	0.03	0.90	<0.01	0.68
Heptanoate, %	0.033	0.033	0.004	0.96	<0.01	0.22

[1] Overall effect of the treatment (Trt), time (T), and their interaction (Trt×T)

Total VFA production decreased in both groups after dry-off but at 0 DFD was lower in NR compared with CTR (92.2 vs 57.9 ± 4.23 mmol/L in CTR and FR, respectively; $P < 0.01$; Fig. 6.7), but was not different at calving. With regards to molar proportions of individual VFA, acetate increased after dry-off in both groups and at 0 DFD was higher in NR compared with CTR (61.4 vs 72.2 ± 0.89% in CTR and FR, respectively; $P < 0.01$). Other VFA (propionate, butyrate, valerate, isovalerate, hexanoate, and heptanoate; $P < 0.01$) had the opposite trend, increasing after dry-off and being higher in CTR at 0 DFD. No difference between groups was observed in isobutyrate

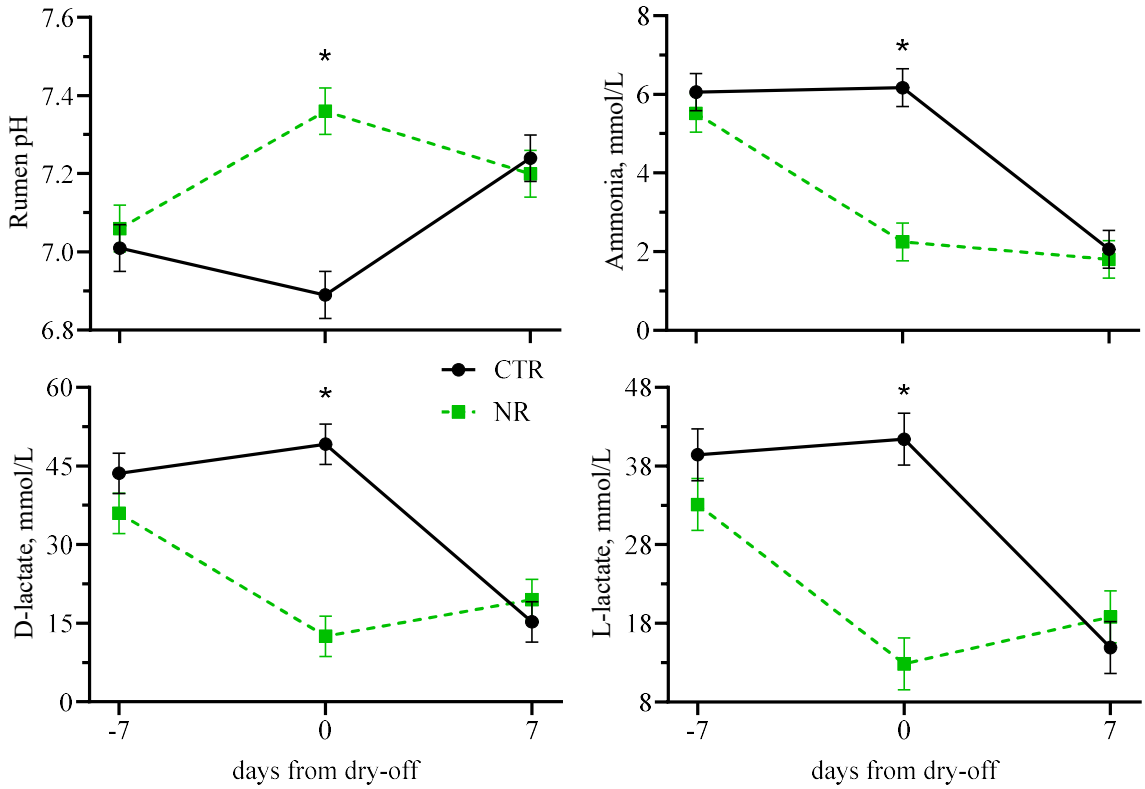

Figure 6.6. Rumen fluid pH, ammonia-N, D- and L-lactate from –7 days to 7 days from dry-off in high producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk.

. Around calving, acetate tended to have different trends between groups (Trt×T; $P = 0.08$), with initially a slower decrease in vcompared with CTR. Propionate had the opposite trend (Trt×T; $P = 0.04$), with a slower increase in NR at 7 DFC.

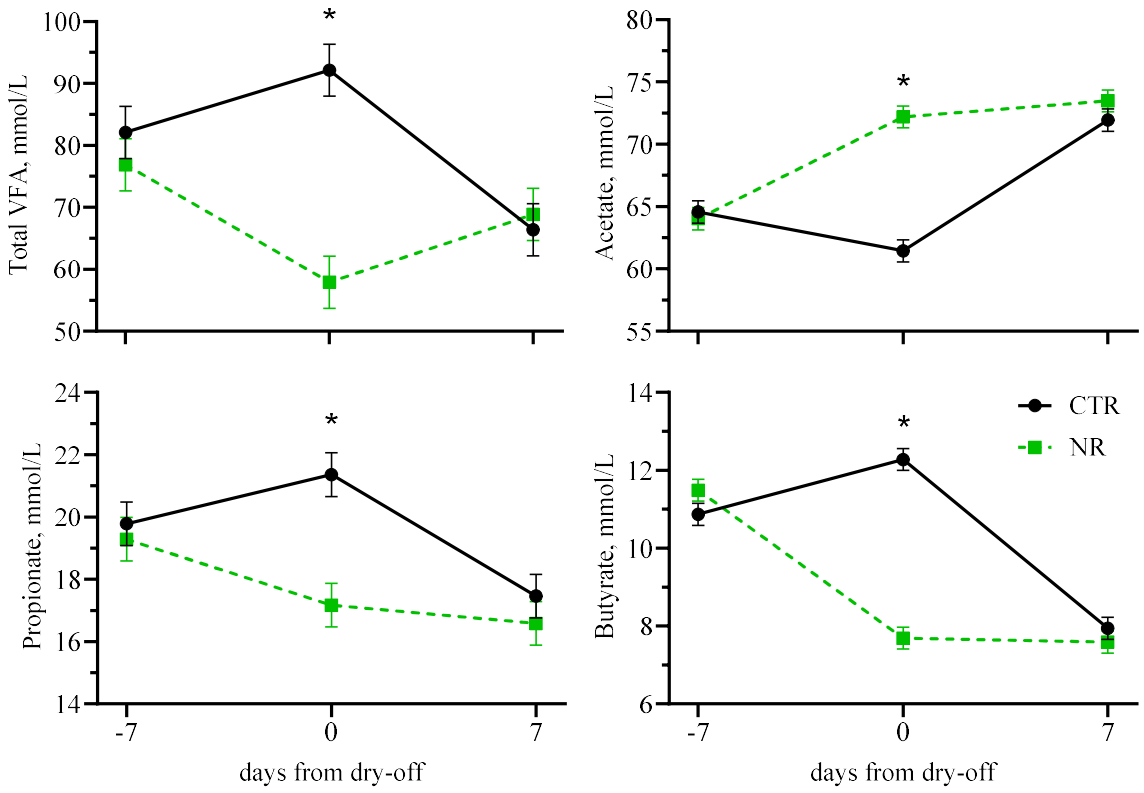

Figure 6.7. Rumen fluid total VFA concentration and molar proportions of acetate, propionate, and butyrate from –7 days to 7 days from dry-off in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk.

6.4.5 Plasma metabolic markers and hormones

Glucose concentration dropped (–10%) at –3 and 0 DFD in NR while it was stable in CTR (4.77 vs 4.28 ± 0.09 mmol/L at –3 DFD and 4.63 vs 4.17 ± 0.09 mmol/L at 0 DFD in CTR and FR, respectively; $P < 0.01$; Fig. 6.8). After dry-off and in the transition period, no difference was noted. Similarly, urea level decreased before dry-off in NR but not in CTR (3.28 vs 5.43 ± 0.32 mmol/L at –3 DFD and 3.32 vs 5.31 ± 0.32 mmol/L at 0 DFD in CTR and FR, respectively; $P < 0.01$), and at 1 DFD (2.92 vs 5.46 ± 0.32 mmol/L). Subsequently, urea concentrations were not different between groups. Creatinine was overall higher around dry-off in NR compared with CTR (92.1 vs 101.9 ± 1.78 µmol/L in CTR and FR, respectively; Trt, $P < 0.01$), in particular from –3 to 7 DFD (Trt×T, $P < 0.01$). At calving, its values were similar between groups.

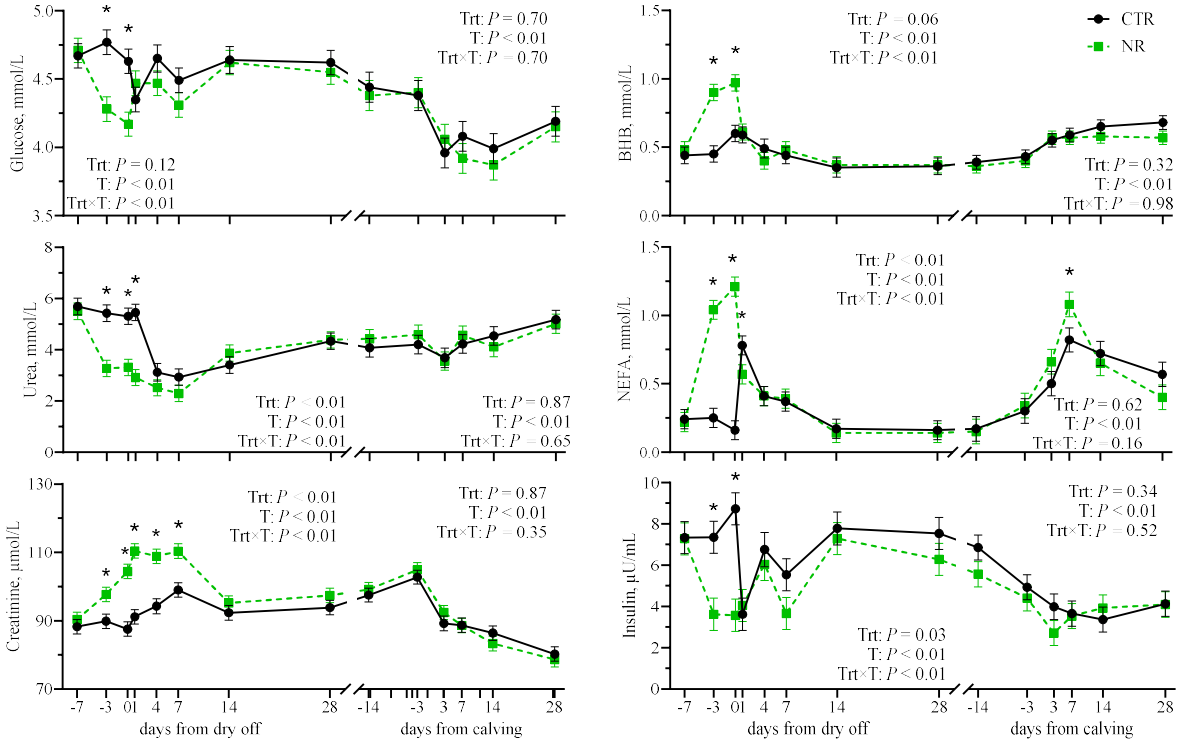

Figure 6.8. Plasma concentrations of glucose, urea, creatinine, BHB, NEFA, and insulin from –7 days to 7 days from dry-off and from –14 days to 28 days from calving in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off. Differences between groups ($P \leq 0.05$) are denoted with an asterisk. Significance levels of the main effects of the models are reported.

Conversely, plasma BHB steadily increased before dry-off in NR but not in CTR (0.45 vs 0.90 ± 0.06 mmol/L at –3 DFD and 0.60 vs 0.97 ± 0.06 mmol/L at 0 DFD in CTR and FR, respectively; $P < 0.01$). NEFA had a similar trend (0.25 vs 1.04 ± 0.07 mmol/L at –3 DFD and 0.16 vs 1.21 ± 0.07 mmol/L at 0 DFD in CTR and FR, respectively; $P < 0.01$), but at 1 DFD their level increased in CTR and decreased in NR (0.78 vs 0.57 ± 0.07 mmol/L; $P < 0.01$). Afterward, no main differences were observed, even though NEFA were higher in NR compared with CTR at 7 DFC (0.82 vs 1.08 ± 0.09 mmol/L; $P = 0.05$).

Insulin dropped in NR during the restriction, while it remained pretty constant in CTR (Trt×T; $P < 0.01$). After dry-off, its concentration in the group decreased as well but no difference was noted between groups. At calving, circulating insulin was not affected by treatment.

6.4.6 Milk FA profile

Outcomes of PC analysis and the eigenvectors between all the variables considered are shown in Fig. 6.7. The first 3 PC explained over 70% of the total variance in each time point. No clear separation between groups was observed at –7 DFD. Moreover, PC1 discriminated very efficiently

among the samples at –3 and 0 DFD according to dietary treatment. The PC eigenvectors revealed that medium and long chain FA (C18:1n9c, C17:1, C17:0, C16:1, C20:1n9, C18:2n6c) were the predominant variables with a negative effect on PC1, and short chain FA (C8:0, C12:0, C14:0, C10:0, C6:0, C13:0, C10:1) were the predominant variables with a positive effect on PC1.

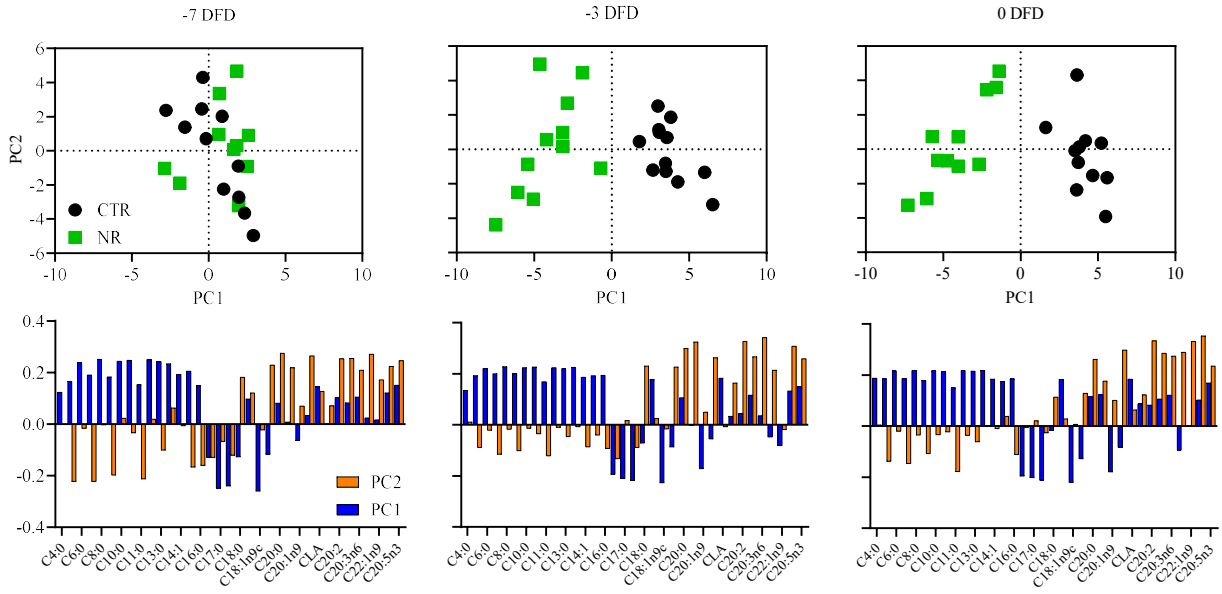

Figure 6.9. Principal component (PC) analysis and eigenvectors on milk fatty acids at –7, –3, and 0 days from dry-off (DFD) in high-producing dairy cows fed either ad libitum ryegrass hay (NR) or lactation diet (CTR) from –7 to 0 days from dry-off.

6.5 Discussion

The dry-off phase has been gaining importance in recent years. Protocols including different intensities of feed restriction are used in commercial farms to reduce excessive milk yield in some cows, but the impact of these practices has not yet been fully explored. The current study investigated the effect of a feed restriction strategy at dry-off on milk production and metabolic responses up to the subsequent early lactation.

6.5.1 Impact at dry-off

The qualitative feed restriction adopted in the present study was effectively able to reduce milk yield in the last week of lactation (–65% compared to the control group). On the last day before dry-off, NR cows produced on average ~8 kg/d, far less than the safety threshold of 15 kg/d proposed by Vilar and Rajala-Schultz (2020). The milk reduction achieved is consistent with previous studies adopting a similar protocol (Ollier et al., 2014, 2015), whereas a more moderate feed restriction resulted in a lower drop (~30%; Larsen et al., 2021). In our trial, the restriction was approximately

equal to 40% of the lactation diet, comparable to the intensities used in other studies (Leduc et al., 2021; Cattaneo et al., 2022a).

Although we did not measure intramammary pressure and milk leakage, these low production levels should ensure mitigation of the risk of these phenomena (Dingwell et al., 2004). Conversely, the still high yield in the control group (~22 kg/d) was linked to increased intramammary pressure, glucocorticoid production, risk of milk leakage and entrance of pathogens into the teat canal, and delayed involution (Bertulat et al., 2013; Silanikove et al., 2013). Nevertheless, despite being numerically higher in FR, lying time after dry-off was not significantly different. High milk yield, and the consequently increased udder engorgement, volume, and intramammary pressure, usually make lying less comfortable (Zobel et al., 2013). However, other authors reported the lack of association between lying time after dry-off and milk production level before, particularly in multiparous cows (Chapinal et al., 2014; Bach et al., 2015). Although the sample size was likely low to detect behavioral differences, we would have expected a reduced lying time in CTR cows.

At the same time, cows lost weight (~20 kg) but not BCS. Therefore, the weight loss was mostly due to the lower weight of rumen content and digesta caused by the likely decreased intake. We could not accurately measure feed intake in the drying off, but the greater volume of the hay compared with the lactation diet likely limited the intake capacity increasing gut distension (Allen, 1996). Surprisingly, BCS was not different between groups, since we would have expected a decrease in NR cows. However, the limited duration of the restriction, paired with the limited sample size, likely prevented the achievement of significant differences. Moreover, the hormonal framework in late lactation could partly account for this fact. In similar intake deficit conditions, early lactating cows mobilize a relevant amount of body reserves to support milk synthesis (Drackley, 1999). Nevertheless, after calving the mammary gland is metabolically prioritized (Bell and Bauman, 1997), circulating insulin is low and cows are insulin-resistant (De Koster and Opsomer, 2013). This metabolic status sustains milk production, mobilizing body reserves and redirecting nutrients available toward the mammary gland at the expense of peripheral tissues, despite deficient feed intake. In contrast, late lactating cows have higher insulin (Vicini et al., 1991) and the mammary gland is no longer a priority. Thus, cows responded to feed restriction dropping mammary synthesis, preserving body reserves, and sparing energy for the fetus. In our study, insulin was higher in late lactation compared with the postpartum phase. As expected (Leduc et al., 2021), insulin dropped during the restriction. In similar experimental conditions, Odensten et al. (2005) observed a drop in plasma insulin during feed restriction, which was more marked when a lower quality forage (i.e. straw) was fed, compared with silage.

Milk composition reflected the change in yield, with a steady increase in fat and protein concentration and a decrease in lactose. These variations are consistent with a decrease in mammary epithelial cell function, which is related to nutrient restriction (Dessauge et al., 2011). The fat and protein content increase could be also partly due to a concentration effect related to the reduced milk fluid volume, a consequence of the reduced lactose, which is the major milk osmotic component (Kronfeld, 1982). Nevertheless, other factors were likely involved. Protein usually decreases in response to feed restriction (Leduc et al., 2021), due to lower dietary and microbial protein availability (which in our study was confirmed by the lower blood urea levels), but increases during mammary involution (Hurley, 1989), as a result of the increase in immunoglobulins, serum albumin, and lactoferrin, despite the decrease in milk-specific proteins (α-lactalbumin, β-lactoglobulin, and caseins). Thus, the rise in milk protein content might suggest a loss of mammary epithelium integrity, which may be interpreted as a marker of anticipated involution. Fat content could have been affected by both rumen fermentation and body fat mobilization. Feeding only hay increased the acetate production in the rumen, decreasing that of propionate. Acetate is a substrate for *de novo* milk FA synthesis (Urrutia and Harvatine, 2017). However, NR cows had a reduced proportion of *de novo* FA, suggesting that other mechanisms were involved in fat variations. At the same time, in fact, despite the lack of significance in BCS, a certain degree of body reserve mobilization happened, as confirmed by the higher levels of NEFA and BHB. This is the typical metabolic framework of feed restriction (Leduc et al., 2021), which is also accompanied by low circulating insulin. In the present study, insulin markedly decreased during restriction but did not reach the values observed postpartum. The increase in the markers of catabolism was relevant, but of a reduced intensity compared with the first days after calving, never reaching the threshold of subclinical ketosis (Duffield, 2000). Concurrently, a higher amount of mobilized fatty acids was likely available in the bloodstream for mammary gland uptake and incorporation into the milk fat. This was confirmed also by the milk FA analysis, pointing out a greater proportion of preformed and long-chain FA in NR cows. These FA classes increase when energy balance is negative and fat reserves are mobilized (Stoop et al., 2009; Gross et al., 2011). Another possible explanation might be related to a decline in the activity of acetyl-CoA carboxylase (Bauman et al., 1974; Hurley, 1989), which limits the synthesis of short- and medium-chain FA.

The decrease in lactose content was caused by a combination of concurrent factors. First, the reduced energy availability and the consequent low glycemia limited the glucose availability for the mammary gland. Moreover, lactose synthetase activity declines during involution (Bauman et al., 1974; Hurley, 1989). At the same time, the lower lactose content might suggest a decrease in prolactin, which regulates its secretion (Boutinaud et al., 2016). Finally, the higher protein content and the lower lactose might also point out a loss of integrity of the mammary epithelium (Zhao et al.,

2019). The mammary epithelium maintains the ionic gradient between milk and interstitial fluid. Tight junctions permeability increases with involution allowing the paracellular passage of blood components into milk, and vice versa (Stelwagen and Singh, 2014).

Daily rumination time drops similarly in the NR group in the first few days of restriction and in the control group immediately after dry-off. This drop was in agreement with previous observations at dry-off by Abuelo et al. (2021). Therefore, its main driver was the stress arising from diet and group change, rather than the milking cessation itself. The increase after dry-off in the NR group was likely a result of the milking cessation, from a time budget standpoint. Decreasing the time spent in the milking operations made available more time for lying (Chapinal et al., 2014), similarly to what happens, in the opposite way, at the onset of lactation (Huzzey et al., 2005; Cattaneo et al., 2020), and times spent lying and rumination are associated (Schirmann et al., 2012). Rumen pH increased as a result of hay feeding. Long forages require longer chewing time than concentrates and stimulate saliva secretion, which has a buffer effect on VFA production and rumen pH (Mertens, 1997). The lower total VFA production, rumen lactate, and urea (consistent with the plasma levels) highlighted also a lower intensity of rumen fermentation. Moreover, the lower nitrogen level both in the rumen fluid and in the blood was consistent the lower protein supply, given that they are closely interrelated (Marini and Van Amburgh, 2003). Besides, feed restriction at dry-off decreased protozoa number in the rumen, which are responsible for ammonia production (Odensten et al., 2005). Besides, creatinine greatly increased in NR cows around dry-off. Creatinine is a breakdown metabolite of creatine phosphate produced as a result of muscle creatine catabolism. Thus, its blood concentration can serve as a marker of muscle mass or protein catabolism. However, creatinine increase due to muscle catabolism is usually paired to an increase in plasma urea concentration (Keogh et al., 2015). Conversely, we had a decrease in plasma urea, that may be explained by a reduced flux from the rumen paired with a reduced utilization of amino acids as gluconeogenetic source. Higher creatinine can also indicate a decrease in renal filtration ability or increased liver synthesis, as previously reported in a feed restriction trial performed during peripartum period (Shahzad et al., 2014).

Although the restriction was short, it may have somewhat impacted welfare (Zobel et al., 2015). We did not directly measure these behavioral responses, but similar studies reported that comparable feed restriction protocols were associated with signs of hunger, such as increased vocalizations (Valizaheh et al., 2008; Franchi et al., 2019), and stress (i.e. increased cortisol; (Odensten et al., 2007).

6.5.2 Impact on ensuing transition and early lactation periods

To evaluate the success of dry-off protocols is essential to assess also the impact on the subsequent lactation. In the present study, cows that were nutrient restricted at dry-off had a slightly

lower milk production in the first 2 weeks of the new lactation. Milk yield is influenced by previous dry period mammary redevelopment (Capuco et al., 1997; Zhao et al., 2019), which might have been impacted by nutrient restriction. Different types of stressors can impair mammary involution. Heat stress, for instance, has a detrimental effect on autophagy in the first stages of dry period (Wohlgemuth et al., 2016), eventually reducing milk yield in the subsequent lactation (Ouellet et al., 2020). Cows feed restricted during early-lactation did not show any carryover effect at refeeding on milk production, despite a significantly greater daily mammary epithelial cell exfoliation rate during restriction (Herve et al., 2019), but data about mammary cell activity at the resumption of lactation after feed restriction at dry-off are lacking. Therefore, further studies on this aspect would be required. Another possible explanation might be related to the lower DMI observed in this period in cows that underwent feed restricted at dry-off. However, this difference in DMI remains unclear and requires further investigations, focusing particularly on inflammatory and liver conditions. Accordingly, NEFA were also higher in NR cows at the onset of lactation. Despite the lower DMI, given the concurrently lower milk yield, we would not have expected a rise in NEFA concentration. Considering that other markers of metabolism and circulating insulin were unaltered, rather than a higher degree of mobilization, it might be possible to hypothesize that cows which were nutrient restricted had also a lower ability to oxidize NEFA after calving, leading to the increase of their plasmatic level. The reasons behind this different liver function require to be investigated. Consistent with the lower yield and DMI, NR cows had also a slightly higher rumen pH 7 days after calving, pointing to a lower fermentation intensity, as supported by the numerically lower total VFA production.

6.6 Conclusions

Feed restriction was confirmed to be an effective solution to reduce milk production before dry-off in a short time. Low glucose and urea plasma levels, paired with increased NEFA and BHB, pointed out that feed restriction led, as expected, to lower energy availability and increased fat mobilization. Hay feeding also altered rumen fermentation pattern, with an increase in acetate proportion and a decrease in propionate and butyrate proportions. As a result of the latter and the increased permeability of the mammary epithelium (which is a hallmark of mammary involution), milk fat and protein content increased, and lactose declined. The analysis of milk fatty acid profile revealed that body reserve mobilization, and partly rumen fermentations, was the driver of the increased fat content, because of the lower proportion of short-chain fatty acids. Concurrently, rumination time dropped as a result of the drastic diet changes.

In the subsequent early lactation, milk yield and DMI were slightly lower in restricted cows, but fertility was not different. Further investigations are needed to precisely determine the long-lasting effects (if present) of feed or nutrient restriction at dry-off.

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Chapter 7: General Discussion

The dry-off has been proven to be a complex and challenging phase of the lactation cycle for dairy cows. Alongside milking cessation, it involves drastic changes in physiology, daily routine, diet, and social interactions. As a result, inflammatory response and behavioral alterations point out that it also represents a potentially stressful event. Many factors can exacerbate these responses and, among them, the spread of selective dry-cow therapy (i.e. drying-off healthy cows without antibiotic treatment) and the high milk yield before dry-off are probably the most impactful. The success of the dry-off can establish the outcomes of the ensuing transition period and lactation.

In the first introductory chapter, existent literature has been explored to provide an overview of the dry-off, especially focusing on high-producing cows, and find gaps in knowledge about these aspects.

In the second chapter, it has been investigated how previous inflammatory conditions can affect the adaptation to the transition period. Particularly, we used the albumin-to-globulin ratio as a proxy of the systemic inflammatory status. Cows with different albumin-to-globulin ratio before dry-off kept this difference up to early lactation, meaning that animals with a better condition at dry-off had also a reduced metabolic and inflammatory response at calving and improved productivity and fertility. Therefore, this work highlighted how the dry-off can influence future performance and health.

In the third chapter, we assessed the inflammometabolic profile in healthy cows that were selectively treated at dry-off. To sum up, in cows with low SCC at dry-off antibiotic therapy could be avoided, with only minor transient differences in plasma biomarkers. If udder health is carefully monitored, selective dry-cow therapy can represent a viable approach to decrease antibiotic use in the dairy sector. Further research is needed to evaluate the impact of selective dry-cow therapy on cows with microbial contamination in the udder, despite having low SCC, which might not be detected in commercial settings and can adversely affect the success of the dry period.

Considering that, despite being mild, we noted some alterations in plasma biomarkers in cows dried off without antibiotic therapy, in the fourth chapter, we investigated whether the supplementation of a nutraceutical (*Aloe arborescens* Mill.) could improve the transition from lactation to the dry period in cows that did not with antibiotic treatment at dry-off. Results showed that *Aloe* administration improved liver function but increased the production of ROM. Moreover, cows that received *Aloe* at dry-off had a milder inflammatory response after calving. Consistently, milk yield in the subsequent lactation was higher, whereas milk composition, SCC, and mastitis

incidence were not affected by *Aloe* treatment. Thus, the importance of improving cows' condition even before dry-off to obtain healthier cows during the transition period was confirmed, particularly in cows not treated with antibiotics at milking cessation.

In the fifth chapter, the leukocyte gene expression was evaluated to explore at the molecular level what happens at dry-off. Previous findings were confirmed, highlighting that dry-off alters blood biomarkers of nutrient metabolism, inflammation, and oxidative stress. Particularly, peripheral leukocyte's antimicrobial and antioxidant capacities were impaired. In particular, we compared the responses of high- and low-producing cows. Previous studies showed that the response to dry-off was intensified in those animals. Indeed, in high-producing cows (> 15 kg/d before dry-off) the inflammatory response after dry-off was also exacerbated, as supported by the upregulated *IL-8* and *IL-18*, supporting the previous findings. Therefore, the importance of the transition from lactation to the dry period, in particular in high-yielding cows, was again confirmed.

Finally, in the sixth chapter, we evaluated a nutrient restriction strategy at dry-off. Since it has been shown that high milk yield at dry-off might have detrimental effects, feed restriction is one of the available strategies to reduce production levels before miking cessation. In this study, we confirmed the effectiveness of the dietary treatment in reducing yield. Moreover, mammary involution appeared to be anticipated. At the same time, a relevant metabolic response has been observed in those animals, and feed intake and milk yield in the ensuing early lactation were slightly reduced. Further research is needed to better understand the mechanism underlying these variations, which might include, among others, inflammation, liver, or rumen function.

To conclude, the dry-off confirmed to be a pivotal stage of the cow's life, with relevant implications in the subsequent lactation. Stressors in late gestation, in fact, affect metabolism, inflammation, and immune system after calving. Improving cow condition at dry-off and mitigating inflammatory phenomena at this time might ameliorate the adaptation to the transition period, boost productivity, and enhance animal health and welfare in these critical phases.

Future research would be required to investigate the impact of systemic inflammation or nutrient restriction at dry-off on subsequent inflammatory conditions and immune system functionality, up to early lactation. Considering that the nutritional interventions assessed in this dissertation might affect particularly liver, rumen epithelium, and mammary gland, studies addressing the changes in transcriptome or methylome in these tissues during and after the restriction could help understand further the mechanisms implied in the observed responses.

Overview of Scientific Publications

Cattaneo, L., V. Lopreiato, E. Trevisi, and A. Minuti. 2020. Association of postpartum uterine diseases with lying time and metabolic profiles of multiparous Holstein dairy cows in the transition period. Vet. J. 263:105533. doi:10.1016/j.tvjl.2020.105533.

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