Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Veterinarie

Ciclo Ventinovesimo

Settore Concorsuale: 07/G1

Settore Scientifico Disciplinare: AGR18

Strategies to improve transition dairy cows health and milk quality

Presentata da: Dott.ssa Ludovica Maria Eugenia Mammi

Coordinatore Dottorato

Prof. Arcangelo Gentile

Supervisore

Prof. Andrea Formigoni

Esame finale anno 2018

Table of Contents

1. Introduction

1.1. The transition period

One of the most critical moment in the dairy cow lactation cycle is the so-called *"transition period",* that consist in the passage through the event of calving from a pregnant non-lactating status (dry period) to a non-pregnant lactating one (lactation period). As defined by Grummer, the transition period is identified with the 3 weeks before calving and the 3 weeks after, and it is characterised by important endocrine changes that lead to parturition and to the onset of lactation (Grummer, 1995).

In this period, several simultaneous factors contribute to worse this situation: endocrine and metabolic variations related to the end of pregnancy, mainly represented by immune suppression and increase of nutritional demands, coincide with changes of diet, environment and management. This situation was defined by Ingvartsen (2006) as "physiological imbalance" (PI), where homeoretic mechanisms of the animals are not able to adapt to the new demands (Ingvartsen, 2006). This expression effectively outlines a situation where a physiological event, like calving, is actually critical for the animal because of the suppression of the innate immune system and the nutritional deficit.

Immune suppression in the peripartum period has a multifactorial aetiology.

In order to start the process of delivery at the end of pregnancy, progesterone decreases suddenly from 7-8 ng/ml to almost 0 ng/ml while at the same time estrogens and cortisol increase (estrogens from 300 to 6000 pg/ml and plasma cortisol from 4-8 ng/ml to 30 ng/ml) (Goff & Horst, 1997; Chew *et al*, 1977). These rapid modifications in hormone concentrations, especially the dramatic increase of estrogens and cortisol, are considered to be responsible for the

suppression of the innate immune system in the prepartum period, in particular for the reduction of neutrophil and lymphocytes functions (Clemens *et al*, 1979; Wyle & Kent, 1977; Goff & Horst, 1997). Indeed, adhesion, phagocytic and migration capability of neutrophil and mitogen activity of leucocytes are impaired around parturition (Kehrli Jr. *et al*, 1989a, 1989b; Mallard *et al*, 1997).

Endocrine variations anyway, are not the only responsible for immune suppression. In addition to that, the immune system is considerably affected by the negative energy and protein balance, oxidative stress and hypocalcemia that characterise the first weeks after calving.

The innate immune system is activated by the inflammatory condition typical of the peripartum, and therefore its nutritional demands are increased. At the same moment, foetal metabolism and later on the onset of lactation require high nutrients supply that cannot be satisfied because of the coincident depression of feed intake. As widely reported in literature, in fact, dry matter intake starts to decline during the last trimester of pregnancy, reaching its nadir during the day of calving and taking from 8 to 22 weeks to reach the maximum (Ingvartsen & Andersen, 2000).

On the other hand, milk production start to increase rapidly after calving and nutrients are prioritized towards the mammary gland creating a considerable gap between nutrients demands and their availability (Goff & Horst, 1997; Sordillo *et al*, 2009; Ingvartsen, 2006; Leroy *et al*, 2008). The first direct consequence of the Negative Energy Balance (NEB) after calving is ketosis.

Ketosis is a metabolic disease characterised by high blood, milk and urine, concentration of ketone bodies (acetoacetate, b-hydroxybutyrate (BHBA) and acetone).

When glucose demands exceed gluconeogenesis supply lipids are mobilised from body reserve and fatty acids absorbed from mitochondria of liver cells, in order to let their acid carbons being used by extra-hepatic tissues as energy source, in place of glucose (Ingvartsen, 2006).

If glucogenic substrates are low, as in the case of severe NEB, ketogenesis increase: NEFA are only partially oxidized to ATP thus resulting in the production of ketones. When BHBA exceed physiological levels in blood (<1.2 mmol/l) we talk about subclinical (1.2<>2.5 mmol/l) or clinical (>2.5 mmol/l) ketosis, whether clinical signs are present or not (Duffield, 2000). Being high levels of blood ketones toxic for the organisms, the main clinical symptoms of ketosis are reduction of the appetite, reduced milk production and immune system depression (Duffield *et al*, 2009).

Clinical or subclinical ketosis, usually occurs in the first weeks after partum, and it is associated with increased risk of displaced abomasum, metritis and major duration and severity of mastitis, besides reduced reproductive performance (LeBlanc, 2010).

Together with ketosis, NEB is also characterised by an increase of blood NEFA (non-esterified fatty acids) concentration that further decrease immune efficiency. This is particularly evident in overconditioned animals, as a consequence of excessive mobilizations of lipids body reserves (Lacetera *et al*, 2005). When present in excess, NEFAs exert a negative effect on the innate immune system, resulting in an overwhelming inflammatory response, both in human and periparturient cows (Wood *et al*, 2009; Bernabucci *et al*, 2005; Sordillo *et al*, 2009). This condition contributes to the development and protracting of pro-inflammatory diseases like metritis and mastitis (Goff, 2006).

A prolonged inflammatory status is favoured by the imbalance between antioxidant mechanisms and the production of reactive oxidant species (ROS), commonly known as Oxidative Stress (OS). OS is an hallmark of transition period and it is responsible for cellular and tissue injuries as well as for the reduction of functional capabilities of leukocytes (Sordillo & Aitken, 2009).

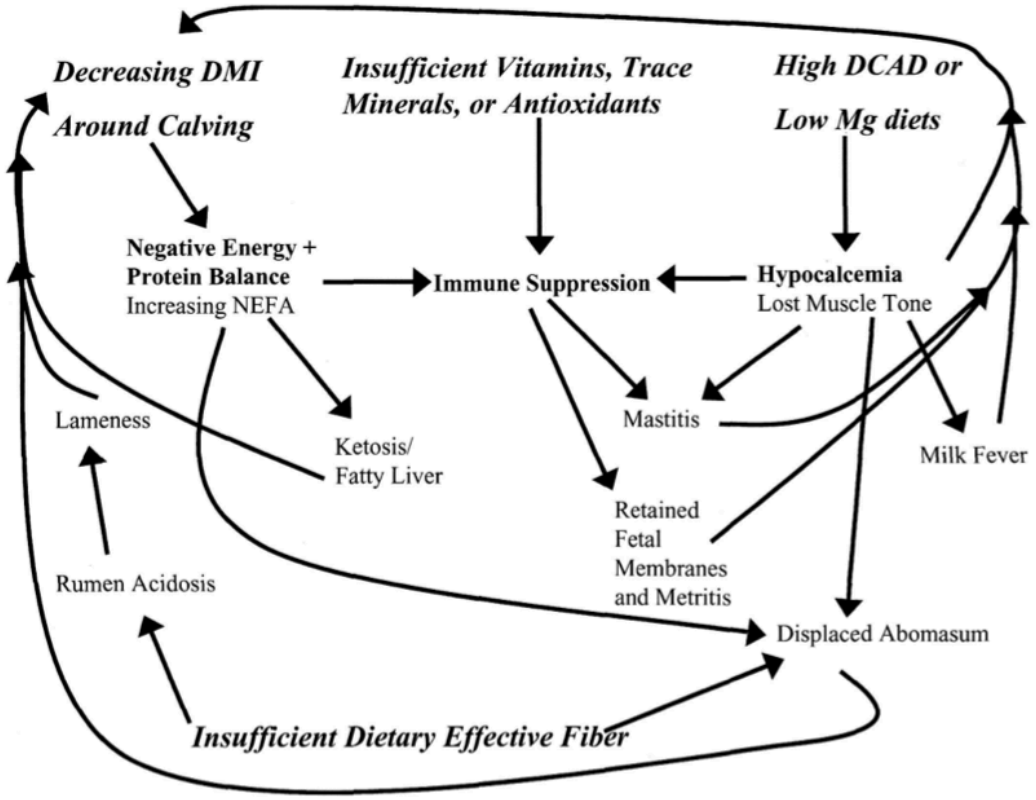
In the peripartum, the activation of maternal immune response plays an important role in the breakdown of foetal-maternal junction of the placenta. Therefore impaired leucocytes chemotaxis and phagocytic activity, together with antioxidants imbalance, substantially contribute to the retention of foetal membranes (RFM) (Beagley *et al*, 2010; Kimura *et al*, 2002). Indeed, investigators suggest that the mechanisms that lead to RFM start with an antioxidant imbalance at placenta level and found that vitamin E supplementation during dry period reduce oxidative stress and increase neutrophils activity thus decreasing the incidence of RFM (Bourne *et al*, 2007). Blood calcium level has an important role too, in the pathogenesis of placenta retention. Indeed, subclinical hypocalcemia resulting from the unbalanced uptake of calcium from blood to the mammary gland, contribute to reduce immune efficiency, prevents skeletal and smooth muscles contractions and inhibits nerve functions. The reduced effectiveness of muscles contractions can lead to dystocia and prolonged labour thus facilitating the retention of foetal membranes (Beagley *et al*, 2010).

Actually, hypocalcemia is very common in high producing dairy cows after calving and it is responsible besides milk fever and RFM, of several problems including displaced abomasum, mastitis and metritis.

Moreover, the lacking of calcium availability for immune cells reduce the general capability of the immune system to cope with infections (Goff, 2006).

All these aspects, summarized in Figure 1, convert the transition period in a very critical moment for dairy cows, that can compromise the entire lactation and career of the animals, leading in the worst cases to the culling of the animal before the 60 Days In Milk (DIM). More than 15% of culling, indeed, occurs during transition period and up to the 50% of cows experiences a disease during the first weeks of lactation (Ingvartsen, 2006; LeBlanc *et al*, 2006; Dechow & Goodling, 2008).

Figure 1 *Interrelationship between nutrition and disease in the periparturient dairy cow (Goff, 2006).*



1.2. Consequences of diseases on milk production and quality

All the aspects presented above have important effects not only on bovine health, but also on milk production and its quality.

Milk production losses as direct consequence of diseases has been widely reported in literature. While some diseases, like mastitis, results directly in a decrease of milk production because of the involvement of the mammary gland, some others reduce milk production as a consequence of altered metabolism and inflammatory status.

As a consequence of negative energy balance, ketosis reduces milk yield by 1.9 kg/d and 3.3 kg/d when BHBA higher than 1400 μmol/l and higher than 2000 μmol/l in the 1st and 2nd week after calving, respectively. In addition, animals with BHB >1800 μmol/l in week 1 after calving were reported to have reduced projected production for the entire lactation (Duffield *et al*, 2009). Apparently, effects of ketosis on milk production are not consistent if it is considered the whole lactation. While during ketosis milk production decreases, 305-d milk production was shown to be higher in cows that had hyperketonemia in the first weeks after calving. This apparent contradiction is explained by the fact that high producing cows have more risk to develop ketosis, because they experience a more severe negative energy balance and consequently higher (Duffield *et al*, 2009). However, when estimated milk production was considered, ketotic cows had a depression in their lactation curves and even if their total 305-d production was higher than healthy cows, it was calculated that without ketosis in the whole lactation they would have produced up to 200 kg more (Detilleux *et al*, 1994). In his study Detilleux calculated in 44.2 kg the milk lost in 100 days of lactation for 17 days of hyperketonemia. Antanaitis et al. (2015) estimated a milk loss of about 10 kg for every day of ketosis. Comparing two group of cows, healthy and ketotic,

they found a production loss of 1.1% for sick cows between day 9 and day 12 before ketosis was diagnosed and up to 22% of milk less than healthy cows in the day of diagnosis (Antanaitis *et al*, 2015).

Besides milk production, hyperketonemia affects also milk composition and quality.

Milk fat percentage increase by 0.22 to 0.48% in ketotic cows and milk protein decrease by 0.09 to 0.22% (Duffield *et al*, 2009; Vanholder *et al*, 2015). The increase of the fat to protein ratio of milk is indeed one of the peculiar sign of ketosis, used by the automatic milking systems to detect ketotic cows.

As reported by Koeck, in healthy cows, fat to protein ratio is around 1.2, while it increases to 1.4 and 1.6 in cows with subclinical and clinical ketosis (Koeck *et al*, 2014). Antanaitis, moreover, reported that hyperketonemia increases milk conductivity by 3.1-3.4% since 2 days before symptoms (Antanaitis *et al*, 2015)

Immune suppression, on the other hand, increases the risk and the duration of mastitis that together with ketosis, negatively affect milk production and its composition. Mastitis are well known to reduce milk yield and quality. Numerous studies are present in literature that clarify their impact on the quantity and quality of milk and dairy products although estimation of milk losses due to clinical mastitis vary among different studies (Seegers *et al*, 2003; Halasa *et al*, 2007; Sharif & Muhammad, 2008).

Seegers et al. (2003) proposed a value of 375 kg of milk lost for a single case of mastitis while Schukken et al. (2009a) calculated milk loss considering different etiology of mastitis. These authors estimated a loss of 304 kg and 228 kg, in multiparous and primiparous cows respectively during the 50 days following a gram-negative mastitis, while for gram-positive cases milk loss was estimated to be around 128 kg in multiparous animals and 92 in primiparous (Schukken *et al*,

2009). Despite the source of infection, it was calculated by Rueg that for every increase of unit in somatic cell score, milk loss is around 91 and 181 kg per lactation, for primiparous and multiparous cows, respectively (Ruegg, 2017).

Besides milk production, mastitis reduce milk quality by increasing Somatic Cell Count (SCC) and worsening its composition. Milk produced by cows with high somatic cells count is characterized by reduced content of lactose and increased pH (Bobbo *et al*, 2017).

Despite total proteins are apparently not influenced by SCC, true casein and casein number ({casein/protein}*100) is lower than in healthy milk (Bobbo *et al*, 2017; Seegers *et al*, 2003). Indeed, protein content considers also non-proteic nitrogen and therefore include peptones resulted by the increased proteolityc activity of infected milk and inflammatory proteins derived from blood as a consequence of augmented permeability of the blood-milk barrier (Bobbo *et al*, 2017; Seegers *et al*, 2003).

Actually, somatic cells increase casein degradation with a consequent reduction of α- and β- caseins that are fundamental for the cheese making process (Summer *et al*, 2015).

All these changes contribute to worse coagulation properties of milk. High SCC and higher pH are responsible of reduced clotting ability and curd firmness due to the reduction of the enzymatic activity involved in the coagulation process (Swaisgood, 1982). In particular, Bobbo et al. (2017) found that contagious pathogens compared to others, are more effective in worsening milk coagulation time and curd firmness.

The lower casein number of milk high in somatic cells, in addition, is related to the decreased recovery of fat and protein in the curd, that are lost in the whey during the cheese making process.

All these aspects together contribute to relate high SCC to a lower cheese yield (Bobbo *et al*, 2016; Summer *et al*, 2015)

For these reasons, the consequences of high SCC and mastitis are even more serious for those farmers involved in dairy transformation, and in particular for those that produce milk for PDO cheese production, like Parmigiano Reggiano. In this production process, infact, feedstuff, management and milk processing are strictly regulated by European Union and producers are allowed to use only unpasteurised, raw milk without the addition of any additive (Consorzio del Formaggio Parmigiano Reggiano, 2011; European Union, 2006; Mordenti *et al*, 2017). For this reason, high quality milk produced by healthy cows is fundamental and the economic impact of disease and poor quality milk on this kind of productions is considerable.

1.3. Strategies to improve cows health

1.3.1. Management and environment

In the last decades, efforts to understand and improve health of dairy cows during transition period have been remarkable.

Starting from a correct nutritional management of early lactating cows, passing through the attention for cow comfort for animals in lactation and during dry period and calving, strategies that aim to reduce incidence of disease during transition are several and different.

As shown in the first paragraph of this work, most of diseases occurring in the early lactation are related to low energy availability to sustain milk production as well as the immune system. Excessive lipid mobilization from body reserve leads to metabolic disorders, like fatty liver and ketosis, in addition to exacerbate immune depression.

Overcondition at calving is one of the main risk factor for the development of the so called “fat cow syndrome”, existing a positive relation between fatness at calving and post partum lipid mobilization (Ingvartsen, 2006; Morrow, 1976). A massive mobilization of body reserve increases feed intake depression, contributing to worse the negative energy balance. Considering that during dry period is not possible to act on body weight to avoid the risk of prepartum mobilization, body condition of cows needs to be controlled during the entire lactation (Ingvartsen, 2006).

Depression of feed intake is almost physiological around calving, but its extent is worsen by several factors like inflammatory status, blood NEFA concentration, nutritional strategy and environment (Ingvartsen, 2006).

High digestibility of diet, passage rate and proper physical form should be assured in fresh cows in order to maximize dry matter intake and assure a good rumen environment (Fustini *et al*, 2017).

Stressful situation like overcrowding and social conflicts among animals contribute to increase fat mobilization and cortisol, resulting in a more severe immune suppression and higher risk of metabolic disorders. During transition, cows experience several changes in a very short period and changes of group expose animals to face with a new environment and new mates. These situations can induce a stress response, especially in low ranking cows, like primiparous and sick animals. Separating fresh primiparous from multiparous cows, for example, can improve dry matter intake of those animals up to 14%, because of a reduction of social stress (Konggard & Krohn, 1978; Grant & Albright, 2001).

For the same reason, it is extremely important to allow enough feed bunk space to permit the contemporary access of all cows to the manger avoiding aggressive behaviour and displacements. Different studies indicate in 0.75 m the optimal space per cow at the feed bunk, and found that when space/cow is between 0.2-0.5 m aggressive and stressful behaviour increase, while when space is below or equal 0.2 m/cow even feed intake is depressed (Friend & Polan, 1974; Friend *et al*, 1977; Manson & Appleby, 1990) .

Another important aspect that needs to be considered in order to reduce stress in cows is the climatic environment. Heat stress is known to negatively impact cows' health. Temperature of 25-26°C have been considered for years the cut off above which dairy cows start suffering. However, an indicator of negative environmental conditions was introduced in the late 50s in humans: the THI (Temperature and Humidity Index) (Thom, 1959). This index considers the combination between temperature and humidity of the air, and nowadays can be recorded in farms with

proper instruments directly inside the pens, at 150-200 cm from soil. This is extremely important because animals produce heat and humidity and therefore the measure of THI outside the pens is inaccurate.

In literature, the threshold value of THI for heat stress ranges from 64 to 76. This value comes out from the combination of temperature between 21-36°C and a humidity between 5 to 95%. Even if most of the authors consider 68 as threshold value for heat stress in dairy cows (Polsky & Keyserlingk, 2017), even with THI around 65, cows start to reduce their resting time, decrease feed intake and rumination activity and increase water consumption (Bernabucci *et al*, 2014; Chen *et al*, 2016; Herbut & Angrecka, 2018). At THI value of 73, conception rate is significantly reduced (Schuller *et al*, 2014). If THI persist over these thresholds for the majority of the day (>17h) a decrease in milk production and protein content was observed. This was particularly evident in multiparous and high yielding cows. Lactating cows indeed produce more heat than heifers and dry cows, and therefore high producing animals are more susceptible to heat stress (Bernabucci *et al*, 2014; Ingvartsen, 2006). Reduced lying time and DMI for long periods have important consequences on animals' health that can persist for several days and months later, like the appearance of lameness and metabolic disease, more than the reduced milk production. Therefore the application of cooling systems, like provision of shade, fences, sprinkler and showers are strongly recommended (Polsky & Keyserlingk, 2017).

1

1.3.2. Nutrition

Together with good management conditions, proper nutritional strategies are essential during the transition period.

Adequate energy supply is essential for the functionality of the immune system. As previously described, immune cells actions involved in the inflammatory response (i.e. phagocytosis, respiratory burst, secretion of cytokines) require high energy utilisations, therefore energy requirements during inflammation increase considerably. For these functions immune cells can use different source of energy, like glucose, fatty acids or glutamine (Calder, 2013; Newsholme *et al*, 1986). Glucose uptake by cells is regulated by the expression of surface transporters GLUT (Calder *et al*, 2007). Their expression is partially reduced in resting immune cells in the early lactation period, thus contributing to the partitioning of glucose towards the mammary gland and explaining the depression of leucocytes activity in this period (O'Boyle *et al*, 2012; Sordillo, 2016).

Fatty acids play an important role, too, in the modulation of inflammatory response, by altering composition of membrane phospholipids and regulating intracellular signalling (Sordillo, 2016). Thus, several authors suggest the possibility to modulate immune and inflammatory response by supplementing cow diet with long chain polyunsaturated fatty acids (Sordillo, 2016).

Adequate micronutrients supply it is fundamental to assure both production efficiency and immune competence.

Micronutrients are known to be essential for the immune system, but their availability is reduced around calving, due to the depression of feed intake and their increased utilisation during metabolic stress (Sordillo & Mavangira, 2014).

As described in table 1, their role is pivotal because of their antioxidant capabilities and their involvement in several enzymatic complexes that act in the

inflammatory response. Some of the most effective micronutrients radical scavengers are tocopherols, gluthatione and ascorbic acid, while selenium, copper, zinc, manganese and iron are essential for the functioning of all the anti-inflammatory enzymes systems (Sordillo & Aitken, 2009).

Deficiencies of vitamin E and selenium, indeed, are responsible of increased incidence of mastitis, retained foetal membranes as well as oxidative damages of tissues. Supplementation of these nutrients during dry period is indeed recommended by several authors (Bourne *et al*, 2007; Politis, 2012; Sordillo, 2016).

Table 1. *Different mode of actions of vitamins and minerals described by Sordillo, 2016.*

Nutrient	Active component	Function
Vitamin A	β-Carotene	Prevents fatty acid peroxidation chain reaction
Vitamin C	Ascorbic acid	Radical scavenger
Vitamin E	α-Tocopherol	Disrupts fatty acid peroxidation chain reaction
Selenium	Thioredoxin reductase	Redox signaling and reduces reactive oxygen species (ROS)
Selenium	Glutathione peroxidase	Redox signaling and reduces ROS
Copper	Ceruloplasmin	Oxidase activity; peroxyl radical scavenger
Copper-zinc	Superoxide dismutase	Converts cytosol superoxide to H_2O_2
Zinc	Metallothionein	Cysteine rich radical scavenger
Manganese	Superoxide dismutase	Converts mitochondrial superoxide to H_2O_2
Iron	Catalase	Converts H_2O_2 to water

1.3.3. Immunomodulants

Since antimicrobial resistance is one of the emerging issues for human health, with about 700.000 deaths every year in the world, according to European Medicine Agency evaluation, restrictions to the use of antibiotics in animal productions were issued by different authorities all over the world, as well as by the European Community (European Commission, 2011). AMR can occur naturally through adaption to the environment, but the pace of AMR spread is now on the uptick due to inappropriate and excessive use of antimicrobials.

Therefore, new strategies to have healthier and productive animals without using antimicrobial treatments have been promoted. In this context, a variety of substances able to modulate and stimulate the immune system by different pathways were introduced in farm animals, including dairy cows. A molecule with a specific immune stimulant action (peg-bovigrastim) has been recently introduced in the dairy industry with the aim to modulate the animal innate immune system. This drug acts like an endogenous colony stimulating factors (CSF) promoting the production, differentiation and function of mononuclear leucocytes. Administering by injection to dry cows 10 days before calving reduces the risk of new mammary infections in the first 8-10 weeks of lactation (Ruiz *et al*, 2017).

Some other components naturally present in feedstuffs or added to the ration are supposed to be effective in improving immune system.

European Union regulates the authorisation process for animal feed additives that need to comply with the specific criteria defined in the Regulation (EC) No 1831/2003 (Regulation, 2003).

Among these, additives defined as immunomodulants, are those substances able to benefit animal welfare and include probiotics, prebiotics, plant extracts, animal by

products, like lactoferin and antibodies, and micronutrients, like vitamins and minerals (EFSA, 2015). In recent years, the number of products that aim to improve immune system of farm and companions animals is increased considerably.

From a recent review edited by EFSA, probiotics resulted to be the most effective in the bovine species. Probiotics are defined as living microorganisms able to enhance host microbiota properties (Fuller, 1992), commonly known to balance intestinal microflora, they are now recognized as immune modulators (EFSA, 2015). Their main action is to exclude pathogens present in the gut from competition for nutrients by colonizing and strengthening intestinal epithelial barrier. In addition, probiotics interact with pattern recognition receptors, like Toll-like receptors (TLR) present in the intestinal tract, that are responsible of the activation of signals that modulates different gene expression profile (Kang & Hyeog, 2015).

Probiotics most used in the bovine species, are *Lactobacillus spp*. and *Entreoccous spp*., which were shown to reduce inflammatory microbes in the gut of supplemented animals and increase bacteriocines and hydrogen peroxidase. In calves, *Enteroccoccus spp.* increase the local intestinal mucosal immunity and improve the resistance of the animals to stressful events (Bayatkouhsar *et al*, 2013; Roodposhti & Dabiri, 2012), while Saccharomyces supplementation showed an improvement in neutrophil chemiotaxis and respiratory burst activity against E. Coli. (Magalhães *et al*, 2008).

Prebiotics, instead, are defined as substances able to promote the growth and/or the activity of a particular microorganism and thus improving host's welfare (Gibson & Roberfroid, 1995; Roberfroid, 2007). The most frequently used in ruminant productions are mannanoligosaccharides (MOS), glucan and yeast cell

wall (YCW) (EFSA, 2015).

Plant extracts include several numbers of vegetable products, like essential oils, herbs and spices, like Curcumin and Soybean derivate, whose effects have been tested on rearing calves and lactating cows showing improvements of innate immune system but whose mode of action is still unclear (Oh *et al*, 2013; Kwon *et al*, 2011). Among these, polyphenols have been deeply investigated on monogastric animals and humans, showing interesting effects on the oxidative status and on the immune and inflammatory responses (Liu *et al*, 2018). The action of these substances in ruminants, instead, is still unclear with only few researches that report contrasting results about their inhibitory effects on some bacteria in vitro and their potential influence on rumen microbiome (McSweeney *et al*, 2001; Ramos-Morales *et al*, 2018).

Overall, according to EFSA report, literature about the effects of natural feed additives in ruminants is lacking and the mode of action of the majority of these substances is still not well understood (EFSA, 2015).

Despite that, the addition of immuomodulant supplements to dry and fresh cows diets is one of the several strategies used nowadays to ameliorate transition dairy cows' health.

Among these, OmniGen-AF® received particular attention from researchers and industries for its ability to enhance the innate immune system. OmniGen-AF® is a complementary feed composed mainly by dried inactivated brewer's yeast cells wall, wheat fiber, bentonite and purified diatomaceous earth. Diet supplementation with this additive tended to improve neutrophil functions of heifers by increasing mRNA expression of neutrophil adhesion molecule L-selectine, thus enhancing migration and adhesion ability of neutrophils. Supplemented heifers experienced a reduction of inflammation at calving, with

lower production of reactive oxygen species and fewer cases of new intra-mammary infections (Nace *et al*, 2014). Another study reported a greater leucocytes infiltration in endometrium of fresh cows after supplementation, combined with higher milk production and similar blood haptoglobine level compared to control cows. According to the authors, these results suggest an increase capability of neutrophils of supplemented animals to migrate from blood to the site of infection and overall an improved immune competence of these cows (Brandão *et al*, 2016).

These effects are probably due to the interactions between PAMP (pathogen associated molecular pattern) contained in yeasts, and toll-like receptors of intestinal epithelium. This interaction exerts a positive stimulus for the expression of adhesion molecules of neutrophils, like L-selectine, and for the secretion of citokines, thus enhancing neutrophils activity (Wang *et al*, 2007; Iwasaki & Medzhitov, 2004).

1.3.4. Monensin

Monensin is a carboxylic polyeter ionophore commonly used as feed additive in ruminants to alter rumen fermentation in order to improve energy efficiency (Russell & Strobel, 1989).

This molecule was discovered in 1968 and later on used as anticoccidial treatment in avian species. Since 1975, monensin was introduced as feed additive in bovine nutrition, as growth promoter (Rumensin®, Elanco Animal Health, USA), thanks to its positive effects on ruminants energy metabolisms, well described both in beef and dairy cattle (Goodrich *et al*, 1984; Ipharraguerre & Clark, 2003; Duffield *et al*, 2012).

However, in 2006 the European Community prohibited the use of antimicrobials as growth promoters, including monensin, allowing its use only on the avian species as anticoccidial treatment. In the end, in 2013 the European Medicines Agency (EMA) approved the use of a monensin controlled release capsule (Kexxtone, Elanco Animal Health, UK) as treatment for the prevention of ketosis in dairy cows.

The ability of monensin to improve efficiency of energy utilization in ruminants is due to its effects on rumen microflora. Indeed, this molecule alters ions exchange through the inner and outer membranes of microbial cells. In this way, it reduces the prevalence of protozoa and gram positive population and promotes gram negative proliferation, whose are mainly responsible for propionate production (Russell & Strobel, 1989). Increased propionate production and the consequent shift of acetate and propionate ratio in favour of propionate is the main effect of monensin supplementation. Considering that glucose produced by propionate represents the main part (21-46%) of energy production in cows, the increase

production of propionate in the rumen, actually improve energy metabolism of cows (Russell & Strobel, 1989; McGuffey *et al*, 2001).

Moreover, a recent study investigated the kinetic of gluocose metabolism in transition cows after monensin supplementation and reported an improved utilization of propionate in glucose production through the gluconeogenesis process and a reduction in the glucose contribution from other sources in supplemented animals (Markantonatos & Varga, 2017).

According to the authors, these results suggest that monensin effects are not limited to the increased rumen propionate production, but include also an improved efficiency of its conversion to glucose (Markantonatos & Varga, 2017; McCarthy *et al*, 2015b).

These results agree with other studies that reported reduced NEFA and BHBA plasma concentration and decreased incidence of ketosis, displaced abomasum and mastitis, suggesting an improved energy balance in cows supplemented with monensin (Duffield *et al*, 1998; McCarthy *et al*, 2015b; Duffield *et al*, 2008b).

As a consequence of a better energy balance, other studies reported higher dry matter intake and milk production in the first weeks of lactation for monensin supplemented cows compared to cows fed a control diet (McCarthy *et al*, 2015a).

2. References

- Antanaitis, R., V. Žilaitis, A. Kučinskas, J. Juozaitienė, and K. Leonauskaitė. 2015. Changes in cow activity, milk yield, and milk conductivity before clinical diagnosis of ketosis and acidosis. Vet. Ir Zootech. 70.
- Bayatkouhsar, J., A.M. Tahmasebi, A.A. Naserian, R.R. Mokarram, and R. Valizadeh. 2013. Effects of supplementation of lactic acid bacteria on growth performance, blood metabolites and fecal coliform and lactobacilli of young dairy calves. Anim. Feed Sci. Technol. 186:1–11. doi:10.1016/j.anifeedsci.2013.04.015.
- Beagley, J.C., K.J. Whitman, K.E. Baptiste, and J. Scherzer. 2010. Physiology and Treatment of Retained Fetal Membranes in Cattle. J. Vet. Intern. Med. 24:261–268. doi:10.1111/j.1939-1676.2010.0473.x.
- Bernabucci, U., S. Biffani, L. Buggiotti, A. Vitali, N. Lacetera, and A. Nardone. 2014. The effects of heat stress in Italian Holstein dairy cattle. J. Dairy Sci. 97:471–486. doi:10.3168/jds.2013-6611.
- Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J. Dairy Sci. 88:2017–2026.
- Bobbo, T., C. Cipolat Gotet, G. Bittante, and A. Cecchinato. 2016. The nonlinear effect of somatic cell count on milk composition, coagulation properties, curd firmness modeling, cheese yield, and curd nutrient recovery. J. Dairy Sci. 99:5104–5119.
- Bobbo, T., P.L. Ruegg, G. Stocco, E. Fiore, M. Gianesella, M. Morgante, D. Pasotto, G. Bittante, and A. Cecchinato. 2017. Associations between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein

composition, and cheese-making traits in dairy cows. J. Dairy Sci. 100:4868–4883. doi:10.3168/jds.2016-12353.

- Bourne, N., R. Laven, D.C. Wathes, T. Martinez, and M. McGowan. 2007. A meta-analysis of the effects of Vitamin E supplementation on the incidence of retained foetal membranes in dairy cows. Theriogenology 67:494–501. doi:10.1016/j.theriogenology.2006.08.015.
- Brandão, A.P., R.F. Cooke, F.N. Corrá, M.B. Piccolo, R. Gennari, T. Leiva, and J.L.M. Vasconcelos. 2016. Physiologic, health, and production responses of dairy cows supplemented with an immunomodulatory feed ingredient during the transition period. J. Dairy Sci. doi:10.3168/jds.2015-10621.
- Calder, P.C. 2013. Feeding the immune system. Proc. Nutr. Soc. 72:299–309. doi:10.1017/S0029665113001286.
- Calder, P.C., G. Dimitriadis, and P. Newsholme. 2007. Glucose metabolism in lymphoid and inflammatory cells and tissues. Curr. Opin. Clin. Nutr. Metab. Care 10:531–540. doi:10.1097/MCO.0b013e3281e72ad4.
- Chen, J.M., K.E. Schütz, and C.B. Tucker. 2016. Cooling cows efficiently with water spray: Behavioral, physiological, and production responses to sprinklers at the feed bunk. J. Dairy Sci. 99:4607–4618. doi:10.3168/jds.2015-10714.
- Chew, B.P., H.F. Keller, R.E. Erb, and P.V. Malven. 1977. Periparturient Concentrations of Prolactin, Progesterone and the Estrogens in Blood Plasma of Cows Retaining and not Retaining Fetal Membranes1 ,2. J. Anim. Sci. 44:1055–1060. doi:10.2527/jas1977.4461055x.
- Clemens, L.E., P.K. Siiteri, and D.P. Stites. 1979. Mechanism of immunosuppression of progesterone on maternal lymphocyte activation during pregnancy. J. Immunol. 122:1978.

- Consorzio del Formaggio Parmigiano Reggiano. 2011. Specification of the Parmigiano Reggiano cheese.
- Dechow, C.D., and R.C. Goodling. 2008. Mortality, Culling by Sixty Days in Milk, and Production Profiles in High- and Low-Survival Pennsylvania Herds. J. Dairy Sci. 91:4630–4639. doi:10.3168/jds.2008-1337.
- Detilleux, J.C., Y.T. Gröhn, and R.L. Quaas. 1994. Effects of Clinical Ketosis on Test Day Milk Yields in Finnish Ayrshire Cattle. J. Dairy Sci. 77:3316–3323. doi:10.3168/jds.S0022-0302(94)77272-6.
- Duffield, T. 2000. Subclinical Ketosis in Lactating Dairy Cattle. Vet. Clin. North Am. Food Anim. Pract. 16:231–253.
- Duffield, T.F., K.D. Lissemore, B.W. McBride, and K.E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. J. Dairy Sci. 92:571–580. doi:10.3168/jds.2008-1507.
- Duffield, T.F., A.R. Rabiee, and I.J. Lean. 2012. Overview of Meta-Analysis of Monensin in Dairy Cattle. Vet. Clin. North Am. Food Anim. Pract. 28:107–119. doi:10.1016/j.cvfa.2011.12.009.
- Duffield, T.F., A.R. Rabiee, and I.J. Lean. 2008. A Meta-Analysis of the Impact of Monensin in Lactating Dairy Cattle. Part 3. Health and Reproduction. J. Dairy Sci. 91:2328–2341. doi:10.3168/jds.2007-0801.
- Duffield, T.F., D. Sandals, K.E. Leslie, K. Lissemore, B.W. McBride, J.H. Lumsden, P. Dick, and R. Bagg. 1998. Physiology and management:-Efficacy of Monensin for the Prevention of Subclinical Ketosis in Lactating Dairy Cows. J. Dairy Sci. 81:2866–2873.
- EFSA. 2015. Review of Immune Stimulator Substances/agents That Are Susceptible of Being Used as Feed Additives: Mode of Action and Identification of End-Points for Efficacy Assessment. European Food Safety

Authority. Accessed April 1, 2018. https://www.efsa.europa.eu/it/supporting/pub/905e.

- European Commission. 2011. Communication from the Commission to the European Parliament and the Council. Action plan against the rising threats from Antimicrobial Resistance.
- European Union. 2006. European Union (EU): Council Regulation (EC) No. 510/2006 of 20 March 2006 on the Protection of Geographical Indications and Designations of Origin for Agricultural Products and Foodstuffs. Accessed January 24, 2018. http://www.wipo.int/wipolex/en/text.jsp?file_id=126989.
- Friend, T.H., and C.E. Polan. 1974. Social Rank, Feeding Behavior, and Free Stall Utilization by Dairy Cattle1. J. Dairy Sci. 57:1214–1220. doi:10.3168/jds.S0022-0302(74)85040-X.
- Friend, T.H., C.E. Polan, and M.L. McGilliard. 1977. Free Stall and Feed Bunk Requirements Relative to Behavior, Production and Individual Feed Intake in Dairy Cows1. J. Dairy Sci. 60:108–116. doi:10.3168/jds.S0022-0302(77)83835-6.
- Fuller, R. 1992. History and development of probiotics. Springer, Dordrecht.
- Fustini, M., A. Palmonari, G. Canestrari, E. Bonfante, L.M.E. Mammi, M.T. Pacchioli, G.C.J. Sniffen, R.J. Grant, K.W. Cotanch, and A. Formigoni. 2017. Effect of undigested neutral detergent fiber content of alfalfa hay on lactating dairy cows: Feeding behavior, fiber digestibility, and lactation performance. J. Dairy Sci. 100:4475–4483. doi:10.3168/jds.2016-12266.
- Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401–1412. doi:10.1093/jn/125.6.1401.

- Goff, J.P. 2006. Major Advances in Our Understanding of Nutritional Influences on Bovine Health. J. Dairy Sci. 89:1292–1301.
- Goff, J.P., and R.L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders1, 2. J. Dairy Sci. 80:1260–1268.
- Goodrich, R.D., J.E. Garrett, D.R. Gast, M.A. Kirick, D.A. Larson, and J.C. Meiske. 1984. Influence of monensin on the performance of cattle. J. Anim. Sci. 58:1484–1498.
- Grant, R.J., and J.L. Albright. 2001. Effect of Animal Grouping on Feeding Behavior and Intake of Dairy Cattle. J. Dairy Sci. 84:E156–E163. doi:10.3168/jds.S0022-0302(01)70210-X.
- Grummer, R.R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J. Anim. Sci. 73:2820–2833. doi:10.2527/1995.7392820x.
- Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29:18–31. doi:10.1080/01652176.2007.9695224.
- Herbut, P., and S. Angrecka. 2018. Relationship between THI level and dairy cows' behaviour during summer period. Ital. J. Anim. Sci. 17:226–233. doi:10.1080/1828051X.2017.1333892.
- Ingvartsen, K.L. 2006. Feeding- and management-related diseases in the transition cow. Anim. Feed Sci. Technol. 126:175–213. doi:10.1016/j.anifeedsci.2005.08.003.
- Ingvartsen, K.L., and J.B. Andersen. 2000. Integration of metabolism and intake regulation: a review focusing on periparturient animals. J. Dairy Sci. 83:1573–1597.

- Ipharraguerre, I.R., and J.H. Clark. 2003. Usefulness of ionophores for lactating dairy cows: a review. Anim. Feed Sci. Technol. 106:39–57. doi:10.1016/S0377-8401(03)00065-8.
- Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5:987–995. doi:10.1038/ni1112.
- Kang, H.J., and I. Hyeog. 2015. Probiotics as an Immune Modulator. J. Nutr. Sci. Vitaminol. 61:s103–s105.
- Kehrli Jr., M.E., B.J. Nonnecke, and J.A. Roth. 1989a. Alterations in bovine lymphocyte function during the periparturient period. Am. J. Vet. Res. 50:215–220.
- Kehrli Jr., M.E., B.J. Nonnecke, and J.A. Roth. 1989b. Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50:207–214.
- Kimura, K., J.P. Goff, M.E. Kehrli, and T.A. Reinhardt. 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. J. Dairy Sci. 85:544–550. doi:10.3168/jds.S0022-0302(02)74107-6.
- Koeck, A., J. Jamrozik, F.S. Schenkel, R.K. Moore, D.M. Lefebvre, D.F. Kelton, and F. Miglior. 2014. Genetic analysis of milk β-hydroxybutyrate and its association with fat-to-protein ratio, body condition score, clinical ketosis, and displaced abomasum in early first lactation of Canadian Holsteins. J. Dairy Sci. 97:7286–7292. doi:10.3168/jds.2014-8405.
- Konggard, S.P., and C.C. Krohn. 1978. Performance of First-Calf Heifers in Two Different Grouping Systems. RepNatInst AnimSci.
- Kwon, I.H., M.H. Kim, C.-H. Yun, J.Y. Go, C.H. Lee, H.J. Lee, W. Phipek, and J.K. Ha. 2011. Effects of Fermented Soybean Meal on Immune Response of Weaned Calves with Experimentally Induced Lipopolysaccharide

Challenge. Asian-Australas. J. Anim. Sci. Asian-Australas. J. Anim. Sci. 24:957–964. doi:2011.24.7.957.

- Lacetera, N., D. Scalia, U. Bernabucci, B. Ronchi, D. Pirazzi, and A. Nardone. 2005. Lymphocyte functions in overconditioned cows around parturition. J. Dairy Sci. 88:2010–2016.
- LeBlanc, S.J. 2010. Monitoring Metabolic Health of Dairy Cattle in the Transition Period. J. Reprod. Dev. 56:S29–S35.
- LeBlanc, S.J., K.D. Lissemore, D.F. Kelton, T.F. Duffield, and K.E. Leslie. 2006. Major advances in disease prevention in dairy cattle. J. Dairy Sci. 89:1267–1279.
- Leroy, J., T. Vanholder, A. Van Knegsel, I. Garcia-Ispierto, and P. Bols. 2008. Nutrient Prioritization in Dairy Cows Early Postpartum: Mismatch Between Metabolism and Fertility? Reprod. Domest. Anim. 43:96–103. doi:10.1111/j.1439-0531.2008.01148.x.
- Liu, Y., C.D. Espinosa, J.J. Abelilla, G.A. Casas, L.V. Lagos, S.A. Lee, W.B. Kwon, J.K. Mathai, D.M.D.L. Navarro, N.W. Jaworski, and H.H. Stein. 2018. Non-antibiotic feed additives in diets for pigs: A review. Anim. Nutr. doi:10.1016/j.aninu.2018.01.007.
- Magalhães, V.J.A., F. Susca, F.S. Lima, A.F. Branco, I. Yoon, and J.E.P. Santos. 2008. Effect of feeding yeast culture on performance, health, and immunocompetence of dairy calves. J. Dairy Sci. 91:1497–1509. doi:10.3168/jds.2007-0582.
- Mallard, B.A., L.C. Wagter, M.J. Ireland, and J.C.M. Dekkers. 1997. Effects of growth hormone, insulin-like growth factor-I, and cortisol on periparturient antibody response profiles of dairy cattle. Vet. Immunol. Immunopathol. 60:61–76. doi:10.1016/S0165-2427(97)00118-9.

- Manson, F.J., and M.C. Appleby. 1990. Spacing of dairy cows at a food trough. Appl. Anim. Behav. Sci. 26:69–81. doi:10.1016/0168-1591(90)90088-U.
- Markantonatos, X., and G.A. Varga. 2017. Effects of monensin on glucose metabolism in transition dairy cows. J. Dairy Sci. 100:9020–9035. doi:10.3168/jds.2016-12007.
- McCarthy, M.M., T. Yasui, C.M. Ryan, G.D. Mechor, and T.R. Overton. 2015a. Performance of early-lactation dairy cows as affected by dietary starch and monensin supplementation. J. Dairy Sci. 98:3335–3350. doi:10.3168/jds.2014-8820.
- McCarthy, M.M., T. Yasui, C.M. Ryan, S.H. Pelton, G.D. Mechor, and T.R. Overton. 2015b. Metabolism of early-lactation dairy cows as affected by dietary starch and monensin supplementation. J. Dairy Sci. 98:3351–3365. doi:10.3168/jds.2014-8821.
- McGuffey, R.K., L.F. Richardson, and J.I.D. Wilkinson. 2001. Ionophores for dairy cattle: current status and future outlook. J. Dairy Sci. 84:E194–E203.
- McSweeney, C.S., B. Palmer, D.M. McNeill, and D.O. Krause. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91:83–93. doi:10.1016/S0377-8401(01)00232-2.
- Mordenti, A.L., N. Brogna, and A. Formigoni. 2017. Review: The link between feeding dairy cows and Parmigiano-Reggiano cheese production area. Prof. Anim. Sci. 33:520–529. doi:10.15232/pas.2016-01602.
- Morrow, D.A. 1976. Fat Cow Syndrome. J. Dairy Sci. 59:1625–1629. doi:10.3168/jds.S0022-0302(76)84415-3.
- Nace, E.L., S.C. Nickerson, F.M. Kautz, S. Breidling, D. Wochele, L.O. Ely, and D.J. Hurley. 2014. Modulation of innate immune function and phenotype

in bred dairy heifers during the periparturient period induced by feeding an immunostimulant for 60 days prior to delivery. Vet. Immunol. Immunopathol. 161:240–250. doi:10.1016/j.vetimm.2014.08.013.

- Newsholme, P., R. Curi, S. Gordon, and E.A. Newsholme. 1986. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. Biochem. J. 239:121–125.
- O'Boyle, N.J., G.A. Contreras, S.A. Mattmiller, and L.M. Sordillo. 2012. Changes in glucose transporter expression in monocytes of periparturient dairy cows. J. Dairy Sci. 95:5709–5719. doi:10.3168/jds.2012-5327.
- Oh, J., A.N. Hristov, C. Lee, T. Cassidy, K. Heyler, G.A. Varga, J. Pate, S. Walusimbi, E. Brzezicka, K. Toyokawa, J. Werner, S.S. Donkin, R. Elias, S. Dowd, and D. Bravo. 2013. Immune and production responses of dairy cows to postruminal supplementation with phytonutrients. J. Dairy Sci. 96:7830–7843. doi:10.3168/jds.2013-7089.
- Politis, I. 2012. Reevaluation of vitamin e supplementation of dairy cows: Bioavailability, animal health and milk quality. Animal 6:1427–1434. doi:10.1017/S1751731112000225.
- Polsky, L., and M.A.G. von Keyserlingk. 2017. Invited review: Effects of heat stress on dairy cattle welfare. J. Dairy Sci. 100:8645–8657. doi:10.3168/jds.2017-12651.
- Ramos-Morales, E., G. Rossi, M. Cattin, E. Jones, R. Braganca, and C.J. Newbold. 2018. The effect of an isoflavonid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the rumen simulation technique. FEMS Microbiol. Ecol. 94. doi:10.1093/femsec/fiy009.

- Regulation, O. 2003. Regulation (EC) No 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition. Off. J Eur Union 29–43.
- Roberfroid, M.B. 2007. Inulin-type fructans: functional food ingredients. J. Nutr. 137:2493S–2502S.
- Roodposhti, P.M., and N. Dabiri. 2012. Effects of Probiotic and Prebiotic on Average Daily Gain, Fecal Shedding of Escherichia Coli, and Immune System Status in Newborn Female Calves. Asian-Australas. J. Anim. Sci. 25:1255–1261. doi:10.5713/ajas.2011.11312.
- Ruegg, P.L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. J. Dairy Sci. 100:10381–10397. doi:10.3168/jds.2017-13023.
- Ruiz, R., L.O. Tedeschi, and A. Sepúlveda. 2017. Investigation of the effect of pegbovigrastim on some periparturient immune disorders and performance in Mexican dairy herds. J. Dairy Sci. 100:3305–3317. doi:10.3168/jds.2016-12003.
- Russell, J.B., and H.J. Strobel. 1989. Effect of ionophores on ruminal fermentation. Appl. Environ. Microbiol. 55:1.
- Schukken, Y.H., J. Hertl, D. Bar, G.J. Bennett, R.N. González, B.J. Rauch, C. Santisteban, H.F. Schulte, L. Tauer, F.L. Welcome, and Y.T. Grohn. 2009. Effects of repeated gram-positive and gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. J. Dairy Sci. 92:3091–3105. doi:10.3168/jds.2008-1557.
- Schuller, L.K., O. Burfeind, and W. Heuwieser. 2014. Impact of heat stress on conception rate of dairy cows in the moderate climate considering different temperature-humidity index thresholds, periods relative to breeding, and heat

load indices. Theriogenology 81:1050–1057. doi:10.1016/j.theriogenology.2014.01.029.

- Seegers, H., C. Fourichon, and F. Beaudeau. 2003. Production effects related to mastitis and mastitis economics in dairy cattle herds. Vet. Res. 34:475–491.
- Sharif, A., and G. Muhammad. 2008. Somatic cell count as an indicator of udder health status under modern dairy production: A review. Pak. Vet J 28:194–200.
- Sordillo, L.M. 2016. Nutritional strategies to optimize dairy cattle immunity. J. Dairy Sci. doi:10.3168/jds.2015-10354.
- Sordillo, L.M., and S.L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128:104–109. doi:10.1016/j.vetimm.2008.10.305.
- Sordillo, L.M., G.A. Contreras, and S.L. Aitken. 2009. Metabolic factors affecting the inflammatory response of periparturient dairy cows. Anim. Health Res. Rev. 10:53–63. doi:10.1017/S1466252309990016.
- Sordillo, L.M., and V. Mavangira. 2014. The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. Anim. Prod. Sci. 54:1204–1214.
- Summer, A., P. Franceschi, P. Formaggioni, and M. Malacarne. 2015. Influence of milk somatic cell content on Parmigiano-Reggiano cheese yield. J. Dairy Res. 82:222–227. doi:10.1017/S0022029915000102.
- Swaisgood, H.. 1982. Chemistry of milk proteins. Applied Science Publishers, New York.
- Thom, E.C. 1959. The Discomfort Index. Weatherwise 12:57–61. doi:10.1080/00431672.1959.9926960.

- Vanholder, T., J. Papen, R. Bemers, G. Vertenten, and A.C.B. Berge. 2015. Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. J. Dairy Sci. 98:880–888. doi:10.3168/jds.2014-8362.
- Wang, Y., S.B. Puntenney, J.L. Burton, and N.E. Forsberg. 2007. Ability of a commercial feed additive to modulate expression of innate immunity in sheep immunosuppressed with dexamethasone. animal 1:945. doi:10.1017/S1751731107000365.
- Wood, L., H. Scott, M. Garg, and P. Gibson. 2009. Innate immune mechanisms linking non-esterified fatty acids and respiratory disease. Prog. Lipid Res. 48:27–43. doi:10.1016/j.plipres.2008.10.001.
- Wyle, F.A., and J.R. Kent. 1977. Immunosuppression by sex steroid hormones. Clin. Exp. Immunol. 27:407.

3. Aim of the study

Despite the large numbers of studies, the problems occurring in dairy cows around calving are far from being solved, thus leaving the transition period to be a big issue for dairy farmers and researchers.

In light of the arguments presented in the introduction, it is clear that multiple and different factors determine the success of cow transition from dry to lactating period.

Moreover, considering the overwhelming issue of antimicrobial resistance for human health and for its implications for both food safety and food security, the development of efficient strategies, capable to reduce the incidence of disease and to increase animal resilience towards pathogens is therefore essential. The FAO/OIE/WHO identified strategies for reducing the use of antimicrobials in dairy cattle. Among these, are: genetic selection of more resistant and resilient animals, improving animal welfare and following animal health and management guidelines, increasing the use of vaccines and the use of natural feed supplements like probiotics, prebiotics and immunomodulators.

A comfortable and healthy environment represents the base for the fulfilment of animals' requirements, in terms of rest, nutrition and welfare, but despite recommendations about lying and feeding space, overstocking remains a common situation in dairy herds, especially in dry cows areas. In addition, the whole impact of overstocking on animal physiology is not clear yet, and researchers suggest that stress related indicators may be altered in such conditions, thus influencing other physiological pathways (Huzzey *et al*, 2012).

Therefore, according to these recommendations, the main goal of this thesis was to evaluate through 2 different studies the impact of management and feeding strategies on the health and welfare of transition dairy cows.

On the other hand, with the last study it was investigated the effect of a treatment for the prevention of ketosis on the quality of milk and cheese production. This treatment, in fact, ameliorates the energy metabolism of fresh cows, but the widespread of its use, especially during summer period when the risk of ketosis is higher, created great concern among dairy industry, because of the potential negative impact on Protected Designation of Origin (PDO) cheese quality, like Parmigiano Reggiano.

4. Experimental studies

4.1. Study 1: Published paper

Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion

M. Fustini*, G. Galeati*[1], G. Gabai†, L. E. Mammi*, D. Bucci*, M. Baratta‡, P. A. Accorsi* and A. Formigoni*

*Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, 40062 Ozzano dell'Emilia (BO), Italy

†Department of Comparative Biomedicine and Food Science, University of Padua, 35020 Legnaro (PD), Italy.

‡ Department of Veterinary Science, University of Turin, 10095 Grugliasco (TO), Italy

ABSTRACT

Stressful situations trigger a number of changes such as the secretion of cortisol (C) and dehydroepiandrosterone (DHEA) from the adrenal cortex, in response to adrenocorticotropic hormone (ACTH). The aim of this study was to verify whether overstocking during the dry period (from 21±3 d to the expected calving until calving) affects DHEA and C secretion and behavior in Holstein Friesian cows. Twenty-eight cows were randomly divided into two groups (14 animals each), balanced for number of lactations, BCS (body condition score) and expected date of calving. Cows in the far-off phase of the dry period (from 60 to 21 d before the expected calving date) were

housed together in a bedded-pack. Then, animals from 21±3 d prior to the expected calving until calving were housed in pens with the same size but under different crowding conditions due to the introduction of heifers (interference animals) into the pen. Control condition (CTR) had two animals per pen with 12.0 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 4,8 m^2 for each animal. On d -30±3, -21±3, - 15±3, -10±3, -5±3 before and 10, 20, 30 after calving, blood samples were collected from each cow for the determination of plasma DHEA and C concentrations by RIA. Rumination time (min/d), activity (steps/h), lying time (min/d) and lying bouts (bouts/d) were individually recorded daily. In both groups, there was an increase in DHEA before calving and after parturition the concentration declined rapidly. Overstocking significantly increased DHEA concentration compared to CTR group at d -10 (1.79±0.09 vs 1.24±0.14 pmol/ml) while an increase of C was observed at d -15 (3.64±0.52 vs 1.64±0.46 ng/ml). OS group showed significantly higher activity (step/h), compared with CTR group. Daily lying bouts tended to be higher for OS group compared with CTR group in the first week of treatment. The overall results of this study documented that overstocking during the dry period was associated with a short term changes in DHEA and cortisol but these hormonal modifications did not influence cow behavior.

Key words: dairy cattle, cortisol, dehydroepiandrosterone, overstocking, dry period

INTRODUCTION

Stressful situations trigger a number of changes such as activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis. As a consequence, the adrenal cortex, in response to adrenocorticotropic hormone (ACTH), starts to secrete both cortisol and dehydroepiandrosterone (DHEA). Cortisol and DHEA are produced in different sections of the adrenal cortex; the zona fasciculata secretes cortisol while the zona reticularis secretes DHEA and its sulfated metabolite dehydroepiandrosterone sulfate (DHEA-S) (Nguyen and Conley, 2008). In female primates, DHEA and DHEA-S are also produced in the ovary (Sirinathsinghji and Mills, 1983) and in primates and rodents DHEA is produced within the central nervous system and in peripheral nerves (Baulieu, 1998).

Cortisol stimulates the mobilization of the energy needed to overcome stressors; DHEA and DHEA-S are androgen precursors that have been shown to exert antioxidative and anti-inflammatory effects (Kalimi et al., 1994; Maninger et al., 2009) and to play a protective and regenerative role (Theorell, 2009; Maninger et al., 2009).

In humans, an acute psychosocial stress induces a DHEA and DHEA-S increase (Izawa et al., 2008; Lennartsson et al., 2012) while long-term psychosocial stress negatively affects both steroids levels (Izawa et al., 2012; Lennartsson et al., 2013). Elevated levels of DHEA and DHEA-S in response to the stressor have been found in both men and women, along with significantly increased ACTH, cortisol, heart rate and blood pressure. Modifications in DHEA release in response to stressors have been observed also in the bovine species. A 23% decrease in serum DHEA and 65% higher cortisol:DHEA ratio were observed in lame cows compared to sound cows (Almeida et al., 2008), and a 1.6-fold DHEA decrease was observed in the plasma of transportation-stressed bulls (Buckham Sporer et al., 2008).

In cows, as in most non-primate mammals, circulating DHEA-S is significantly lower than DHEA, which can be considered an indicator of the P450c17 enzyme activity and the most important circulating precursor of ectopic androgen and estrogen synthesis. Conversely, DHEA-S contribution as an androgen reservoir is rather limited (Feher et al., 1977; Marinelli et al., 2007). In the bovine, DHEA concentrations are quite variable between individuals in both female (Marinelli et al., 2007) and male (Simontacchi et al., 2005) animals.

Increased stocking density is a common practice among dairy producers; the behavioral consequences of this practice are well documented while the physiological ones have still not been thoroughly investigated. Fregonesi et al. (2007a) observed in dairy cows a linear reduction in lying time as freestall stocking density increased while Huzzey et al. (2006) observed a linear reduction in feeding time as stocking density at the feed bunk was increased. Moreover, increased aggressive displacements are often observed at the overstocked feed bunk or freestalls (Huzzey et al., 2006; Fregonesi et al., 2007b); these competitive environments can make it difficult for some cows to gain access to feed.As for the physiological consequences of overstocking, previous works have shown that cows regrouped into a high stocking density group (Friend et al., 1977) or subjected to overcrowding in the resting area (Friend et al., 1979) presented a higher cortisol response to ACTH challenge compared with cows that were not regrouped or overcrowded, respectively.

In contrast to cortisol, DHEA and DHEA-S have received little attention within the stress research area of domestic animals and no studies have investigated so far the effect of overcrowding on DHEA secretion.

Therefore, the aim of this study was to verify whether overstocking during the dry period affects DHEA and cortisol (C) secretion and the behaviors of activity, rumination, resting and lying time in Holstein Friesian cows.

MATERIALS AND METHODS

Animals, housing and diet

Twenty-eight Holstein dairy cows were enrolled in this experiment. All animals were housed at the farm of the University of Bologna (Ozzano Emilia, Italy) and used according to EEC animal care guidelines. The experimental procedures had been approved by the Ethical Committee of Bologna University.

Animals were randomly divided into two groups (14 animals each), balanced for number of lactations (1.35±1.31), BCS (body condition score, 3.58±0.35) and expected date of calving. Cows in the far-off phase of the dry period (60 to 21 d before the expected calving date) were housed together in a bedded-pack and received water and grass hay ad libitum. From 21±3 d until calving animals were housed in two bedded-pack groups where they had ad libitum access to water and were fed daily using total mixed ration (TMR). After calving, cows were housed together in a bedded pack area for the first 2 weeks of lactation and then moved to a free-stall pen in a group composed with overall 20 cows for the rest of lactation. TMR were fed approximately at 7 am for lactating cows and 9 am for dry cows. TMR samples were collected weekly throughout the study and analyzed for the chemical composition according to the following methods: dry matter (DM) was determined by gravimetrically drying the sample at 103°C to a constant weight, crude protein (CP), neutral detergent fibre (aNDFom), acid detergent fiber (ADF) and lignin (ADL) were determined according to Mertens (2002), and AOAC 973.18, respectively. Starch was determined according to AOAC official method (AOAC 996.11) and ether extract according to AOAC 920.390020. Diet composition and analysis for both dry period and lactation are shown in Table 1.

Experimental design, blood sampling and hormone assays

Animals from 21 d prior to the expected calving until calving were housed in pens with the same size (24.0 m^2 in total with 15.5 m^2 of resting area and 8.5 m^2 of feeding area) but in different crowding conditions due to the introduction of heifers into the pen (interference animals) having a body weight of 500-550 Kg. In particular, control condition (CTR) had two animals per pen with 12.0 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 4,8 m^2 for each animal. Interference animals were part of the group during the far off dry period, in order to avoid social stress at the introduction. Bunk space was 3.3 meters long and design with a neck rail allowing a space of 1.65 m/head for control animal and 0.66 m/head for OS animal. Resting area was a deep-bedded pack with straw added twice a d.

On d -30±3, -21±3, - 15±3, -10±3, -5±3 before and 10, 20, 30 d after calving blood samples were collected from each cow from the jugular vein for the determination of plasma DHEA and C concentrations. Blood samples were collected before the morning feeding while cows were restrained in individual self-locking headlocks adjacent to the feed bunk. The needles used were 20 gauge and samples were collected into evacuated heparinized tubes. The utmost care was taken to minimize stress during sample collection. The utmost care was taken to minimize stress during sample collection.

After collection, blood samples were placed immediately on ice and centrifuged at 1200 x *g* for 20 min at 4°C. Plasma was harvested and stored at -20°C until steroids were measured. Plasma cortisol concentration was determined using a validated RIA as previously described (Tamanini et al, 1983). The sensitivity of the assay was 4.3 pg/tube, and the intra- and inter-assay coefficients of variation were 5.4% and 8.6%, respectively. Cortisol plasma levels are expressed as ng/mL.

Plasma DHEA was measured by a microtiter RIA method previously described (Gabai et al., 2004), using a commercial anti-DHEA-7-carboxymethyloxime-BSA (Biogenesis, Poole, UK) that showed the following cross-reactions: DHEA 100%, 5α-androstane-3ß, 17 ß -diol 6.3%, androstenedione 1.3%, testosterone 0.1%, other related compounds less than 0.05%. The antiserum was used at a working dilution of 1:20,000. The tracer was [1,3,6,7 3H]DHEA (Perkin-Elmer Life Sciences; specific activity: 71 Ci/mmol; 30 pg/well). The standard curve was made by serially diluting (1.56–200 pg/well) a solution of DHEA (Sigma, Milan, Italy). The detection limit of the assay was 1.56 pg/well (software Riasmart; Perkin-Elmer Life Sciences). The results of the intra- and inter-assay precision test, expressed as coefficients of variation (CV), were 3.7 and 7.2%, respectively.

Body condition score

At enrolment (60 d before calving), three weeks before calving, at calving and at 5 weeks of lactation, all cows were scored for body condition (1=emaciated and 5=obese; 0.25-unit increments, as described by Edmonson et al., 1989) and locomotion (1=normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997). Cows with locomotion score ≥3 were considered lame. Body condition score and locomotion score were performed by the same observer for the whole experiment in order to avoid inter-observer variability.

Behaviour Monitoring

Rumination time was recorded using the Hi-Tag rumination monitoring system (SCR Engineers Ltd., Netanya, Israel). This rumination sensor includes a microphone that detects the rumination sounds, a motion sensor, a microprocessor, a storage unit and a battery. The sensor is fixed on a collar and placed on the left side of

the cow's neck. To guarantee the correct position of the tag a counter weight was placed on the bottom of the collar. The data were sent to a PC via antenna. A software (Data Flow software, SCR Engineers Ltd.) analyses the rumination time as minutes of 2 h (Schirmann et al., 2009), and calculates the rumination time of the last 24 h.

The cows were also equipped with another sensor (Pedometer Plus; S.A.E. Afikim) that monitored 3 parameters: activity (steps/h), lying time (min/d), and lying bouts (switching between standing and lying; Higginson et al., 2009). The tag was attached to a leg band on the right rear leg of each cow and the data were accumulated and transmitted to management software (AfiFarm; S.A.E. Afikim) each time the cows passed an antenna located in the milking parlor and under the water throughs. Behavioral data were collected every d but for statistical analysis the data were averaged per week.

Clinical Examination and Definitions of Diseases

All cows were examined at 1, 3±1, 10±1 d in milk (DIM) for diagnosis of retained foetal membrane, metritis, and acute metritis. Retained foetal membrane was defined as retention of foetal membrane after 24 h postpartum. Metritis was reffered to cows with watery, pink or brown, and fetid uterine discharge. Cows with symptoms of metritis, rectal temperature >39.5°C, or anorectic, or depressed were considered to have acute metritis (Sheldon et al., 2006). All cows were observed once daily for displacement of abomasum and twice daily for mastitis throughout their lactation.

Production parameters

After calving, cows were milked twice daily at 07.30 and 19.30 h and individual yield of milk (AfiFlo milk meters, S.A.E. Afikim), concentrations of fat,

true protein, and lactose (AfiLab on-line real-time milk analyzer, S.A.E. Afikim) were recorded by the Afikim milking system. The AfiLab system is calibrated once monthly with data on milk composition from 90 cows analyzed by the ARAER Laboratoty (Modena, IT). Concentrations of milk components from each milking were used to calculate the daily yields of fat, protein, and lactose after adjusting for milk production during each milking. The ECM yield (energy correct milk) was calculated as [(0.327 × milk yield) + (12.95 × fat yield) + (7.2 × protein yield)] (Orth, 1992). Daily values were averaged into weekly means for statistical analyses.

Statistical Analysis

The experiment had a randomized switch-back design with pen as the experimental unit. Seven replicates were used, six of them had a pregnant heifer (nulliparous animal) and a cow (parous animal) together and one replicate had only cows. All statistical analysis were conducted using SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC). Data were tested for non-normality by using the Shapiro test. Binomial dependent variables were analyzed by logistic regression using GLIMMIX procedure with a binary distribution. Continuous data were analyzed by ANOVA for repeated measures using the MIXED procedure. The structure of covariance (autoregressive, unstructured, or compound symmetry) was chosen according to the Bayesian Akaike information criteria. In all models, treatment (OS vs Control), replicate (1 to 7), and parity (nulliparous vs parous) were included as fixed effect. For analysis of repeated measurements variables, time and the interaction between treatment and time were included in the model as fixed effect. Only the independent variables with $P < 0.10$ were retained in the model. Cortisol data were handled by log transformation to match normality.

RESULTS AND DISCUSSION

To our knowledge, this is the first study that demonstrates the difference in time-course variation of DHEA and cortisol secretion in response to overstocking during the dry period in Holstein Friesian cows. In both groups, before calving, an increase in DHEA was observed which tended to be more evident in the overstocked group, although the difference between groups was significant only at -10 d. Then, DHEA concentrations rapidly declined after parturition. Overstocking significantly (P=0.0049) increased DHEA concentration compared to CTR group at d -10 (1.79±0.09 vs 1.24±0.14 pmol/ml) while an increase of C was observed (P=0.022) at d -15 (3.64±0.52 vs 1.64±0.46 ng/ml) (Figure 1). No correlation was found between DHEA and C.

In primates and rodents, it is generally accepted that DHEA is secreted mainly by the adrenal cortex and the ovary (Baulieu, 1998), and peripheral tissues are able to metabolize this steroid into active androgens and estrogens (Labrie, 1991). In pregnant primates and horses, placenta can utilize circulating DHEA to synthesize estrogens (Strauss et al., 1996).

In humans, DHEA and DHEA-S levels significantly increase in response to acute psychological stress (Lennartsson et al., 2012) and it has been suggested that these steroids play a protective role during the stress reaction, antagonizing the effects of cortisol (Hechter et al., 1997; Morgan et al., 2004). The stress-induced DHEA and DHEA-S increase has likely behavioral and emotional effects. Studies on mice showed antidepressant, anxiolytic, anti-aggression, and memory-enhancing effects of DHEA-S (Melchior and Ritzmann, 1994).

In cattle , stressful situations are associated with a decrease in circulating DHEA, as suggested by observations in lame cows (Almeida et al., 2008) and in transportation-stressed bulls (Buckham Sporer et al., 2008). In the late pregnant cow,

Marinelli et al. (2007) suggested that the placenta is the most important source of DHEA; placenta utilizes mainly the D5 steroidogenic pathway to produce estrogen (Geisert & Conley 1998). Previous works (Gabai et al., 2004; Marinelli et al., 2007) indicate that the DHEA placental secretion increases in late pregnancy, probably depending upon the tissue mass (Geisert & Conley 1998), and suddenly decreases after parturition. Therefore, the DHEA increased observed in the OS group approximately five d following a significant increase in plasma cortisol was quite surprising. Indeed, adrenal DHEA has been reported being secreted synchronously with cortisol during night and day (Rosenfeld et al., 1971), and the delay in DHEA secretion in respect to cortisol was unexpected. A possible explanation resides in the stimulating glucocorticoid effect on the placental CYP17 enzyme in the cow (Gross and Williams, 1988; Shenavai et al., 2012) that, in turn, could speed up the conversion of pregnenolone into DHEA.

Walking is associated with an increase in plasma cortisol concentrations (Coulon et al., 1998) and, likely, the OS cows, which displayed the greater number of steps per hour and thus were more active, experienced higher cortisol concentrations during the pre-partum period, possibly resulting in the higher cortisol concentrations observed on d -15. The suitability of blood cortisol as a stress biomarker in livestock is in doubt because of its variability and as blood sampling is an invasive technique that can cause the activation of the HPA (Mormede et al., 2007). Therefore, the intrinsic variability in plasma cortisol could have masked the greater HPA activation associated with OS and increased walking. Moreover, it is possible that the cows' HPA axis responded to increased walking during the first d of the OS treatment and then animals adapted. Indeed, Coulon et al. (1998) observed that cortisol concentrations were higher on d 1 and 8 in cows that walked in comparison with cows that remained at the barn, but the difference was not anymore evident after 20 d.

Recent study conducted by Silva and coauthors (Silva et al., 2016), evaluated the effects of prepartum stocking density on serum cortisol and hair cortisol concentration of Jersey cows. Treatments consist in 80% or 100% headlock stocking density. In this study serum and hair cortisol concentrations were not affected by treatment.

As glucocorticoids can alter placental steroidogenesis (Gross and Williams, 1988; Shenavai et al., 2012), it is possible that the modified endocrine milieu affects pregnancy length. However, in this experiment the increased plasma DHEA observed in OS cows was not associated with differences in pregnancy length, (CTR = 279.9 ± 5.0 d OS = 278.7 ± 4.2 d; $P = 0.32$) although d dry tended to be lower for OS animals (CTR = 55.6 ± 12.6 d, OS = 48.6 ± 3.0 d). At the beginning of the experimental period d of gestation (CTR = 258.8 ± 5.3 d, OS = 257.7 ± 4.7 d; $P = 0.35$) were not different among treatments.

About calving difficulties there were not major differences. Calf weights were not different ($P = 0.46$) among treatments (CTR = 41.5 ± 3.7 d, OS = 41.7 ± 4.3 d) and no animals carried twins. Overall incidence of peripartum diseases was not different between CTR and OS treatments. No animals has displaced abomasum and mastitis in the first 5 weeks after calving. One cow had metritis in the OS group while no cows in CTR group. Body condition score and lameness score were not affected by treatment. Current recommendation for feed bunk space for prepartum freestall-housed dry cows is to provide a minimum of 0.76 m of linear bunk space per cow (Nordlund et al., 2006). In the present study, control cows had 1.2 m of bunk space per cow and OS cows had only 0.66 m of bunk space, which should be adequate to limited bunk space. Reducing linear feeding space has been observed to increase competition at the feed bunk (Huzzey et al., 2006 Collings et. al., 2011). However, the results of these studies, while showing more cow displacements from the feed bunk, have variable effects on feeding behaviour (Collins et al., 2011, Huzzey, 2012). In a

study on lactating cows, a reduction in feeding time was observed in multiparous cows (Proudfoot et al., 2009) and, in other studies, the competitively fed cows had fewer meals per d with a tendency of larger and longer meals (Olofsson, 1999; Hosseinkhani et al., 2008). Olofsson (1999) found that competition slightly increased the DMI of dairy cows, and this increase was driven by an increase in feeding rate. Based on these studies, it is not surprising to have little or no effect on DMI with the feed bunk restriction used in the current study.

Rumination times were not different in OS animals in the current analysis (Table 2). This parameter can be a key indicator of DMI, therefore animals in both groups had similar rumen activities and more than likely similar intakes.

Total minutes of lying time per d was not different among OS and CTR groups (Table 2). In some studies, lying time has been shown to decrease with increased stocking density (Krawczel et al., 2012; Lobeck-Luchterhand et al., 2015); however, other studies using late lactation or dry cows showed no differences (Collings, 2011; Huzzey et al., 2012). It is consistent that dry cows with more available time throughout the d (Grant and Albright, 2001) would have sufficient hours available to allow for a normal number of lying hours. Lying time has a higher priority than eating for cows, when these two behaviors are restricted (Munksgaard et al., 2005). This could explain why the resting time did not change although the space was consistently lower in OS animal (3.3 m^2 of bedded area versus 7.8 m^2 for control animals). The time budgets of prepartum cows tend to be interrupted less than lactating dairy cows, because the animals are not moved outside the pen for milking and do not have cycling activity with estrus behavior. Both groups, however, showed a daily lying time lower than the recommended 12 h/d (Munksgaard et al., 2005). Comfort of the bedding surface could be an important factor in determining daily lying time (Fregonesi et al., 2007b). In a study with either 9 or 4.5 $m^2$of bedded area

per cow there was no difference in lying time (Fregonesi and Leaver, 2002). Animals could better tolerate overcrowding when open pack area is present compared with stall barn, since they can lie down at the same time staying closer to one another. Using free stall type bedding, lying time linearly decreased when stocking density increased from 100% to 150% (Fregonesi et al., 2007a). In same conditions, Krawczel et al. (2012) reported that lying time was reduced for stocking densities of 131 and 142% compared with 100 or 113%.

Mean lying bouts tended to be higher in OS group the first week of overcrowding, indicating an adjustment period was occurring (Table 3). Animals had a resting time that is more disrupted, considering that the daily lying time were divided in more bouts. After this first week, the behavior was similar in OS and control animals. Competition at the feed bunk generally caused an increase in standing time in multiparous transition cows (Proudfoot et al., 2009) and in midlactation cows (Olofsson, 1999; Huzzey et al., 2006). The importance of this is determined by the overall DMI of the animals. Excessive standing time is a risk factor for developing lameness conditions such as claw horn lesions (Greenough and Vermunt, 1991; Singh et al., 1993). Avoiding excessive standing is important throughout lactation, but in particular during transition when animals are subjected to many endocrine and metabolic changes (Goff and Horst, 1997).

As for the activity behavior, the OS group showed significantly higher activity (step/hour), compared with CTR group, as reported in Table 2. This difference could indicate the increased need of movement in the pen and represents another evidence of stress occurring in this phase. An increased number of animal displacements and animal movement would be expected with overcrowding and feed bunk restriction (Collings, 2011; Huzzey 2012) and the related stress could be expected to alter parameters being measured in this study.

Energy corrected milk production were not different among treatments (Table 4). Among cows, treatment did not differ regarding previous lactation 305-d mature equivalent milk yield (CTR = 10.2 ± 231.1 kg, OS = 10.0 ± 191.7 kg; $P = 0.39$) so we can assume that there were no interference effect of the genetic potential. A recent study (Silva et al., 2014) reports no difference in yield of ECM when cows are overcrowded. It would be expected that the minimal differences in cow behavior and rumination, as observed in this study, would not carry through to any differences in DMI or early lactation milk production.

The overall results of this study documented that overstocking during the dry period is associated with a short term changes in DHEA and cortisol but these hormonal modifications do not influence cow behavior.

ACKNOWLEDGMENTS

This work was s upported by a MIUR-COFIN grant (prot. 2010YBP4LZ_003). We thank Dr. Laura Da Dalt and Dr. Sara Speroni for skilled technical assistance.

REFERENCES

- Almeida, P. E., P. S. D. Weber, J. L. Burton, A. J. Zanella. 2008. Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions. Dom Anim Endoc 34: 89–99.

- Baulieu, E. E. 1998. Neurosteroids: a novel function of the brain. Psychoneuroendocrinology 23:963–87.

- Buckham Sporer K. R., L. Xiao, R. J. Tempelman, J. L. Burton, B. Earley, M. A. 2008. Crowe. Transportation stress alters the circulating steroid environment and neutrophil gene expression in beef bulls. Vet Immunol Immunopathol 121:300–320.

- Coulon, J. B., P. Pradel, T. Cochard, and B. Poutrel. 1998. Effect of Extreme Walking Conditions for Dairy Cows on Milk Yield, Chemical Composition, and Somatic Cell Count. J. Dairy Sci. 81:994–1003.

- Collings, L. K., D. M. Weary, N. Chapinal, and M. A. von Keyserlingk. 2011. Temporal feed restriction and overstocking increase competition for feed by dairy cattle. J. Dairy Sci. 94:5480–5486.

- Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 72:68–78.

- Feher, T., L. Bodrogi, K. G. Feher, E. Poteczin, and I. S. Kolcsey. 1977. Free and solvolysable dehydroepiandrosterone and androsterone in blood of mammals under physiological conditions and following administration of dehydroepiandrosterone. Acta Endocrinol. 85:126–33.

- Fregonesi, J. A., and J. D. Leaver. 2002. Influence of space allowance and milk yield level on behaviour, performance and health of dairy cows housed in strawyard and cubicle systems. Livest. Prod. Sci. 78:245–257.

- Fregonesi, J. A., C. B. Tucker, and D. M. Weary. 2007a. Overstocking reduces lying time in dairy cows. J. Dairy Sci. 90:3349–3354.

- Fregonesi, J. A., D. M. Veira, M. A. G. von Keyserlingk, and D. M. Weary. 2007b. Effects of bedding quality on lying behavior of dairy cows. J. Dairy Sci. 90:5468–5472.

- Friend, T. H., F. C. Gwazdauskas, and C. E. Polan. 1979. Change in adrenal response from free stall competition. J. Dairy Sci. 62:768–771.

- Friend, T. H., C. E. Polan, F. C. Gwazdauskas, and C. W. Heald. 1977. Adrenal glucocorticoid response to exogenous adrenocorticotropin mediated by density and social disruption in lactating cows. J. Dairy Sci. 60:1958–1963.

- Gabai, G., L. Marinelli, C. Simontacchi, and G. Bono. 2004. The increase of plasma C195 steroids in subcutaneous abdominal and jugular veins of dairy cattle during pregnancy is unrelated to estrogenic activity. Steroids 69:121–127.

- Geisert, R. D., and A. J. Conley. 1998. Secretion and metabolism of steroids in subprimate mammals during pregnancy. In: Bazer FW, Conn PM, editors. The endocrinology of pregnancy. Totowa, NJ: Humana Press Inc., p. 291–318.

- Goff, J. P., and R. L. Horst. 1997. Physiological changes at parturition and their relationships to metabolic disorders. J. Dairy Sci. 80:1260–1268.

- Grant, R. J., and J. L. Albright.. 2001. Effect of animal grouping on feeding behaviour and intake of dairy cattle. J. Dairy Sci. 84:156–163.

- Greenough, P. R., and J. J. Vermunt. 1991. Evaluation of subclinical laminitis in a dairy herd and observations on associated nutritional and management factors. Vet. Rec. 128:11-17.

- Gross, T. S. and W. F. Williams. 1988. In-vitro steroid synthesis by the placenta of cows in late gestation and at parturition. J. Reprod. Fertil. 83:565-573.

- Hechter, O., A. Grossman, and R. T. Chatterton Jr. 1997. Relationship of dehydroepiandrosterone and cortisol in disease. Medical Hypotheses 49: 85–91.

- Higginson, J. H., K. E. Leslie, S. T. Millman, and D. F. Kelton. 2009. Evaluation of the Pedometry Plus system for the detection of pedometric activity and lying behaviour in dairy cattle. J. Dairy. Sci. 92(E-Suppl. 1):346.

- Hosseinkhani, A., T. J. DeVries, K. L. Proudfoot, R. Valizadeh, D. M. Veira, and M. A. G. von Keyserlingk. 2008. The effects of feed bunk competition on the feed sorting behavior of close-up dry cows. J. Dairy Sci. 91:1115–1121.

- Huzzey, J. M., T. J. DeVries, P. Valois, and M. A. G. von Keyserlingk. 2006. Stocking density and feed barrier design affect the feeding and social behavior of dairy cattle. J. Dairy Sci. 89:126–133.

- Huzzey, J. M., D. V. Nydam, R. J. Grant, and T. R. Overton. 2012. The effects of overstocking Holstein dairy cattle during the dry period on cortisol secretion and energy metabolism. J. Dairy Sci. 95(8):4421-4433.

- Izawa, S., N. Sugaya, K. Shirotsuki, K. C. Yamada, N. Ogawa, Y. Ouchi, Y. Nagano, K. Suzuki, and S. Nomura. 2008. Salivary dehydroepiandrosterone secretion in response to acute psychosocial stress and its correlations with biological and psychological changes. Biol. Psychol. 79:294-298.

- Izawa, S., K. Saito, K. Shirotsuki, N. Sugaya, and S. Nomura. 2012. Effects of prolonged stress on salivary cortisol and dehydroepiandrosterone: a study of a two-week teaching practice. Psychoneuroendocrinology 37(6):852-8.

- Kalimi, M., Y. Shafagoj, R. Loria, D. Padgett, and W. Regelson. 1994. Anti-glucocorticoid effects of dehydroepiandrosterone (DHEA). Mol. Cell. Biochem. 131:99-104.

- Krawczel, P. D., L. B. Klaiber, R. E. Butzler, L. M. Klaiber, H. M. Dann, C. S. Mooney, and R. J. Grant. 2012. Short-term increases in stocking density affect the lying and social behavior, but not the productivity, of lactating Holstein dairy cows. J. Dairy Sci. 95:4298-4308.

- Labrie, F.1991. At the cutting edge: intracrinology. Mol. Cell. Endocrinol.78:C113–8.

- Lennartsson, A. K., M.M. Kushnir, J. Bergquist, I. H. Jonsdottir. 2012. DHEA and DHEA-S response to acute psychosocial stress in healthy men and women. Biol. Psychol. 90:143-149.

- Lennartsson, A. K., T. Theorell, M. M. Kushnir, J. Bergquist, and I. H. Jonsdottir. 2013. Perceived stress at work is associated with attenuated DHEA-S response during acute psychosocial stress. Psychoneuroendocrinology 38:1650-1657

- Lobeck-Luchterhand, K. M., P. R. Silva, R. C. Chebel, and M. I. Endres. 2015. Effect of stocking density on social, feeding, and lying behavior of prepartum dairy animals. J. Dairy Sci. 98(1):240-9.

- Maninger, N., O. M. Wolkowitz, V. I. Reus, E. S. Epel, and S. H. Mellon. 2009. Neurobiological and neuropsychiatric effects of dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS). Front. Neuroendocrinol. 30:65–91.

- Marinelli, L., E. Trevisi, L. Da Dalt, M. Merlo, G. Bertoni, and G. Gabai. 2007. Dehydroepiandrosterone secretion in dairy cattle is episodic and unaffected by ACTH stimulation. J. Endocrinol. 194(3):627-35.

- Melchior, C. L., and R. F. Ritzmann. 1994. Dehydroepiandrosterone is an anxiolytic in mice on the plus maze. Pharmacol. Biochem Behav. 47:437–441.

- Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217–1240.

- Morgan 3rd, C. A., S. Southwick, G. Hazlett, A. Rasmusson, G. Hoyt, Z. Zimolo, and D. Charney. 2004. Relationships among plasma dehydroepiandrosterone sulfate and cortisol levels, symptoms of dissociation, and objective performance in humans exposed to acute stress. Arch. Gen. Psychiatry 61:819–825.

- Mormede, P., S. Andanson, B. Auperin, B. Beerda, D. Guemene, J. Mamkvist, X. Manteca, G. Manteuffel, P. Prunet, C. G. van Reenen, S. Richard, and I. Veissier. 2007. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. Physiol. Behav. 92:317-339

- Munksgaard, L., M. B. Jensen, L. J. Pedersen, S. W. Hansen, and L. Matthews. 2005. Quantifying behavioural priorities—Effects of time constraints on behaviour of dairy cows, *Bos taurus*. Appl. Anim. Behav. Sci. 92:3–14.

- Nordlund, K., N. Cook, and G. Oetzel. 2006. Commingling dairy cows: Pen moves, stocking density, and health. 39th Proc. Am. Assoc. Bovine Pract., St. Paul, MN. Bovine Practitioner, Opelika, AL; (Pages 36–42).

- Nguyen, A. D., and A. J. Conley. 2008. Adrenal androgens in humans and nonhuman primates: production, zonation and regulation. Endocr. Dev. 13:33—54.

- Olofsson, J. 1999. Competition for total mixed diets fed for ad libitum intake using one or four cows per feeding station. J. Dairy Sci. 82:69–79.

- Orth, R. 1992. Sample Day and Lactation Report. DHIA 200 Fact Sheet A-2. Mid-States DRPC, Ames, IA.

- Proudfoot, K. L., D. M. Veira, D. M. Weary, and M. A. G. von Keyserlingk. 2009. Competition at the feed bunk changes the feeding, standing and social behavior of transition dairy cows. J. Dairy Sci. 92:3116–3123.

- Rosenfeld, R. S., L. Hellman, H. Roffwarg, E. D. Weitzman, D. K. Fukushima, and T. F. Gallagher. 1971. Dehydroisoandrosterone is secreted episodically and synchronously with cortisol by normal man. J. Clin. Endocrinol. Metab. 33:87–92.

- Schirmann, K.,M. A. G. von Keyserlingk, D. M. Weary, D. M. Veira, and W. Heuwieser. 2009. Technical note: Validation of a system for monitoring rumination in dairy cows. J. Dairy Sci. 92:6052–6055.

- Sheldon, I. M., G. S. Lewis, S. LeBlanc, and R. O. Gilbert. 2006. Defining postpartum uterine disease in cattle. Theriogenology 65:1516–1530

- Shenavai, S., S. Preissing, B. Hoffmann, M. Dilly, C. Pfarrer, C. G. Ozalp, C. Caliskan, K. Seyrek-Intas, and G. Schuler. 2012. Investigations into the mechanisms controlling parturition in cattle. Reproduction 144:279–292.

- Silva, P. R. B., A. R. Dresch, K.S. Machado, J. G. N. Moraes, K. Lobeck-Luchterhand, T. K. Nishimura, M. A. Ferreira, M. I. Endres, and R. C. Chebel. 2014. Prepartum stocking density: Effects on metabolic, health, reproductive, and productive responses. J. Dairy Sci. 97:5521–5532

- Silva, P. R. B., K. M. Lobeck-Luchterhand, R. L. A. Cerri, D. M. Haines, M. A. Ballou, M. I. Endres, and R. C. Chebel. 2016. Effects of prepartum stocking density on innate and adaptive leukocyte responses and serum and hair cortisol concentrations. Vet. Immunol. Immunopathol. 169:39–46.

- Simontacchi, C., T. Perez de Altamirano, L. Marinelli, R. Angeletti, and G. Gabai. 2005. Plasma steroid veriations in bull calves repeatedly treated with testosterone, nortestosterone and oestradiol administered alone or in combination. Vet. Res. Communication 28:467-477.

- Singh, S.S., W. R. Ward, J. W. Lautenbach, J. W. Hughes, and R. D. Murray. 1993. Behaviour of first lactation and adult dairy cows while housed and at pasture and its relationship with sole lesions. Vet. Rec. 133:469-474.

- Sirinathsinghji, D. J. S., and I. H. Mills. 1983. Effect of human pituitary luteinizing hormone administration on plasma levels of dehydroepiandrosterone, androstenediol and their sulphates and testosterone in women with secondary amenorrhoea. J. Endocrinol. 98:201–10.

- Sprecher, D. J., D. E. Hostetler, and J. B. Kaneene. 1997. A lameness scoring system that uses posture and gait to predict dairy cattle reproductive performance. Theriogenology 47, 1179–1187.

- Strauss J. F. 3rd, F. Martinez, and M. Kiriakidou. 1996. Placental steroid hormone synthesis: unique features and unanswered questions. Biol. Reprod. 54:303–11.

- Tamanini, C., N. Giordano, F. Chiesa, and E. Seren. 1983. Plasma cortisol variations induced in the stallion by mating. Acta. Endocrinol. 102:447–50.

- Theorell, T. 2009. Anabolism and catabolism. In: Sonnentag, S., Ganster, D.C., Perrewe, P.L. (Eds.), Current Perspectives on Job-Stress Recovery (Research in Occupational Stress and Well Being), vol. 7:249-276.

Table 1. Ingredients and chemical composition of the rations.

Composition	TMR Dry period	TMR Lactation
Ingredients (% of DM)		
Grass hay[1]	71.0	48.6
Corn ground fine	-	20.0
Sorghum grain meal	-	16.5
Soybean meal	-	7.9
Molasses	-	0.5
Concentrate mix [2]	29.0	-
Vitamins and minerals [3]	-	1.7
Chemical composition (% of DM)		
Crude protein	12.4	14.1
aNDFom	44.7	33.5
ADF	31.5	19.9
ADL	5.8	4.1
Starch	11.1	23.7
Ether extract	3.3	3.5
Ash	5.6	6.7
NEl (Mcal/Kg of DM)	1.5	1.7

[1]Grass hay chemical composition on a dry matter basis was: 8.9% crude protein, 54% aNDFom, 39.9% ADF, 7.5% ADL, 8.8% ash.

[2] Concentrate mix: 48% corn meal, 20% soybean meal, 15% wheat bran, 10% beet pulp, 5% sunflowers meal, 2% mineral mix (4% Ca, 6% P, 4% Na, 10% Mg, 2000 mg/Kg of Zn, 1500 mg/Kg of Fe, 1000 mg/Kg of Mn, 175 mg/Kg of Cu, 150 mg/Kg I, 30 mg/Kg of Se, ,2000000 IU/Kg of vitamin A, 60000 IU/Kg vitamin D3, and 10000 mg/Kg of vitamin E).

[3] The lactating cows vitamins and minerals supplement contained 1.4% Ca, 8,3% P, 16 % Na, 5.5 % Mg, 4000 mg/Kg of Zn, 4000 mg/Kg of Mn, 400 mg/Kg of Cu, 400 mg/Kg I, 40 mg/Kg of Se, 20 mg/Kg of Co,1200000 IU/Kg of vitamin A, 200000 IU/Kg of vitamin D3, and 1000 mg/Kg of vitamin E.

Table 2. Mean ruminating period (total minutes/d) and mean activity (step/h) in response to treatment over the transition period: Week -4 is the pre experimental period, wk -3 to -1 treatment period, wk 1 -2 housed in bedded packed area, wk 3 – 4 housed in free-stall barn.

	Rumination time				Activity			
Weeks before and after calving	Control	OS	SEM	*P*-value	Control	OS	SEM	*P*-value
-4	568.0	564.2	8.1	0.67	75.5	82.5	3.0	0.18
-3	562.0	542.3	8.9	0.21	75.0	109.2	4.7	<0.001
-2	550.7	551.4	9.3	0.98	73.5	109.4	4.6	<0.001
-1	525.1	512.3	12.8	0.58	79.7	113.2	5.2	<0.01
1	489.2	478.3	11.8	0.59	102.9	102.1	5.8	0.85
2	590.9	608.4	9.9	0.28	83.8	90.4	4.1	0.54
3	557.4	572.9	11.0	0.07	81.7	88.4	3.7	0.21
4	554.5	577.0	10.8	0.31	82.7	91.6	4.1	0.29

Table 3. Mean lying period (minutes/d) and mean lying bouts (bouts/d) in response to treatment over the transition period: Week -4 is the pre experimental period, wk -3 to -1 treatment period, wk 1 -2 housed in bedded packed area, wk 3 – 4 housed in free-stall barn.

Weeks before and after calving	Mean Lying period				Mean lying bouts			
	Control	OS	SEM	*P*	Control	OS	SEM	*P*
-4	659.1	672.5	10.9	0.55	14.4	14.9	0.4	0.66
-3	660.7	670.1	12.9	0.87	14.2	16.1	0.5	0.09
-2	672.2	659.9	19.9	0.54	14.3	16.0	0.5	0.2
-1	643.1	630.6	16.4	0.41	15.1	16.6	0.6	0.32
1	683.9	688.1	19.7	0.81	16.6	17.7	0.5	0.42
2	620.0	667.2	18.6	0.41	13.7	14.7	0.5	0.27
3	621.0	607.2	19.3	0.38	13.5	13.6	0.4	0.3
4	624.5	605.7	19.8	0.33	12.7	12.2	0.6	0.42

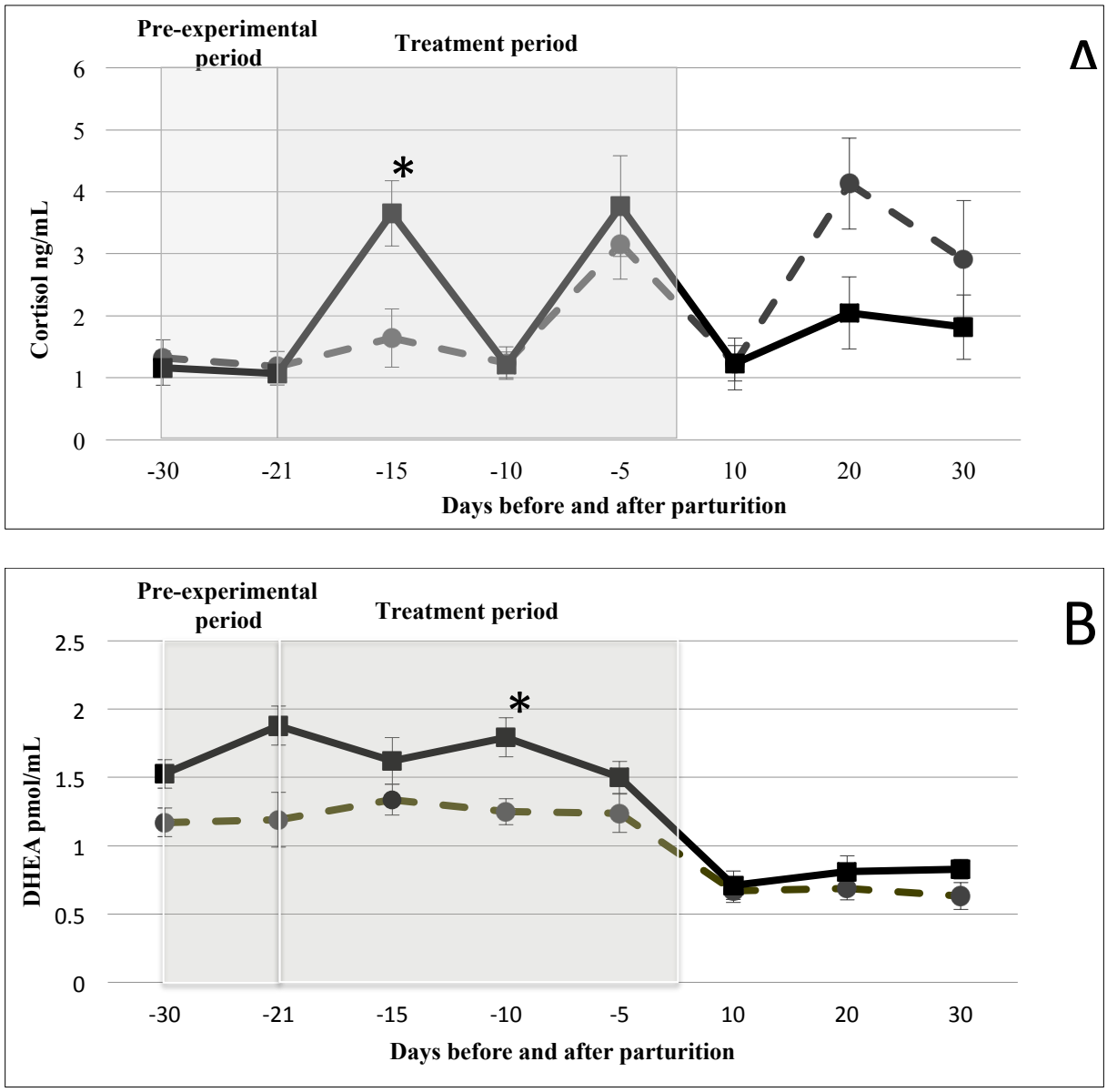
Table 4. Mean ECM yield in response to treatment over the transition period.

Weeks after calving	Control	OS	SEM	*P*-value
1	24.2	21.5	1.3	0.46
2	34.8	32.1	1.6	0.53
3	36.6	33.9	1.5	0.77
4	38.2	36.9	1.4	0.65

Figure captions

Figure 1. Plasma cortisol and DHEA concentrations in CTR (●) and OS (■) group over the transition period.

The asterisk indicates a statistically significant difference between CTR and OS ($P<$ 0.05) group. Values are mean ± SEM.



4.2. Study II

NOTE: This is a pre-refereeing version of a work submitted for publication to Animal Feed Science and Technology journal. Changes resulting by the reviewing process will not be reflected in this document.

Immunomodulant feed supplement to support dairy cows health and milk quality evaluated in Parmigiano Reggiano cheese production.

L.M.E. Mammi[a*], A. Palmonari[a], M. Fustini[a], D. Cavallini[a], G. Canestrari[a], J. D. Chapman[b], D.J. McLean[b] and A. Formigoni[a].

[a] Department of Veterinary Medicine, Università di Bologna, Bologna, Italy

[b] Phibro Animal Health Corporation, Quincy, IL 62305, USA

ABSTRACT

The effects of an immunomodulant feed supplement (OmniGen-AF®) were evaluated on cow health and composition and quality of milk produced for Parmigiano Reggiano cheese. One hundred-ninety primiparous and multiparous Holstein and crossbred dairy cows, were randomly assigned to either a control (CTR, n=95) or a group fed 55g/h/d of the supplement (TRT, n=95), from dry off through 150 days in milk (DIM). Individual milk yield (MY) was recorded daily, and individual milk quality was analyzed monthly. Casein content in the milk of primiparous TRT cows increased after 90 DIM and in general, milk cheese-making properties were enhanced from cows fed the supplement. Daily feeding of the supplement did not produce any negative effect on long ripened hard cheese production and quality. All health events were recorded. TRT cows had fewer health related events (-21% compared to CTR group). Multiparous TRT cows tended to have a lower somatic cell score (SCS) in the

first 60 DIM than CTR (-0.6 pts). The incidence of clinical mastitis was observed to be lower in the TRT Holsteins cows than CTR (4 vs 11 cases). The supplemented cows had a lower culling rate within 60 DIM (1% TRT vs 7.4% CTR) and time of culling (DIM) occurred later in TRT cows compared to CTRs (102.6 and 57 DIM for TRT and CTR, respectively). These results suggest that cow health and milk quality can be improved through an appropriate nutritional strategy and the use of an immunomodulator supplement like OmniGen-AF®. This combined nutritional strategy could have important implications for strictly regulated products, like Parmigiano Reggiano cheese or other milk products from organic certified farms. This strategy may provide management options when animal health and welfare control is fundamental to ensure high quality products and could satisfy consumer's expectations from these kinds of agricultural food chains.

INTRODUCTION

Impact of diseases such as mastitis, ketosis, metritis and retained fetal membranes on cow health, milk production and milk quality has been widely demonstrated. In particular, high somatic cell count (>300.000 cells/ml) is related to a lower production and lower milk quality for cheese production, due to the degradation of caseins operated by the proteases secreted by the somatic cells (Gröhn et al., 2004; Larsen et al., 2004; Barbano et al., 1991). The reduction in casein content affects cheese yield as well as the ripening attitude of cheese (Marino et al., 2005). This aspect is particularly important for cheeses like Parmigiano Reggiano and other Protected Designation of Origin (PDO) products that require a long period for ripening. The breakdown of caseins by somatic cell proteases is greatly reduced by the cooling temperature (Barbano et al., 1991), but Parmigiano Reggiano cheese is made using raw unpastuerized milk stored at a temperature not lower than 18 °C until delivery to

the cheese factory. This step must occur within 4 hours from milking, and cheese is consumed after 18-24 months of ripening. So good animal health and high quality milk is essential. Moreover, the entire production chain is strictly regulated: animal origin, feeding and herd management, additives and milk processing must be in compliance with the specific regulation, listed in the official rules of the Parmigiano Reggiano cheese production (Consorzio del Formaggio Parmigiano Reggiano, 2011). Rations are based on fresh forages and/or dry hay, while silages are not allowed. TMR is frequently prepared without adding water in the mixer wagon, in order to avoid unwanted fermentation (Fustini et al., 2016). Considering that forage : concentrate ratio in the diet must be 50 : 50 at least, good quality forages are necessary to meet the energy requirements of animals and milk quality (Fustini et al., 2017). Because of this, dry matter intake needs to be improved. This requirement could be achieved by selecting highly digestible forages, thus presenting a low amount of indigestible NDF, or reducing the particle size of the diet (Palmonari et al., 2014; Palmonari et al., 2016; Bonfante et al., 2016).

High incidence of pathologies in farms necessarily leads to the use of drugs and consequently, high amounts of milk are wasted due the presence of residues.

Consumer's awareness and concerns about animal health and welfare is increasing and, likewise, the pressure to reduce of the use of antimicrobials in farm animals is increasing and in particular in dairy cattle producing these kinds of products. The concern of antimicrobial resistance in humans is a reoccurring issue and in 2015 the European Commission launched an action plan against animal resistance recommending the importance of an appropriate use of antimicrobials in veterinary medicine (European Commission, 2011).

For all these reasons, in order to reduce disease prevalence in the herds it becomes essential to improve animal capability to resist different stressors including pathogens.

The innate immune system represents the first barrier against incoming pathogens (Janeway and Medzhitov, 2002) and its efficiency is therefore essential for the maintenance of animal's health. For example, the activity of udder leucocyte populations plays a pivotal role determining the evolution of intramammary infections (Sordillo and Streicher, 2002). The impairment of the immune system is therefore hazardous for cow performance and production and this is particularly evident during the peripartum period where cows experience a strong reduction of immune efficiency (Sordillo and Streicher, 2002). Thus, in addition to accurate herd management practices, it is important to supply the adequate nutrients, in order to support, together with milk production and pregnancy, the immune system (Ingvartsen and Moyes, 2013). Therefore, the development of efficient immunomodulatory strategies, safe and effective, is strongly recommended to support animal health and high quality productions (Sordillo and Streicher, 2002).

Some authors have recently reported the ability of a natural feed supplement (OmniGen-AF®) to increase leucocyte activity of supplemented dairy heifers, both for a long (Ryman et al., 2013) or a short period (Nace et al., 2014). OmniGen-AF® is a patented proprietary blend of ingredients demonstrated to support immune function in dairy cattle and other species (Phibro Animal Health Corporation, Quincy, IL, USA). Briefly, it contains a mixture of silicon dioxide, calcium aluminosilicate, sodium aluminosilicate, brewers dehydrated yeast, mineral oil, calcium carbonate, rice hulls, niacin supplement, biotin, d-calcium pantothenate, vitamin B-12 supplement, choline chloride, thiamine mononitrate, pyridoxine hydrochloride, riboflavin-5-phosphate and folic acid. It has been demonstrated that OmniGen-AF® can activate the innate immune system through the up-regulation of L-selectin (CD62L) mRNA expression (Nace et al., 2014; Ryman et al., 2013) and other neutrophil genes (Wang et al., 2009) compared to non-supplemented controls. These effects are potentially exerted by the

additive through the interactions between PAMP (pathogen-associated molecular patterns), contained by yeasts and fungal organisms, and toll-like receptors of animals' gastrointestinal tract (Wang et al., 2007). Toll-like receptors are involved in microbe recognition and signal transduction activation to induce the expression of L-selectin and secretion of inflammatory chemokines (Iwasaki and Medzhitov, 2004). Ultimately, this sequence of events can contribute to the modulation of the adaptive immune response (Janeway and Medzhitov, 2002). As reported in these publications (Nace et al., 2014; Ryman et al., 2013), only heifers were used and no results are available about multiparous cows and involuntary culling. In addition, Ryman and co-workers (2013) evaluated only blood parameters while Nace (2014) reported that their evaluation about health and milk quality was not sufficient due to the number of animals used in the project.

Therefore, the aim of our research was to evaluate the capability of this complementary feed supplement to reduce the incidence of pathologies, culling rate and somatic cell score, while maintaining milk production and quality, of primiparous and multiparous cows in a farm that produced milk for Parmigiano Reggiano cheese.

METERIALS AND METHODS

Animals, housing and feeding

The experimental procedures were approved by the Scientific Ethical Committee for animal experimentation of Bologna University, in accordance with the EU Directive 2010/63/EU for animal experiments.

The experiment was conducted between November 2013 and September 2014, in a Parmigiano Reggiano dairy farm, located in the Po Valley region of Northern Italy. One hundred and ninety Holstein and crossbred cows (Italian Holstein x Montbeliarde x Swedish Red Cattle) with expected calving dates between December 2013 and

March 2014, were used in the study. Cows were paired by parity, breed, expected calving date, body condition score (BCS), somatic cell count (SCC) assessed one week before dry-off and previous milk yield of multiparous cows (Table 1).

Sixty days before expected calving, cows were randomly assigned to either control (CTR, n=95), or treatment group (TRT, n=95). Cows assigned to TRT were fed 55 g/cow per day of the supplement (OmniGen-AF®) from dry-off through 150 DIM. A single batch of supplement was used throughout the entire trial. The supplement was mixed with wheat grain and then added to the mixer wagon in order to guarantee an optimal distribution into the TMR.

From dry off to 150 DIM, cows moved through different boxes: far-off dry, close-up dry (-21 days to calving), fresh (1-10 DIM) and lactating (10-150 DIM) cows. Each box was designed to house two CTR and two TRT groups. In order to avoid any possible overstocking issues (Fustini et al., 2017), the experimental pens for far-off dry, close-up dry and lactating cows (10-150 DIM) were organized to house up to 55 animals. In particular, the four pens for lactating animals were designed to have 75cm width headlocks, and stalls with 260cm length and 130cm width. Pens (n= 4, 2 TRT and 2 CTR) for fresh cows (1-10 DIM) were organized to host 15 animals each. Pens for far-off (n = 4) and close-up dry (n = 4) cows were able to house up to 50 animals. Headlock width was 75cm, while the available space for exercise area and resting area (straw bedded-pack) was 10+8m^2 per cow.

All diets were fed to the CTR and TRT groups as total mixed rations twice a day and the composition of those diets is shown in Table 2. Diets were balanced using software based on CNCPS model (DinamilkTM, v.5). All diets were formulated with feedstuffs and other ingredients as approved by Parmigiano Reggiano feeding regulation (Consorzio del Formaggio Parmigiano Reggiano, 2011). The supplement was fed at 55 g/cow per day and was added on-farm to each respective TRT dry and

lactating cow group TMR. Cows BCS were recorded at dry-off, mid-dry, at calving and every 4 weeks thereafter to 150 DIM. BCS was assessed according to Edmonson method from 1 to 5 (Edmonson et al., 1989).

Cases of mastitis, ketosis, metritis, retained fetal membranes (RFM) and displaced abomasum (DA) were diagnosed by the on-sight veterinarian and recorded daily. Definition of new clinical cases and calculation of incidence rate were made according to the method described by Kelton (Kelton et al., 1998). Reasons and time of culling were recorded on all cows that left the trial.

Feed and milk samples

Feed and TMR samples were collected and analyzed monthly using Near Infrared System (NIRS). Every three months, a TMR sample was analyzed by wet chemistry for the determination of: moisture, crude protein (AOAC, 1990; method 976.06 and 984.13), starch, aNDFom-NDF (Mertens, 2002), with addition of sodium sulfite, ADF and ADL (AOAC, 1990; method 973.18), fat, and ash after 4 h combustion in a muffle furnace 550°C (Vulcan 3-550, Dentsply Neytech, Burlington, NJ). Supplement was analyzed, as well, by wet chemistry at the beginning of the trial, in order to determine its composition.

Feed orts from the CTR and TRT groups were recorded every two days and used to calculate daily average dry matter intake of each pen, by deducting the two days orts from the two days feed offered.

Lactating cows were milked twice a day and individual milk production was recorded daily by Afimilk system (Kibbutz Afikim, Israel) in the milking parlour.

In order to detect any possible effect of OmniGen-AF® on cheese production and quality, milk produced by the TRT and CTR groups was processed separately.

Milk samples were collected from all the multiparous cows one week prior to dry-off, to check the right balance among groups. Individual milk was then sampled at calving, and every 4 weeks thereafter from 30 to 150 DIM. Milk samples were immediately refrigerated at 4°C, and analyzed within 12 hours by a laboratory specialized in milk used for the manufacture of Parmigiano Reggiano cheese (Artest S.P.A., Modena, Italy). Every sample was analyzed for fat, casein, protein and lactose percentage, somatic cell count (cell/ml), pH, titrable acidity (°SH) and coagulation properties: rennet coagulation time and aptitude. Rennet coagulation time and aptitude were evaluated by lactodynamographic analysis (LDG) through the use of formagraph (McMahon and Brown, 1982). Rennet coagulation time (LDG, r') represents the minutes between the addition of rennet in the milk and the widening of the baselines in the graph generated from the analysis (McMahon and Brown, 1982; Bittante et al., 2015), while, LDG type indicates the rennet coagulation aptitude: the combination between time (r'), curd-firming rate and curd firmness. In Parmigiano Reggiano cheese production, types A, B and C are considered to have good coagulation aptitude, D suboptimal while E and F defect clotting ability (Zannoni and Annibaldi, 1981).

The Consortium of Parmigiano Reggiano evaluated all the cheese produced during the trial after 12 months of ripening, following Parmigiano Reggiano regulation.

Statistical analysis

The project design was a completely randomized model, with one dietary treatment (TRT) and one control diet (CTR).

Total milk production, disease incidence, culling rate and relative risk (RR) of culling before 60 DIM were evaluated on all cows included in the trial (n=190, 95 cows/ group). Forty-two cows out of 190 were excluded by the analysis of individual milk production and quality data: animals culled before the end of the trial (n=19), cows with missing values and animals with SCC in milk > 300,000 (n=23). Thus, a total of 148 cows (74 cow/group) were included in the analysis of individual milk data.

Normal distribution of the data was first tested by Shapiro Wilk test and somatic cell count data were transformed into somatic cell score (SCS) according to Shook and Schutz method (Shook and Schutz, 1994). Daily milk production, composition and quality data (fat, casein, protein, LDG time, SCS) were subjected to mixed model procedure with repeated measures with cow to serve as experimental unit, using the model reported in equation 1:

$$Y_{ijkl} = \mu + T_i + L_j + B_k + D_l + (T \times D)_{il} + \varepsilon_{ijkl} \qquad [1]$$

where Y_{ijkl} is the dependent variable; μ is the overall mean; T_i is the effect of treatment (Omnigen-AF or control); L_j is the effect of the number of lactation (j=1 or > 2); B_k is the effect of breed (k=Holstein or crossbreed); D_l is the effect of days in milk (l= 11, 12…, 149, 150); $(T \times D)_{il}$ is the effect of treatment x days interaction and ε_{ijkl} is the random residual error. Treatment, lactation, breed, days in milk and treatment by day interaction were considered as fixed effects. Cow and pen were included as random effects while terms of the repeated structure were days with cow as subject. Given that cows were group fed, individual dry matter intake was not available, therefore DMI data were not subjected to statistical analysis.

Total individual milk production and days in milk at culling were analyzed by the same mixed model excluding the time factor and the repetition over time structure.

Milk coagulation aptitude, health events and culling rates were tested by Fisher's exact test. For all tests, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

All data were processed using JMP® Pro (v. 12.0.1, 2015, SAS Institute, Cary, NC, USA).

RESULTS

Feed analysis and dry matter intake

Analytical characteristics of the supplement resulted as following: ash 800.3, crude protein (N*6.25) 28.2, crude fibre 25.3, aNDF0m-NDF 45.1, crude fat 1.0, Ca 20.0, P 0.8, Na 4.2, Mg 3.8, g/kg as fed. Chemical composition of the rations (Table 3), as well as average group DMI, was not influenced by the treatment neither in the dry nor in lactating cows.

Individual dry matter intake was not statistically analyzed, but the average group dry matter intake was similar (11.2 ± 0.9 and 11.5 ± 0.6 kg for CTR and TRT dry cows respectively; 22.3 ± 1.0 and 22.5 ± 0.8 kg for CTR and TRT lactating cows respectively).

Milk production, composition and quality

Overall, no significant differences (P>0.05) were detected in milk yield through 150 DIM (40.78 kg vs 41.97 kg, for CTR and TRT group, respectively) and no significant interactions were detected between parity, breed and dietary treatment (Table 4). Milk composition was maintained and it did not differ substantially between the groups (Table 5). Fat, protein and lactose content remained similar.

As shown in Figure 1, a significant treatment x time interaction was detected in casein content (g/kg) of milk produced by first calving heifers at 120 and 150 DIM (g/kg, TRT=27.5 vs CTR=26.7, P<0.05 and TRT=28.3 vs CTR=27.4, P<0.05). This

increasing trend is appreciable at 90 DIM (g/kg, TRT=26.7 vs CTR=26.0, P=0.07), while no differences were observed for previous time points (30, 60 DIM). Similar situation was observed on clotting time (LDG, r'. Figure 2) for multiparous cows at 150 DIM (min., TRT=20.9 vs CTR= 23.1, P<0.05).

Milk coagulation aptitude was improved in the TRT group, as reported in Table 6: the number of milk samples with defect clotting aptitude (LDG type E and F) was significantly lower (P<0.01) in milk collected from the TRT cows compared to the CTR (111 vs 146, respectively). Opposite situation was observed in milk samples with suboptimal aptitude (LDG type D), which were less in CTR group (47 vs 75 % for CTR and TRT group, respectively; P<0.01).

Due to the exclusion of unhealthy or culled cows, also the SCS were similar among groups, with a slight improvement appreciable in the TRT group.

Cows fed the TRT diet had numerically lower somatic cell scores (SCS) than CTR cows throughout the trial (TRT, SCS=2.68; CTR, SCS=2.92) and tended (P<0.10) to be on average 0.6 points lower for the multiparous TRT cows during the first 60 DIM (Figure 3).

Throughout the trial, cows fed the TRT diet had a significant (P<0.05) reduction of the milk samples with SCC over 300,000 cells/ml compared to the CTR (123 samples, TRT vs 147 samples, CTR).

BCS, health events and culling rates

The CTR and TRT groups were balanced for BCS at dry off and no differences were detected between the groups over the length of the study.

Incidence of infectious and metabolic diseases for the Holstein and Crossbred cows are reported in Table 7. Holstein TRT cows had fewer cases of clinical mastitis than Holstein cows in CTR group (4 cases vs 11 cases; P<0.05). No difference was

detected among Crossbred cows for mastitis. Incidence rates of the other recorded diseases were not different between the CTR and TRT cows. All clinical mastitis were diagnosed by the veterinarian and milk was sampled for microbiological analysis. The majority of these cases were caused by *Streptococcus uberis* (5 cases in TRT group and 7 cases in CTR group) while a minor part of that was caused by *Staphyloccocus aureus* (3 cases in TRT and 4 cases in CRT group) and *Eschirichia Coli*, (1 cases in TRT group and 2 cases in CRT group) with no difference between the groups in the causing agent. In 3 cases no specific pathogens were detected due to milk samples contamination. Among the animals with mastitis, 6 were culled due to the consequences of the disease: 4 in control group and 2 in the treated.

A discrete amount of animals (19) was culled for health issues before 150 DIM (CTR, n=12; TRT, n=7). Culling rate at 60 DIM was significantly lower ($P<0.05$) in TRT cow groups (1.0%) compared to CTR (7.4%). Holsteins (n=8) were the only breed culled before 60 DIM. Considering all breeds, CTR cows were culled earlier in milk than TRT (57.3 and 102.6 DIM for CTR and TRT group, respectively; $P<0.05$) and the risk of being culled before 60 days of lactation was 7 times higher (RR = 7, $P<0.05$) for cows fed the CTR diet than those on the TRT diets.

DISCUSSION

The impact of disease and high SCC on milk production has been widely demonstrated (Fourichon et al., 1999). A recent study evaluated milk loss relative to SCC and reported that as SCC increased from 200 to 2000 ($x10^3$ cell/ml), the milk production decrease ranged from 0.35 to 4.7 kg/day (Hand et al., 2012). Other authors reported that milk yield can start to decrease several weeks before mastitis becomes evident and will reach the nadir only after clinical diagnosis (Gröhn et al., 2004).

The healthier status of cows in the TRT group supports the lower culling rate in this group. Our results agree with those reported in a study using the same feed supplement in dairy heifers (Eubanks et al., 2012). The authors compared milk production, SCC and the expression of genes associated with the function of circulating immune cells of 83 heifers during transition. In this study, after two weeks of lactation, supplemented heifers produced on average 4 kg/day more milk than control, had a lower prevalence on mastitis (4 vs 13 %) and lower SCC (180 vs 711 $x10^3$ cell/ml). However, an increase in milk production is not always reported with the use of this supplement (Nace et al., 2014). The reduction of somatic cells in milk observed in this experiment could also explain the difference in casein content between TRT and CTR primiparous cows (Figure 1), and the improved coagulation properties of milk from the TRT group that had shorter clotting time (LDG, r', Figure 2). Even the fewer samples with bad (E and F) clotting aptitude (30.0 vs 39.9 %, $P<0.01$, Table 6) could be related to SCC reduction. These characteristics are considered to be very important to obtain a good production of Parmigiano Reggiano cheese.

Somatic cells have detrimental effects on these cheese making properties and final cheese yield (Summer et al., 2015) because of the enzymatic degradation of proteins by proteases (Larsen et al., 2004). Milk type E and F indeed, are characterized by a low reactivity to rennet that is typical of milk with high somatic cell count (Bittante et al., 2012). This aspect is particularly important when milk is processed for PDO cheese production (Bittante et al., 2011) and according to the suggestions for good Parmigiano Reggiano cheese production, tank milk must have low SCC (<300 000 cell/ml) and high fat and casein content, in order to obtain first quality long ripened cheese.

The immune system plays a key role in the ability of animals to resist pathologies. The effectiveness of the immune system is partially related to adequate supply of macro and micronutrients that exert their activity through their antioxidant capabilities (Sordillo, 2016). During the dry and transition period, a nutritional imbalance like impaired energy and availability (Formigoni et al., 2003) and the lack of certain vitamins and minerals can impair host immune defense mechanisms and will lead to an associated higher risk of diseases (Sordillo, 2016; Zhao et al., 2015; Ingvartsen and Moyes, 2013; Formigoni et al., 2011). Although the direct study of the activity of immune cells could give more precise information, the evaluation of the incidence of pathologies is a useful approach to understand immune competence of animals (Sordillo, 2016).

In the study presented here, cows in the TRT group reported a lower number of total clinical cases, and in particular, Holstein TRT cows had fewer clinical cases of mastitis than Holstein controls (11 and 4 in CTR and TRT group, respectively; $P<0.05$). In contrast, no significant difference was found between groups among Crossbred animals. Similar results were reported by others investigating OmniGen-AF® supplementation and incidence of health issues with reports of fewer cases of diseases and a tendency for less new mastitis in supplemented cows (Nace et al., 2014). The efficiency of this additive to reduce new mammary infections was first reported by Rowson (Rowson et al., 2011). In this study the authors described a significant reduction in pathogen DNA concentration in mammary glands of mice fed with the supplement for two weeks prior to infection with bovine-origin bacterial isolates. In addition, they reported increased mRNA expression of myeloperoxidase and major histocompatibility complex in mice fed the supplement indicating a more robust inflammatory response and antigen presentation.

In this study with dry and lactating dairy cows, OmniGen-AF® supplementation also reduced culling rates within 60 DIM (1 vs 7.4 %, $P<0.05$) and extended cows production life. Interestingly, this is the first study to report results about improved involuntary culling with OmniGen-AF® supplementation. It is important to note that all culled animals were Holstein cows while no Crossbred cow was culled before the end of the trial (8 vs 0, $P<0.05$). Our results agree with Heins data (Heins et al., 2006), who reported higher survival rates of Crossbreds compared to pure Holsteins, as well as better reproductive performance. These data suggest a higher disease susceptibility of the Holstein breed compared to Crossbreds and our results suggest a better effectiveness of OmniGen-AF® supplementation to Holstein animals.

Stronger evidence of the activity of OmniGen-AF® in optimizing the immune system could be gained by further research that combines transition cow performance with immunity or stress challenge and direct evaluation of metabolic status and protein markers or immune function.

ACKNOWLEDGMENTS

The authors express their appreciation to the Bonlatte dairy farm team (Castelfranco Emilia, Italy) for their contribution in the realization of the trial and to Phibro Animal Health Corporation for funding the study.

REFERENCES

- Barbano, D.M, Rasmussen, R.R., Lynch, J.M., 1991. Influence of milk somatic cell count and milk age on cheese yield1. J. Dairy Sci. 74, 369–388.
- Bittante, G., Cecchinato, A., Cologna, N., Penasa, M., Tiezzi, F. M., 2011. Factors affecting the incidence of first-quality wheels of Trentingrana cheese. J. Dairy Sci. 94, 3700–3707.
- Bittante, G., Cipolat-Gotet, C., Malchiodi, F., Sturaro, E., Tagliapietra, F., Schiavon, S., Cecchinato, A., 2015. Effect of dairy farming system, herd, season, parity, and days in milk on modeling of the coagulation, curd firming, and syneresis of bovine milk. J. Dairy Sci. 98, 2759–2774.
- Bittante, G., Penasa, M., Cecchinato, A., 2012. Invited review: Genetics and modeling of milk coagulation properties. J. Dairy Sci. 95, 6843–6870.
- Bonfante, E., Palmonari, A., Mammi, L.M.E., Canestrari, G., Fustini, M., Formigoni, A., 2016. Effects of a completely pelleted diet on growth performance in Holstein heifers. J. Dairy Sci. 99, 9724-9731.
- Consorzio del Formaggio Parmigiano Reggiano 2011. Specification of the Parmigiano Reggiano cheese. http://www.parmigianoreggiano.com/consortium/rules_regulation. (Accessed on 10 January 2016)
- Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G., 1989. A Body Condition Scoring Chart for Holstein Dairy Cows. J. Dairy Sci. 72, 68–78.
- Eubanks, V.J., Hurley, D.J., Ely, L.O., Kautz, F.M., Nickerson, S.C., Forsberg, N.E., Wang, Y.Q., Zanzalari, K., Chapman, J., 2012. Pre- and postpartum immunomodulatory effects of a dietary supplement on the immune system of dairy heifers. J. Dairy Sci. suppl. 2, 222.

- European Commission, 2011. Communication from the Commission to the European Parliament and the Council. Action plan against the rising threats from Antimicrobial Resistance.http://ec.europa.eu/dgs/health_foodsafety/docs/communication_amr_2011_748_en.pdf (Accessed on 1 February 2016).
- Formigoni, A., Fusaro, I., Giammarco, M., 2003. Il controllo del bilancio energetico per il contenimento delle patologie mammarie nella bovina ad inizio lattazione. Lar. Anim. Rev. 9, 15–19.
- Formigoni, A., Fustini, M., Archetti, L., Emanuele, S., Sniffen, C.J., Biagi, G., 2011. Effect of an organic source of copper, manganese, and zinc on dairy cattle productive performance, health status, and fertility. Anim. Feed Sci. Technol., 16, 191-198.
- Fourichon, C., Seegers, H., Bareille, N., Beaudeau. F., 1999. Effects of disease on milk production in the dairy cow: a review. Prevent. Vet. Med. 41, 1–35.
- Fustini, M., Heinrichs, A.J., Palmonari, A., Formigoni, A., 2016. CASE STUDY: Farm characteristics and total mixed ration particle size issues on Parmigiano Reggiano farms in northern Italy. Prof. Anim. Sci. 32, 869-873.
- Fustini, M., Galeati, G., Gabai, G., Mammi, L.M.E., Bucci, D., Baratta, M., Accorsi, P.A., Formigoni, A., 2017. Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion. J. Dairy Sci. 100, 620-628.
- Fustini, M., Palmonari, A., Canestrari, G., Bonfante, E., Mammi, L.M.E., Pacchioli, M.T., Sniffen, G.C.J., Grant, R.J., Cotanch, K.W., Formigoni, A., 2017. Effect of undigested neutral detergent fiber content of alfalfa hay on lactating dairy cows: feeding behavior, fiber digestibility, and lactation performance. J. Dairy Sci. 100, 4475–4483.

- Gröhn, Y.T., Wilson, D.J., González, R.N., Hertl, J.A., Schulte, H., Bennett, G., Schukken, Y.H., 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87, 3358–3374.
- Hand, K.J., Godkin, A., Kelton, D.F., 2012. Milk production and somatic cell counts: A cow-level analysis. J. Dairy Sci. 95, 1358–1362.
- Heins, B.J., Hansen, L.B., Seykora, A.J., 2006. Fertility and survival of pure Holsteins versus crossbreds of Holstein with Normande, Montbeliarde, and Scandinavian Red. J. Dairy Sci. 89, 4944–4951.
- Ingvartsen, K.L., Moyes, K., 2013. Nutrition, immune function and health of dairy cattle. Animal. 7, 112–122.
- Iwasaki, A., Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5, 987–995.
- Janeway, C.A., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197–216.
- Kelton, D.F., Lissemore, K.D., Martin, R.E., 1998. Recommendations for recording and calculating the incidence of selected clinical diseases of dairy cattle. J. Dairy Sci. 81, 2502–2509.
- Larsen, L.B., Rasmussen, M.D., Bjerring, M., Nielsen, J.H., 2004. Proteases and protein degradation in milk from cows infected with Streptococcus uberis. Int. Dairy J. 14, 899–907.
- Marino, R., Considine, T., Sevi, A., McSweeney, P.L.H., Kelly, A.L., 2005. Contribution of proteolytic activity associated with somatic cells in milk to cheese ripening. Int. Dairy J. 15, 1026–1033.
- McMahon, D.J., Brown, R.J., 1982. Evaluation of formagraph for comparing rennet solutions 1, 2. J. Dairy Sci. 65, 1639–1642.

- Nace, E.L., Nickerson, S.C., Kautz, F.M., Breidling, S., Wochele, D., Ely, L.O., Hurley, D.J., 2014. Modulation of innate immune function and phenotype in bred dairy heifers during the periparturient period induced by feeding an immunostimulant for 60 days prior to delivery. Vet. Immunol. Immunopathol. 161, 240–250.
- Palmonari, A., Fustini, M., Canestrari, G., Grilli, E., Formigoni, A., 2014. Influence of maturity on alfalfa hay nutritional fractions and indigestible fiber content. J. Dairy Sci. 97, 1–6.
- Palmonari, A., Gallo, A., Fustini, M., Canestrari, G., Masoero, F., Sniffen, C.J., Formigoni, A., 2016. Estimation of the indigestible fiber in different forage types. J. Anim. Sci. 94, 248-254.
- Rowson, A.D., Wang, Y.O., Aalseth, E., Forsberg, N.E., Puntenney, S.B., 2011. Effects of an immunomodulatory feed additive on the development of mastitis in a mouse infection model using four bovine-origin isolates. Animal. 5, 220–229.
- Ryman, V.E., Nickerson, S.C., Kautz, F.M., Hurley, D.J., Ely, L.O., Wang, Y.Q., Forsberg, N.E., 2013. Effect of dietary supplementation on the antimicrobial activity of blood leukocytes isolated from Holstein heifers. Res. Vet. Sci. 95, 969–974.
- Shook, G.E., Schutz, M.M., 1994. Selection on Somatic Cell Score to improve resistance to mastitis in the United States. J. Dairy Sci. 77, 648–658.
- Sordillo, L.M., 2016. Nutritional strategies to optimize dairy cattle immunity. J. Dairy Sci. 99, 1-16.
- Sordillo, L.M., Streicher, K.L., 2002. Mammary gland immunity and mastitis susceptibility. J. Mammary Gland Biol. Neoplas. 7, 135–146.

- Summer, A, Franceschi, P., Formaggioni, P., Malacarne, M., 2015. Influence of milk somatic cell content on Parmigiano-Reggiano cheese yield. J. Dairy Res. 82, 222–227.
- Wang, Y.Q., Puntenney, S.B., Burton, J.L., Forsberg, N.E., 2007. Ability of a commercial feed additive to modulate expression of innate immunity in sheep immunosuppressed with dexamethasone. Animal. 1, 945.
- Wang, Y.Q., Puntenney, S.B., Burton, J.L., Forsberg, N.E., 2009. Use of gene profiling to evaluate the effects of a feed additive on immune function in periparturient dairy cattle. J. Anim. Physiol. Anim. Nutr. 93:66–75.
- Zannoni, M., Annibaldi, S., 1981. Standardization of the renneting ability of milk by formagraph-I. Scienza e Tecnica Lattiero Casearia. 2, 79–94.
- Zhao, X.J., Li, Z.P., Wang, J.H., Xing, X.M., Wang, Z.Y., Wang, L., Wang, Z.H., 2015. Effects of chelated Zn/Cu/Mn on redox status, immune responses and hoof health in lactating Holstein cows. J. Vet. Sci. 16, 439.

Table 1. Description of control (CTR) and treatment (TRT) group composition: number of cows, breed, parity, body condition score (BCS), previous milk yield and somatic cell score (SCS) evaluated at dry off. Presented as average ± standard deviation.

	Group	
Measure	CTR	TRT
Animals, n	95	95
Primiparous	32	31
Multiparous	63	64
Holstein	61	63
Crossbred	34	32
Parity, n	2.1 ± 1.15	2.2 ± 1.18
BCS[1], points	3.5 ± 0.4	3.4 ± 0.3
Milk yield, kg/day[2]	32.8 ± 6.6	33.1 ± 5.3
SCS[3], points	4.7 ± 2.3	4.5 ± 2.0

[1] BCS: Body Condition Score (Edmonson et al., 1989).

[2] Previous lactation averaged milk yield of multiparous cows.

[3] Somatic Cell Score.

Table 2. Description of dietary ingredients of lactating and dry cows rations. TRT group was supplemented with 55 g/day per cow of OmniGen-AF®

	Stage of lactation		
Ingredient, kg (as fed)	Lactating	Far-off dry	Close up dry
Wheat straw	--	3	3
Grass hay (1st cut)	2.5	5	5
Alfalfa hay	7.5	--	--
Grass hay	--	At pleasure	At pleasure
Barley meal, fine	2	--	--
Wheat meal, fine	1.5	1.5	1.5
Corn	6	--	--
Corn meal	3	--	--
PAM[1]	3.5	--	--
Dry mix[2]	--	1	3
Enerfeed 4[3]	1	--	--
Nectar[4]	1	--	--
Water	--	3	--

[1] supplementary feeding for lactating cows (g/kg a.f): C.P 330, C.F 115, crude fat 38, ash 97, Na 6. Ingredients: soybean meal, sunflower meal, wheat bran, soy hulls, soybean whole, Ca carbonate, NaCl, Na-bicarb, MgOX, vit. premix (VitA = 120000 IU/kg, VitD = 8000 IU/kg, VitE = 400 mg/kg), micro minerals (Zn = 500 ppm, Mn = 500 ppm, Cu = 75 ppm, I = 45 ppm, Se = 5 ppm).

[2] supplementary feeding for dry cows (g/kg a.f): C.P 303, crude fat 37, C.F 0, ash 105. Ingredients: sunflower meal, soybean meal, wheat bran, corn gluten feed, soybean whole, barley, cane molasses, Ca carbonate, NaCl, Na-bicarb, MgOX, vit. premix (VitA = 30000 IU/kg, VitD = 6000 IU/kg, VitE = 1000 mg/kg, VitB1 = 15 mg/kg, VitB2 = 15 mg/kg, VITB6 = 11 mg/kg, VITB12 = 0.04 mg/kg), micro minerals (Zn = 1500 ppm, Mn = 1500 ppm, Cu = 250 ppm, I = 100 ppm, Se = 15 ppm).

[3] molasses (g/kg a.f): moisture 280 g/kg, sugars 240, C.P 75, crude fat 2, C.F 1, ash 90, Na 9.

[4] supplementary feeding for lactating cows (g/kg a.f): C.P 310, C.F 50, crude fat 103, ash 65, Na 2.6. Ingredients: soybean whole, soybean meal, glicerine, lineseed, Ca carbonate, NaCl, Na-bicarb, MgOX, vit. premix (VitA = 100000 IU/kg, VitD = 4000 IU/kg, VitE = 250 mg/kg, VitB1 = 10 mg/kg, VitB2 = 12 mg/kg, VITB6 = 18 mg/kg, VITB12 = 0.02 mg/kg, Nicot. Ac. = 6000 mg/kg, Coline = 2000 mg/kg, L-Carn. = 280 mg/kg), micro minerals (Zn = 150 ppm, Mn = 150 ppm, Cu = 25 ppm, I = 35 ppm, Se = 5 ppm).

Table 3. Averaged chemical composition (g/kg dry matter, DM) of lactating and dry cow rations fed to control (CTR) or supplemented cows (TRT).

	CTR[1]			TRT[2]		
Item, g/kg DM	Far-off dry	Close-up	Lactating	Far-off dry	Close-up	Lactating
Dry matter, g/kg	721	751	885	695	777	892
Crude Protein,	109	143	172	95	150	172
Starch	24	197	291	33	192	281
aNDFom-NDF[3]	610	402	269	66	397	267
ADF	358	204	205	373	204	211
ADL	66	40	35	67	38	37
E.E.	16	25	26	16	26	27
Ash	95	87	89	95	93	93

[1] CTR: control group.

[2] TRT: treatment group. Animals were supplemented with 55 g/day per cow of OmniGen-AF®.

[3] aNDFom-NDF: alpha-amylase treated NDF, ash corrected.

Table 4. Least square means of daily individual milk production (kg) and number of cows (n) of supplemented (TRT, n = 74) and control (CTR, n = 74) cows divided for parity and breed from 11 to 150 DIM.

	Cows, n		Milk, kg			
Parity	CTR[1]	TRT[2]	CTR[1]	TRT[2]	sem	P-value
Primiparous						
Holstein	23	21	35.2	36.2	0.89	0.63
Crossbred[3]	8	8	33.6	34.6	0.89	0.55
Multiparous						
Holstein	21	29	45.5	46.5	0.89	0.71
Crossbred[3]	22	16	43.9	44.9	0.89	0.53

[1] CTR: control group.

[2] TRT: treatment group. Animals were supplemented with 55 g/day per cow of OmniGen-AF®.

[3] Crossbred: Italian Holstein X (Swedish Red Cattle X Montbeliarde).

Table 5. Least square means of composition and characteristics of milk produced by supplemented (TRT, n = 74) and control (CTR, n = 74) groups, sampled at calving, and every 4 weeks thereafter from 30 to 150 DIM.

	Group			
Item	CTR[1]	TRT[2]	sem	P-value
Composition, g/kg				
Fat	28	28	0.09	0.97
Protein	34	34	0.03	0.91
Casein	26	26	0.02	0.53
Lactose	49	50	0.02	0.48
Titrable acidity, °SH	3.8	3.8	0.04	0.85
LDG[3], r', min	19.6	18.5	0.69	0.74
SCS[4], points	2.8	2.5	0.26	0.63

[1] CTR: control group.

[2] TRT: treatment group. Animals were supplemented with 55 g/day per cow of OmniGen-AF®.

[3]LDG: milk clotting time evaluated through lactodynamographic analysis.

[4] SCS: somatic cell score.

Table 6. Number (n) and percentage (%) of milk samples with good (A, B, C), suboptimal (D) and defect (E, F) coagulation aptitude, evaluated through lactodynamographic analysis (LDG). Individual milk was sampled at calving, and every 4 weeks thereafter from 30 to 150 DIM in both TRT and CTR groups (74 cows per group).

	Group			
LDG, type	CTR[1]	TRT[2]	sem	P-value
A, B				
n	173	184	4.33	0.87
%	47.3	49.7	1.18	0.64
C				
n	0	0	0	-
%	0	0	0	-
D				
n	47^{B}	75^{A}	1.41	< 0.01
%	12.8^{B}	20.3^{A}	0.77	< 0.01
E, F				
n	146^{A}	111^{B}	3.63	< 0.01
%	39.9^{A}	30^{B}	1.35	< 0.01

[A,B] Values within a row with different superscripts differ significantly at $P<0.01$.

[1] CTR: control group.

[2] TRT: treatment group. Animals were supplemented with 55 g/day per cow of OmniGen-AF®.

Table 7. Number of clinical cases recorded in control (CTR) and supplemented (TRT) cows, from calving to 150 DIM or culling, and percentage of animals culled within 60 DIM.

	Group			
Measure	CTR[1]	TRT[2]	sem	P-value
Holstein				
Animals, n	61	63	1.81	0.51
Ketosis	1	1	0.03	0.97
Clinical mastitis	11[a]	4[b]	0.42	< 0.05
Metritis	18	17	0.23	0.63
RFM[3]	8	7	0.11	0.74
Culled, %	7.4[a]	1.0[b]	0.25	< 0.05
Crossbred				
Animals, n	34	32	1.37	0.76
Ketosis	3	2	0.12	0.83
Clinical mastitis	5	5	0.34	0.96
Metritis	5	8	0.32	0.47
RFM[3]	3	3	0.28	0.90
Culled, %	0	0	0	-

[a,b] Values within a row with different superscripts differ significantly at $P<0.05$

[1] CTR: control group

[2] TRT: treatment group. Animals were supplemented with 55 g/day per cow of OmniGen-AF®

[3] RFM: retained fetal membranes.

Figure captions

Figure 1. Effect of feed supplementation during dry and lactation period on casein content (g/kg) of milk produced by primiparous supplemented (TRT) and control (CTR) cows from 11 to 150 DIM, expressed as average value (data point) and standard error (error bars). Casein content differed significantly at DIM 120 and 150. * = $P < 0.05$.

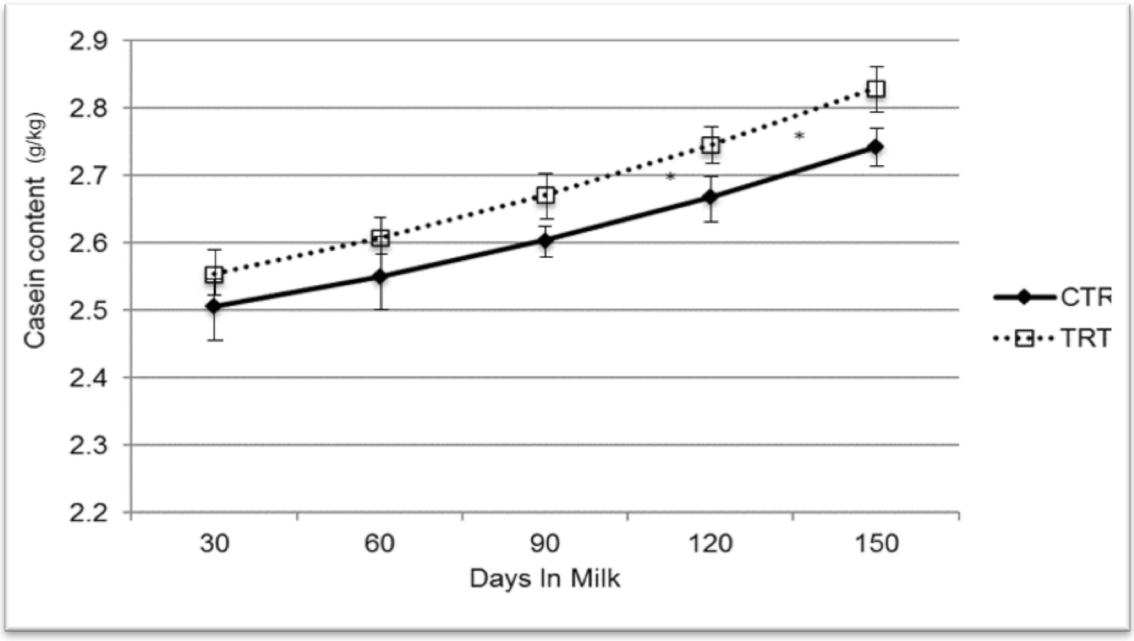


Figure 2. Effect of feed supplementation during dry and lactation period on clotting time (r', minutes) of milk produced by multiparous supplemented (TRT) and control (CTR) cows from 11 to 150 DIM, evaluated by lactodynamographic analysis (LDG), expressed as average value (data point) and standard error (error bars). Clotting time was significantly shorter for TRT multiparous cows at DIM 150.

* = $P < 0.05$.

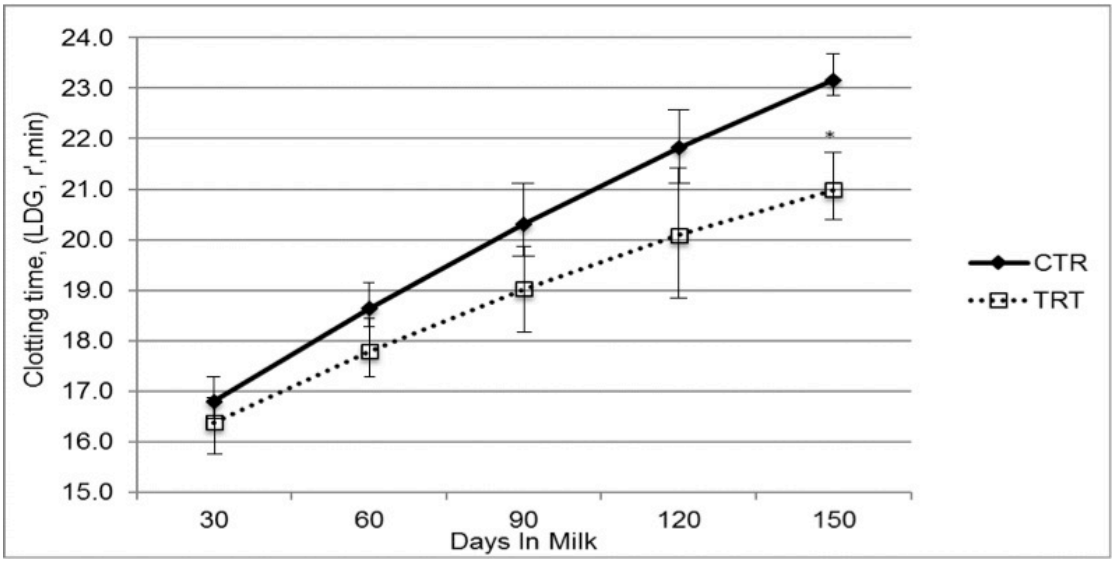
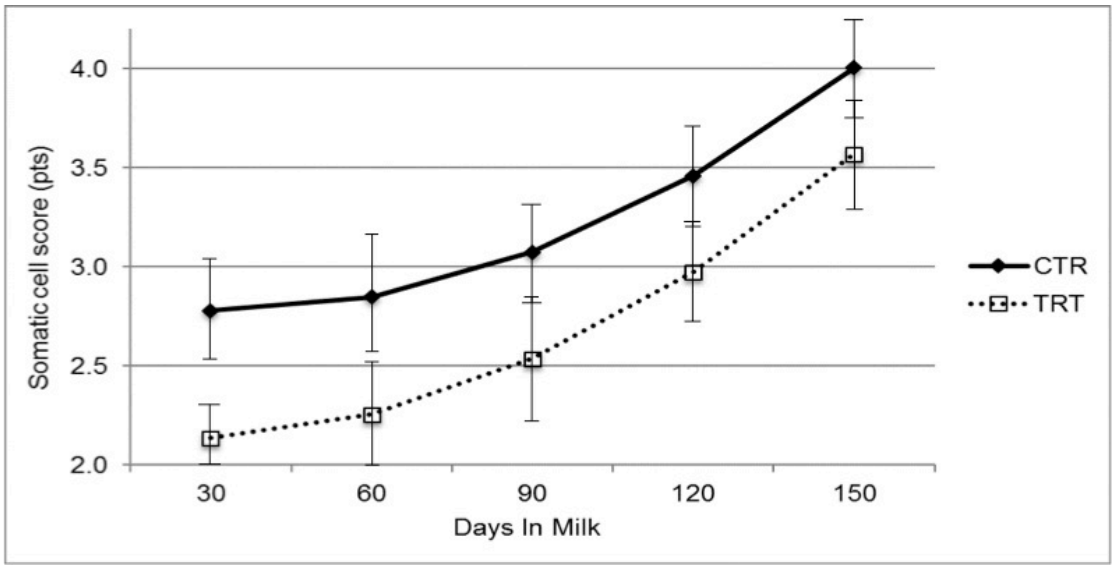


Figure 3. Effect of feed supplementation during dry and lactation period on somatic cell score (SCS) of multiparous supplemented (TRT) and control (CTR) cows from 11 to 150 DIM expressed as average value (data point) and standard error (error bars). SCS tended ($P < 0.1$) to be lower in multiparous TRT cows at 30 and 60 DIM.



4.3. Study III

Use of Monensin controlled release capsule in Parmigiano Reggiano cheese production

L.M.E. Mammi[*1], L. Grazia[†], A. Palmonari[*], G. Canestrari[*], A. Mordenti[*], M. Vecchi[‡], F. Archilei[‡], A. Formigoni[*]

[*] Department of Veterinary Medical Sciences, and

[†] Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy.

[‡] Elanco Italia SpA, Sesto Fiorentino (FI), Italy

ABSTRACT

In this study we investigated the effects of monensin controlled-release capsule (**CRC**) (Kexxtone, Eli Lilly and Company Ltd, United Kingdom) preventative ketosis treatment on traditional cheese making process as well as the final characteristics of Parmigiano Reggiano (**PR**) cheese.

The use of this prevention product to reduce the incidence of ketosis in transition dairy cows was approved by the European Medicines Agency in 2013. There are no previous experiences available concerning the effects of this treatment on prolonged ripening cheeses such as PR. In PR cheese production, feed, feed additives and cow treatments are strictly regulated in order to avoid any possible interference with traditional manufacturing processes.

For these reasons, in one farm where all milk is used for PR cheese production, monensin CRC was administered to 33 cows, 21 days before calving in the monensin

treated group (**TRT**), while untreated cows with similar breed and parity characteristics constituted the control group (**CTR**).

For 20 weeks, milk obtained from each group and whey starter were separately managed and transported in the cheese factory, where 2 cheese wheels per group were produced daily, making 552 PR cheese wheels in total. Morning bulk tank milk composition, cheesemaking properties and whey starter fermentation activities were analyzed twice a week. Every aspect of the cheesmaking process was recorded and the resulting cheese was evaluated after 36 hours, 6, 12 and 18 months from production for yield, texture defects, composition and fatty acids profile. Milk from the two groups differed for somatic cell content (TRT 3.04 *vs* CTR 4.06, Somatic Cell Score p.ts), total bacterial count (TRT 4.08 *vs* CTR 6.08, *1000 UFC/ml), titrable acidity (TRT 3.66 *vs* CTR 3.72, °sh/50ml) and casein content percentage (TRT 2.4 vs CTR 2.5, %). Whey starter parameters were comparable between the two groups. Final cheese composition and organoleptic profile were not influenced by the treatment except for C18:1 content being enhanced (TRT 22.8 vs CTR 20.8, % of fatty acids). Percentage of defected ripened cheese was significantly lower in the treated group, both at x-ray evaluation performed at 6 months (TRT 6.2 vs CTR 12.3, %) and at the Consortium inspection, performed at 12 months of ripening (TRT 1.5 vs CTR 6.5, %). On the other hand, average cheese yield at 18 months of ripening was partially reduced (TRT 7.5 vs CTR 7.7, %).

Overall in this study, the use of monesin CRC had no negative effect on the cheesemaking process, prolonged ripening cheese characteristics, milk composition or whey starter quality.

Key words

Monensin, milk quality, Parmigiano Reggiano, cheese quality

INTRODUCTION

Ketosis is one of the most important diseases in modern herds due to its high incidence and its deep impact on cow health and performance. Recent studies reported that subclinical ketosis (SCK) incidence, within the first 16 days of lactation, varies from 22 to 43% in European and American herds respectively (Suthar *et al*, 2013; McArt *et al*, 2012). Cows affected by subclinical or clinical ketosis have a higher risk of developing pathologies such as displaced abomasum and metritis as well as risk of culling as a consequence of health problems (Suthar *et al*, 2013; McArt *et al*, 2012; Duffield *et al*, 2009). Reproductive performance of these animals is often impaired and milk production reduced (McArt *et al*, 2015) together with changed composition. Indeed, ketosis reduces the protein content of milk on first DHIA test day (Vanholder et al., 2015) and may consequently impair its cheese making properties.

In 2013, the European Medicines Agency (EMA) approved a new treatment for prevention of ketosis in dairy cows: a monensin controlled release capsule (**CRC**) (Kexxtone, Eli Lilly and Company Ltd, United Kingdom).

Monensin is a carboxylic polyether ionophore commonly used as a feed additive in ruminants to alter rumen fermentation in order to improve energy efficiency (Russell & Strobel, 1989). Its effects on energy metabolism are well known and widely described both in beef and dairy cattle (Goodrich *et al*, 1984; Ipharraguerre & Clark, 2003; Duffield *et al*, 2012). Monensin has a selective action on rumen microbes: it alters ion exchange through the inner and outer membranes of microbial cells. In this way it reduces the prevalence of protozoa and gram positive population and promotes gram negative proliferation, that is mainly responsible for propionate production

(Russell & Strobel, 1989). As a consequence, the ratio between acetate and propionate changes in favor of propionate, thereby improving energy metabolism of cows (Russell & Strobel, 1989).

Monensin administration as a feed additive is not allowed in Europe; consequently, its introduction in 2013 as a ketosis prevention product created a concern in the Italian dairy industry that there may be negative effects on the quality of cheese following production.

In recent years, numerous studies have investigated the effects of monensin administration on animal metabolism and performance and regardless of whether or not it is administered as a feed additive or controlled release capsule, the beneficial effects have included reduced NEFA and BHBA plasma concentration, increased propionate production in the rumen and decreased incidence of clinical and subclinical ketosis (Duffield *et al*, 1998). On the other hand, only a few studies have explored the effects on milk quality and these have shown contrasting results. No studies, to our knowledge, have assessed the impact of monensin on cheese quality. Mullins (Mullins *et al*, 2012) did not find any changes in milk production and composition in monensin treated cows, while other authors found a significant reduction in milk fat and protein content percentage (Odongo *et al*, 2007; Duffield *et al*, 2012).

Parmigiano Reggiano cheese is traditionally made with raw, unpasteurized and partially skimmed milk. To produce this kind of cheese, feedstuff, management and milk processing must be in compliance with Parmigiano Reggiano regulations (Consorzio del Formaggio Parmigiano Reggiano, 2011) that implement the European regulation for PDO production (Council Regulation, n 510/2006). Cows are fed without silages and therefore, in order to maintain milk production and composition

and to avoid ruminal disorders, a proper inclusion of high quality hays in the ration is always needed (Fustini *et al*, 2017).

In this specific manufacturing process, milk composition and environmental wild microflora are extremely important (Mordenti *et al*, 2017). Indeed, microbial population of whey starter is fundamental for the quality and the maturation process of the cheese (Coloretti *et al*, 2016). Considering its antimicrobial activity, some have suggested that the administration of monensin might potentially impair cheese composition and quality. Therefore, the main purpose of our study was to evaluate the effect of a mass treatment of dry cows with monensin CRC on Parmigiano Reggiano cheese production.

MATERIALS AND METHODS

Animals, Feeding, Management conditions and Treatment

In the European Union, monensin use is restricted only to cows considered to be at high risk for ketosis. Consequently, the experimental design used in this study resulted in a more extreme scenario in which mass use of monensin controlled release capsule (**CRC**) was required. This is typical of the summer heat stress period, when all cows are considered to be at high risk of ketosis. The treatment, monensin CRC (Kexxtone, Elanco Animal Health, Eli Lilly and Co. Ltd, UK), contained 32.4 g of monensin released continuously in the rumen throughout 95 days, at a daily dose of 335 mg (EMA, 2013).

Cows involved in the study were divided into two groups, Treated (**TRT**) and Control (**CTR**), and housed in two comparable, dedicated pens, with a straw bedded resting area with cubicles. 33 cows received the treatment 21 days before their expected calving date and gradually entered the TRT study group around 10 DIM, once milk became eligible for processing, according to Parmigiano Reggiano regulations (Consorzio del Formaggio Parmigiano Reggiano, 2011).

The percentage of cows in the TRT group within 95 days from treatment administration increased from 50% at the beginning of the trial to a maximum of 80% during the 7^{th} week of study. In the last 5 weeks, the percentage of treated cows gradually decreased until 0. The percentage of cows under treatment throughout the trial is shown in Figure 1.

All health problems were recorded as well as pharmaceutical treatments. Milk from cows treated with antimicrobials during the trial was not used for cheese manufacturing in the experimental groups for a period equal to double the standard withdrawal time in order to avoid any possible interference of the molecule on milk

and whey starter quality. As soon as a cow exited the TRT group, new untreated cows entered, in order to maintain a minimum of 29-30 cows per group and to have at least 1000-1100 kg of milk/day/group, sufficient to produce 2 cheese wheels a day from each group.

During the experiment, both groups received the same TMR, delivered twice a day. The ration was formulated according to Parmigiano Reggiano feeding rules (Consorzio del Formaggio Parmigiano Reggiano, 2011). Samples of TMR were collected monthly and analyzed using NIR equipment for moisture, crude protein, starch, aNDFom (Mertens, 2002), with addition of sodium sulfite, ADF and ADL, fat, and ash after 4 h combustion in a muffle furnace 550°C (Vulcan 3-550, Dentsply Neytech, Burlington, NJ). Ingredients and chemical composition of the diet are shown in Table 1.

Milking and cheese production

Cows of both groups were milked separately, twice a day and milk was stored in separated tanks. Milk and whey starter obtained from the two experimental groups were maintained separately from each other and from the rest of the herd during every phase of the cheese making process using two different copper vats for the cooking procedure and two different comparable tanks for the storage of whey starter.

Each day, 2 cheese wheels per group were produced and marked following Parmigiano Reggiano cheese production standards (Consorzio del Formaggio Parmigiano Reggiano, 2011). Cheese wheels of both groups were stored together in the same traditional ripening rooms for 18 months.

Milk, whey starter and cheese analysis

Every day the amount of milk produced and delivered to the cheese factory by the two groups was recorded. Morning bulk tank milk and whey starter was collected on the same day, twice a week, for a total of 35 samples per group and analyzed by a qualified lab (Artest Spa, Modena, Italy). Milk samples were analyzed for fat, crude protein, casein, total lactose, SCC and urea content, Total Bacteria Count (**TBC**), pH, titrable acidity (°SH/50ml) and clotting time (r') through lactodynamographic analysis (**LDG**). Milk components were measured by mid-infrared analysis (Biggs, 1978) with MilkoScan 6000 FT (Foss Eletric, Hillerød, Denmark). Precalibration procedures were performed according to International Dairy Federation Standards 141C:2000 (IDF, 2000), using total nitrogen for protein expression. Urea content was determined by differential pH-metry with CL-10 Plus (BioControl System, USA) according to ISO14637:2004 and SCC and TBC by flow cytometry (Schmidt-Madsen, 1975) with Combifoss and Bactoscan FC apparatus, respectively (Foss Eletric, Hillerød, Denmark) according to ISO13366-2:2006 and ISO16297:2013. Titrable acidity was determined by Soxhlet-Henkel method (Anonymous, 1963) and pH measurements using a potentiometric technique with Compact Titrator equipped with electrode P/N 53 64 (Crison Instruments, Barcelona, Spain). PH was determined at samples temperature of 25 °C after calibration of pH meter at the same temperature. Coagulation properties were assessed with a Formagraph apparatus (Foss Eletric, Hillerød, Denmark) under isothermal conditions at 35 °C (Annibaldi *et al*, 1977).

Whey starter samples were analyzed for titrable acidity, fermentative activity at 45, 52 and 54 °C. Acidification rate at different temperatures was evaluated by inoculating 1.5 ml of whey in 50 ml of skimmed milk (Oxoid, Termo Fisher Scientific Inc., Monza, Italy). The incubation was carried out at different temperatures (45, 52, and 54 °C) for 4 h. The acidification rate at a specific temperature was expressed as the

difference between the final and initial acidity (Δ°SH.50 mL−1) (Reverberi *et al*, 2009).

Total amount of lactic acid bacteria (**LAB**) of whey starter was determined by dilution of the sample in physiological solution (9 g·L−1 of NaCl). Then, samples were plated in MRS agar (Oxoid, Termo Fisher Scientific Inc., Monza, Italy) and incubated anaerobically at 45 °C for 96 h for thermophilic LAB quantification.

The amount of whole and skimmed milk coming respectively from the milking of the morning and evening in the cooking vat was recorded daily by the cheesemaker and the ratio between them was evaluated.

All cheese wheels produced during the trial were evaluated over different time points during the maturation period. Cheeses were weighed after 36 hours and 18±1 months since production in order to assess cheese yield calculated as kg of cheese/100 kg of milk in the vat. For this purpose, all the milk added and cooked in each copper vat was measured and recorded every day, together with the vat number and the code of the cheese wheel produced in that vat. At 6 months of age, X-ray analysis of all cheese produced was performed by Artest S.p.A. in order to identify internal defects like swellings, splits and "eyes". Defects were classified as "minor", "mild" or "severe" based on their number and severity.

At 12 months of ripening, experts of Parmigiano Reggiano Consortium evaluated every cheese visually and by beating-hammer examination during the mandatory quality inspection as defined in the Consortium marking regulation. Following this inspection, cheese wheels were classified into different categories depending on the presence of surface or texture defects, as prescribed in the Consortium marking regulation: 1st quality cheese, cheese with minor defects, 2nd quality cheese and rejected cheese that cannot be marked as Parmigiano Reggiano cheese (Consorzio del Formaggio Parmigiano Reggiano, 2011)

At the end of the ripening period, 18±1 months, a representative sample of first quality cheese (24/group) were sampled according to IDF sampling procedure (Emmons, 2000) and evaluated for composition, fatty acid profile and organoleptic analysis.

Chemical analysis of cheese was performed by Artest S.p.A. for the determination of moisture (ISO 5534), fat (FIL-IDF 5A:1969) and protein content (UNI 10760:1998), Total and water soluble nitrogen (ISO 27871), volatile fatty acids and ripening index (N sol/N tot *100).

The amount of acetic, propionic and butyric acids was assessed by HPLC analysis (UV detector, SUPELcogEL C-610H 300x7.8mm column, mobile phase: 0.1% w/v phosphoric acid.)

Fatty acids methyl esters were evaluated by the Animal Production and Food Safety laboratory of the Department of Veterinary Medical Sciences, University of Bologna, by capillary gas-chromatography (Antongiovanni *et al*, 2007). Lipids extraction was performed by Folch method (Folch *et al*, 1957) while acid-catalyzed transmethylation was performed according to Stoffel method (Stoffel *et al*, 1959) in order to recover also the free fatty acids component of ripened cheese (Liu, 1994)

Sensory analysis of cheese was performed by CRPA (Research Center for Animal Production, Reggio Emilia, Italy) applying a Quantitative Descriptive Analysis test (QDA) in order to determine the complete sensory profile of cheese considering view, olfaction, taste, aftertaste and structure. The test was conducted according to EN ISO 13299 (EN ISO, 2010), by 12 selected and trained panelists (ISO, 1993 and 1994).

The evaluation was performed by each panelist on two replicates of each sample served at a fixed temperature of 16±2 °C following a blind random order. Parameters evaluated are shown in Table 2. Each feature was evaluated using a graduated scale

from 1 (= absence of sensation) to 7 (= highest intensity of sensation).

Statistical analysis

Summary statistics including mean, standard deviation, minimum and maximum values were calculated for all outcome parameters, stratified on treatment group. Plots of the distribution of the outcome variables, as well as Shapiro-Wilk test, were performed to determine normal distribution. Somatic cell count data were first transformed in linear Somatic Cell Score (**SCS**) (Wiggans & Shook, 1987). One-way ANOVA with treatment as fixed effects were used when the outcome variable was approximately normally distributed. Results of X-ray analysis and Consortium's evaluation were tested using Chi-square test.

For all analysis, level of significance was set for $P \leq 0.05$.

RESULTS AND DISCUSSION

Milk production

Average daily milk production (kg) was 1626.4 ± 220.1 for CTR group and 1154.9 ± 64.5 for TRT group. This difference was due to the different number of animals in the two groups present in the farm throughout the trial: 51.8 ± 7.0 cows in control group and 29.9 ± 1.5 in treated group. This situation was required by the experimental design that aimed to have in the treated group the maximum concentration of cows within 95 days since treatment administration (80%), in order to highlight any possible effects on milk and cheese quality. In this way, control milk exceeded the capacity of the cooking vat, so after the sampling procedure for the analysis, part of this milk was processed separately from the rest of the experimental milk.

Considering the number of cows in each group, average production per head was higher in TRT than CTR group (38.50 ± 1.48 vs 31.37 ± 1.47, kg), but as production performances were not considered among the objectives of the trial, the collection of these data were not included in the experimental design, therefore comparison of individual milk yield cannot be properly analyzed.

Milk and whey starter quality

Results of milk analysis are reported in table 3**.** Overall, bulk tank milk quality did not differ between the groups except for SCS, titrable acidity and casein content percentage. Fat content (%) and coagulation time (LDG, r') were not affected by the treatment. The effect of monensin on milk fat content is inconsistent in the published literature (Duffield *et al*, 2012). Some authors attribute the decrease in milk fat synthesis sometimes observed when using monensin, to a reduction in acetic acid produced in the rumen as consequence of monensin action on ruminal microflora (Ramanzin *et al*, 1997; Van der Werf *et al*, 1998; Phipps *et al*, 2000). Other authors

have found no effect on milk composition (Mullins *et al*, 2012), while Rico (Rico *et al*, 2014) suggested that monensin could interact with dietary component, such as starch or PUFA, when fed at high levels. Thus, the absence of monensin impact on milk fat observed in the current study, could be related to the low dietary inclusion of starch.

Clotting time (LDG, r') was not affected by the treatment. This result is not surprising considering that the overall milk composition was not affected by the treatment. In addition, our results agree with the only other study that considered cheese-making properties of milk. Bertoni and collaborators (Piccioli Cappelli *et al*, 1996) evaluated the effects of monensin, as a feed additive on coagulation properties of milk, showing no effects on coagulation time (r'), curd consistence (a30) or on curd firmness (k20).

Despite differences shown in table 3, titrable acidity and casein content percentage of milk of both groups remained within the optimum range of milk used for Parmigiano Reggiano production (Zannoni & Mora, 1993; Sandri *et al*, 2001; Malacarne *et al*, 2006).

In his meta-analysis Duffield (Duffield *et al*, 2008a) reported heterogeneous results regarding protein content in different studies, with an overall prevalence of studies that reported a decrease in protein percentage and an increase in protein yield in cows treated with monesin.

In our study, the difference in milk protein percentage between the groups was not significant, while the reduction in casein content percentage was. Only few studies, before ours, evaluated the effects of monensin on casein content and they did not show any variation (Gandra *et al*, 2010; Trevisi *et al*, 2015). At the same time, other studies reported a significant reduction in milk protein and fat percentage that was explained by dilution effects due to the increased milk production of monensin treated cows (Phipps *et al*, 2000).

Somatic cells were significantly lower in the treated group and this difference could be related to a better health status of animals treated with monensin (Duffield *et al*, 2008b).

Results of whey starter quality are shown in table 4. No important differences appeared in the activity of treated and control whey starter. The amount of lactic bacteria was not different between the groups and, indeed, the power of acidification of whey starter, here represented by fermentation activities, was not impaired. Fermentative activities are strictly related to the microbial population of whey starters and they were not affected by the treatment, as demonstrated by the high values of acidification rate (Reverberi *et al*, 2009). Titrable acidity of the treated group was slightly lower than the control, but always remained within the optimal range (29-31.5 °SH/50ml) for Parmigiano Reggiano production (Reverberi *et al*, 2009; Gatti *et al*, 2014). These results are extremely important for the dairy industry as, to our knowledge, no previous studies have evaluated the effects of monensin on whey starter quality and activity.

Cheese production and defects.

During the study, 552 cheese wheels were produced, corresponding to 2 "twin" cheese wheels/group/day. As reported in table 5, the weight of twin cheese evaluated at 36 hours and 18±1 months of ripening were significantly lower (P<0.01) in TRT than CTR group (90.8 vs 93.7 kg at 36h and 79.3 vs 81.9 kg at 18 months).

Cheese yield (%), calculated as kg of cheese obtained by 100 kg of milk in the vat, showed the same difference both at 36 hours (8.59 TRT vs 8.85 CTR, %, P <0.05) and after 18 months of ripening (7.5 TRT vs 7.7 CTR, %, P <0.01).

The lower cheese yield of treated group milk could be related to its lower casein content. Cheese yield and casein content of milk are directly proportional (Fossa *et al*,

1994). Formaggioni et al. (2015) proposed a simple predictive formula for Parmigiano Reggiano cheese yield at 24h, including only milk fat and casein content, that has a high correlation with the actual cheese yield (Formaggioni *et al*, 2015).

No early swelling, detectable within 24-48 hours from production, was evident and both the experimental groups showed a very low percentage of defective cheese at 6 and 12 months of ripening.

At X-ray analysis, performed on all cheese at 6 months of ripening, 94% of cheese wheels in the treated group showed no defects, versus 88% of those in the control group. Overall, the treated group showed less ($P<0.05$) minor (6.2 TRT vs 9.4 CTR, %) mild (0 TRT vs 0.4 CTR, %) and severe (0 TRT vs 2.5 CTR, %) defects than the control group.

X-ray analysis has been demonstrated to be a useful non-destructive method to monitor the development of individual cheese during the ripening period (Kraggerud *et al*, 2009).

Similar results were obtained during the subsequent examination of cheese, performed at 12 months of ripening by the Consortium of Parmigiano Reggiano.

98.5% of cheese produced by TRT group showed no defects and was marked as 1st quality cheese compared to 93.5% in the CTR group. In the TRT group, 1.5% of wheels were marked as 2nd quality and none of them were rejected, while in the CTR group, 5.4% were 2nd quality cheese and 1.1% were rejected. At official Consortium evaluation, corrupted cheeses in both groups were less than those recorded by the Consortium of Parmigiano Reggiano in the last three years (2015-2017) of production: 91.5% of 1st category cheese, 7% of 2nd category and 1.5% of rejected cheese (unpublished data, Consortium of Parmigiano Reggiano).

Early swelling occurs rapidly after cheese production and is due to the proliferation of gas-producing bacteria within the cheese, coliform or heterofermentative lactic acid bacteria, and more rarely, yeasts (Walstra et al., 1978).

In particular, these defects become serious in the presence of large microbial populations (10^5–10^6 /ml) and insufficient or slow acidification of milk that may occur as a consequence of a poorly active whey starter, presence of antibiotics, or contamination with phages. In order to avoid these abnormal fermentations and to assure a good ripening process, an active and proper microbial population of whey starter is fundamental (Bergère & Lenoir, 2000).

Cheese composition and sensory analysis.

After 18±1 months of ripening, cheese produced by the two groups had similar composition, consistent with composition of the average of 18 months aged Parmigiano Reggiano cheese (Tosi *et al*, 2008).

Complete results are shown in table 5.

Only slight differences were detected between the two groups: fat percentage was a bit higher in treated cheese while soluble nitrogen and ripening index were slightly lower, but always within the range for 18 months aged Parmigiano Reggiano cheese. The ripening index represents the amount of casein solubilized by proteolytic enzymes, thus this difference is explainable by the lower amount of casein contained in treated milk (Tosi *et al*, 2008).

As shown in table 5, acetic and propionic acids were not different between the groups. Unwanted bacteria produce propionic acid during the aging process and its presence is responsible for texture defects of cheese and undesirable flavors (Bergère & Lenoir, 2000). Also butyric acid producing clostridia are responsible for off-flavors and cheese defects. Their capability to convert lactate into butyrate, acetate, H_2 and CO_2

can lead to the accumulation of gas in the cheese matrices that results in the formation of cracks, slits and eyes (Sheehan, 2011; Brändle *et al*, 2016). During the ripening process, butyric acid is mainly produced by lipolysis facilitated by lipase present in cheese (Brändle *et al*, 2016). In our study, its amount was significantly lower (P<0.001) in TRT cheese than in CTR, but its value remained for both groups within the values typical of 18 months aged Parmigiano Reggiano cheeses (table 5) (Tosi *et al*, 2008).

These differences agree with the results of sensory analysis that showed an overall comparable profile between cheeses with a few exceptions. TRT samples showed a slower ripening process indicated by higher intensity of butter and sweet aroma (p.ts, 3.2 vs 3.0, P<0.01 and 3.5 vs 3.4, P <0.05), lower rind and spicy flavors (p.ts, 2.0 vs 2.1, P <0.05 and 1.8 vs 1.9, P <0.05) and higher elasticity (p.ts, 2.5 vs 2.4, P <0.05). In addition, TRT cheeses had a less intense, negative aroma, such as pungent, acetic and "stall", than CTR cheeses (p.ts, 2.1 vs 2.2, P <0.05).

However, it should be noticed that all these differences were minimal and did not influence the overall sensory profile of resulting cheese which was comparable within typical profiles of 18 month old Parmigiano Reggiano cheese (Zannoni, 2010). Cheese fatty acids (**FA**) profile is shown in table 6.

In the treated group, the percentage of middle-chain fatty acids (C10 to C14) on total FA was reduced (TRT 20.22 vs CTR 21.73, P<0.05) while among long chain fatty acids, C18:1 (TRT 22.77 vs CTR 20.79, P<0.001) and C:17 (TRT 0.64 vs CTR 0.61, P<0.05) were increased. Along with this, unsaturated (**UFA**) and saturated (**SFA**) fatty acid ratios were increased in the treated group (UFA/SFA, TRT 0.42 vs CTR 0.39, P<0.05).

Regardless of treatment or control, cheese fatty acid composition of all samples were in agreement with those reported by other authors for Parmigiano Reggiano cheese (Prandini *et al*, 2007; Mordenti *et al*, 2015).

Even if no other studies, to our knowledge, evaluated the effects of monensin on cheese fatty acid concentration, our results correspond with literature evaluating fatty acid variations in milk produced by cows treated with monensin sodium when administered as a feed additive or as CRC (De Marchi *et al*, 2015; Duffield *et al*, 2008a).

It has to be noticed that fatty acid composition of milk is influenced also by the stage of lactation of cows. In our study, days in milk of the experimental groups were not controlled, therefore it is possible that at least some of the difference in fatty acid profile of cheese between the groups could be due to the presence of a higher percentage of fresh cows in the treated group. Existing literature, however, supports the theory that monensin influences fatty acid concentration in milk by altering ruminal microbiota (Bell et al.,2006; McCarthy et al., 2018).

Odongo and collaborators (Odongo *et al*, 2007) showed an increased concentration of long chain polyunsaturated fatty acids (**PUFA**) and total monounsaturated FA (**MUFA**) in milk by 9 and 5 % respectively, in a group fed TMR + 24 mg of monensin premix per kg of DM compared to a control group. Other studies, as reported by Duffield et al. (2008b), showed the same increase in total C18:1 and PUFA concentrations, a reduction of short and medium-chain fatty acids and a reduction of PUFA/SFA ratio (De Marchi *et al*, 2015; AlZahal *et al*, 2008). The same effects were observed by in vitro studies, reporting a decrease of C18:2 ruminal biohydrogenation by lowering C18:0 production and increasing C18:1 concentration (Jenkins *et al*, 2003; Fellner *et al*, 1997).

In addition, an increase of CLA is reported after monensin supplementation (Duffield et al., 2008a), while in our study, CLA concentration remained similar between the groups (TRT 0.36 vs CTR 0.35, P >0.05). Only few recent researches, on the contrary, reported no (do Prado et al, 2015) or minimal (Akins *et al*, 2014) effects of monensin on milk fatty acid composition.

The rate of ruminal biohydrogenation of unsaturated fatty acids depends primarily on ruminal conditions, including microbial growth, rumen pH, and feed passage rate. Low rumen pH and altered microbial growth contribute to reduce rumen lipolysis and therefore the availability of carboxyl groups for the biohydrogenation of unsaturated fatty acids (Jenkins, 1993). Indeed, ionophores reduce rumen lipolysis, like other antimicrobial compounds known to be active mainly against gram-positive bacteria (Russell & Strobel, 1989; Van Nevel & Demeyer, 1995). However, as reported by Fellner (Fellner *et al*, 1997) these bacteria are not involved in rumen lipolysis neither in the last step of hydrogenation of linoleic acid to stearic. For this reason, it seems to be possible that these molecules exert their effects also against gram negative bacteria, by changing their metabolic properties with a consequent alteration of rumen lipolysis and biohydrogenation (Newbold *et al*, 1993; Odongo *et al*, 2007).

CONCLUSIONS

Milk and whey starter produced during the trial were not affected by the treatment of cows with monensin CRC: the slight differences found in titrable acidity and casein content of milk and in titrable acidity of whey starter agree with the existing literature that relates these effects to the high milk production of treated cows. However, both milk and whey starter maintained the optimum quality for Parmigiano Reggiano cheese production. In particular, fermentative activities of whey starter were not impaired in the treated group at 45°C or at 54°C: this was one of the major initial

concerns, considering the absence of published studies and the importance of whey starter for Parmigiano Reggiano production, in which the use of any other kind of ferments are not allowed.

After ripening, the percentage of defective cheeses in both groups was consistent with values reported by the Consortium of Parmigiano Reggiano for the last three years. Additionally, the treated group cheeses showed less defects than controls. .

Chemical analysis did not highlight any negative influence of the treatment on composition and fatty acid profile. Sensory analysis demonstrated that the treatment did not substantially affect organoleptic characteristics of 18 month aged Parmigiano Reggiano cheese.

In conclusion, high quality cheese production was maintained in both control and the treated group and considering our results, it is possible to state that the preventative treatment of ketosis with monensin CRC of periparturient dry cows did not impair Parmigiano Reggiano cheese quality, composition and sensory characteristics.

ACKNOWLEDGMENTS

The authors want to express their appreciation to the F.lli Caretti dairy farm and cheese factory team (San Giovanni in Persiceto, (BO), Italy) for their essential involvement in the trial and to Elanco Animal Health for funding the study.

REFERENCIES

- Akins, M.S., K.L. Perfield, H.B. Green, S.J. Bertics, and R.D. Shaver. 2014. Effect of monensin in lactating dairy cow diets at 2 starch concentrations. J. Dairy Sci. 97:917–929. https://doi:10.3168/jds.2013-6756.
- AlZahal, O., N.E. Odongo, T. Mutsvangwa, M.M. Or-Rashid, T.F. Duffield, R. Bagg, P. Dick, G. Vessie, and B.W. McBride. 2008. Effects of Monensin and Dietary Soybean Oil on Milk Fat Percentage and Milk Fatty Acid Profile in Lactating Dairy Cows. J. Dairy Sci. 91:1166–1174. http://dx.doi.org/10.3168/jds.2007-0232.
- Annibaldi, S., G. Ferri, and R. Mora. 1977. Nuovo orientamenti nella valutazione tecnica del latte: tipizzazione lattodinamografica [New trends for milk technical evaluation: lactodynamographic standardisation]. Sci. E Tec. Latt. Casearia 28:115–126.
- Antongiovanni, M., S. Banni, A. Buccioni, L. Cercaci, G. Contarini, M. Cutrignelli, G. Lercker, D. Lo Fiego, M. Mele, S. Minieri, A. Nudda, E. Piasentier, and A. Serra. 2007. Metodi Di Analisi per Lo Studio Della Frazione Lipidica Del Latte, Dei Prodotti Di Origine Animale e Degli Alimenti Zootecnici. PLUS PISA UNIVERSITY PRESS, Pisa, Italy.
- Bell, J. A., J. M. Griinari, and J. J. Kennelly. 2006. Effect of safflower oil, flaxseed oil, monensin, and vitamin E on concentration of conjugated linoleic acid in bovine milk fat. J. Dairy Sci. 89:733–748.
- Bergère, J.L., and J. Lenoir. 2000. Cheese manufacturing accidents and cheese defects. Lavoisier Publishing, France.
- Biggs, D.A. 1978. Instrumental infrared estimation of fat, protein, and lactose in milk: collaborative study. J. Assoc. Off. Anal. Chem. 61:1015–1034.

- Brändle, J., K.J. Domig, and W. Kneifel. 2016. Relevance and analysis of butyric acid producing clostridia in milk and cheese. Food Control 67:96–113. https://doi:10.1016/j.foodcont.2016.02.038.
- Coloretti, F., C. Chiavari, M. Nocetti, P. Reverberi, E. Bortolazzo, V. Musi, and L. Grazia. 2016. Whey starter addition during maturation of evening milk: effects on some characteristics of cheese milk and Parmigiano–Reggiano cheese. Dairy Sci. & Technol. 96:185–197.
- Consorzio del Formaggio Parmigiano Reggiano. 2011. Specification of the Parmigiano Reggiano cheese. Accessed Oct. 24 2017. http://www.parmigianoreggiano.com/consortium/rules_regulation_2/default.aspx
- De Marchi, F.E., J.V. Romero, J.C. Damasceno, P.A. Grande, L.M. Zeoula, and S. Dos. 2015. Pelleting in associated with sodium monensin increases the conjugated linoleic acids concentration in the milk of dairy cows fed canola seeds. Asian-Australas. J. Anim. Sci. 28:1095–1104. https://doi:10.5713/ajas.14.0865.
- Duffield, T.F., K.D. Lissemore, B.W. McBride, and K.E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. J. Dairy Sci. 92:571–580. https://doi:10.3168/jds.2008-1507.
- Duffield, T.F., A.R. Rabiee, and I.J. Lean. 2008a. A Meta-Analysis of the Impact of Monensin in Lactating Dairy Cattle. Part 2. Production Effects. J. Dairy Sci. 91:1347–1360. https://doi:10.3168/jds.2007-0608.
- Duffield, T.F., A.R. Rabiee, and I.J. Lean. 2008b. A Meta-Analysis of the Impact of Monensin in Lactating Dairy Cattle. Part 3. Health and Reproduction. J. Dairy Sci. 91:2328–2341. https://doi:10.3168/jds.2007-0801.

- Duffield, T.F., A. Rabiee, and I.J. Lean. 2012. Overview of Meta-Analysis of Monensin in Dairy Cattle. Vet. Clin. North Am. Food Anim. Pract. 28:107–119. https://doi:10.1016/j.cvfa.2011.12.009.
- Duffield, T.F., D. Sandals, K.E. Leslie, K. Lissemore, B.W. McBride, J.H. Lumsden, P. Dick, and R. Bagg. 1998. PHYSIOLOGY AND MANAGEMENT:-Efficacy of Monensin for the Prevention of Subclinical Ketosis in Lactating Dairy Cows. J. Dairy Sci. 81:2866–2873.
- EN ISO, 2010. Sensory analysis. Methodology. General guidance for establishing a sensory profile. EN ISO 13299. *European* Committee for Standardization. Brusselles, Belgium
- EMA, 2013. European Public Assessment Reports (EPAR) European Medicine Agency. Accessed January 2018 http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/veterinary/medicines/002235/vet_med_000267.jsp&mid=WC0b01ac058001fa1c
- Emmons, D.B., 2000. Sampling and analysis. Pages 74–86 in: Practical Guide for Control of Cheese Yield.. International Dairy Federation, Brussels, Belgium.
- Fellner, V., F.D. Sauer, and J.K.. Kramer. 1997. Effect of Nigericin, Monensin, and Tetronasin on Biohydrogenation in Continuous Flow-Through Ruminal Fermenters.pdf. J. Dairy Sci. 80:921–928.
- Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509.
- Formaggioni, P., A. Summer, M. Malacarne, P. Franceschi, and G. Mucchetti. 2015. Italian and Italian-style hard cooked cheeses: Predictive formulas for

Parmigiano-Reggiano 24-h cheese yield. Int. Dairy J. 51:52–58. https://doi:10.1016/j.idairyj.2015.07.008.

- Fossa, E., M. Pecorari, S. Sandri, F. Tosi, and P. Mariani. 1994. The role of milk casein content in the Parmigiano-Reggiano cheese production: chemical composition, rennet coagulation properties and dairy tech- nological behaviour of milk. Sci. E Tec. Latt. Casearia 45:519–535.
- Fustini, M., A. Palmonari, G. Canestrari, E. Bonfante, L.M.E. Mammi, M.T. Pacchioli, G.C.J. Sniffen, R.J. Grant, K.W. Cotanch, and A. Formigoni. 2017. Effect of undigested neutral detergent fiber content of alfalfa hay on lactating dairy cows: Feeding behavior, fiber digestibility, and lactation performance. J. Dairy Sci. 100:4475–4483. https://doi:10.3168/jds.2016-12266.
- Gandra, J.R., F.P. Rennó, J.E. de Freitas Júnior, M.V. dos Santos, and A.P.C. de Araújo. 2010. Productive performance and milk protein fraction composition of dairy cows supplemented with sodium monensin. Rev. Bras. Zootec. 39:1810–1817.
- Gatti, M., B. Bottari, C. Lazzi, E. Neviani, and G. Mucchetti. 2014. Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. J. Dairy Sci. 97:573–591. https://doi:10.3168/jds.2013-7187.
- Goodrich, R.D., J.E. Garrett, D.R. Gast, M.A. Kirick, D.A. Larson, and J.C. Meiske. 1984. Influence of monensin on the performance of cattle. J. Anim. Sci. 58:1484–1498.
- IDF, 1993. Milk: Determination of Nitrogen Content, IDF Standard 20B:1993, Parts 1 and 2. Int. Dairy Fed., Brussels, Belgium.

- IDF, 2000. Whole milk: Determination of milk fat, protein and lactose content – guidance on the operation of mid-infrared instruments. IDF Standard 141C:2000, Int. Dairy Fed., Brussels, Belgium.
- ISO, 1993. Sensory analysis. General guidance for the selection, training and monitoring of assessors. Part 1: Selected assessors. ISO 8586-1. International Organisation for Standardization. Geneva, Switzerland.
- ISO, 1994. Sensory analysis. General guidance for the selection, training and monitoring of assessors. Part 2: Experts. ISO 8586-2. International Organisation for Standardization. Geneva, Switzerland
- ISO, 2004. Cheese and processed cheese products. Determination of fat content. Gravimetric method (Reference method). ISO 1735. International Organisation for Standardization. Geneva, Switzerland.
- ISO, 2004. Cheese and processed cheese. Determination of the total solids content (Reference method). ISO 5534. International Organisation for Standardization. Geneva, Switzerland.
- ISO, 2004. Milk. Determination of urea content. Enzymatic method using difference in pH (Reference method). ISO 14637. International Organisation for Standardization. Geneva, Switzerland.
- ISO, 2006 Milk. Enumeration of somatic cells. Part 2: Guidance on the operation of fluoro-opto-electronic counters. ISO 13366-2. International Organisation for Standardization. Geneva, Switzerland.
- ISO, 2011. Cheese and processed cheese. Determination of the nitrogenous fractions. ISO 27871. International Organisation for Standardization, Geneva, Switzerland.

- ISO, 2013 Milk. Bacterial count: Protocol for the evaluation of alternative methods. International Organisation for Standardization. ISO 16297. Geneva, Switzerland.
- Ipharraguerre, I.R., and J.H. Clark. 2003. Usefulness of ionophores for lactating dairy cows: a review. Anim. Feed Sci. Technol. 106:39–57. https://doi:10.1016/S0377-8401(03)00065-8.
- Jenkins, T.C. 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76:3851–3863.
- Jenkins, T.C., V. Fellner, and R.K. McGuffey. 2003. NUTRITION, FEEDING, AND CALVES-Monensin by Fat Interactions on Trans Fatty Acids in Cultures of Mixed Ruminal Microorganisms Grown in Continuous Fermentors Fed Corn or Barley. J. Dairy Sci. 86:324–330.
- Kraggerud, H., J.P. Wold, M. HøY, and R.K. Abrahamsen. 2009. X-ray images for the control of eye formation in cheese. Int. J. Dairy Technol. 62:147–153. https://doi:10.1111/j.1471-0307.2009.00478.x.
- Liu, K.-S. 1994. Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. J. Am. Oil Chem. Soc. 71:1179–1187.
- Malacarne, M., A. Summer, E. Fossa, P. Formaggioni, P. Franceschi, M. Pecorari, and P. Mariani. 2006. Composition, coagulation properties and Parmigiano-Reggiano cheese yield of Italian Brown and Italian Friesian herd milks. J. Dairy Res. 73:171. https://doi:10.1017/S0022029905001688.
- McArt, J.A.A., D.V. Nydam, and G.R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. J. Dairy Sci. 95:5056–5066. https://doi:10.3168/jds.2012-5443.

- McArt, J.A.A., D.V. Nydam, and M.W. Overton. 2015. Hyperketonemia in early lactation dairy cattle: A deterministic estimate of component and total cost per case. J. Dairy Sci. 98:2043–2054. https://doi:10.3168/jds.2014-8740.

- McCarthy M.M., T. R. Overton, G. D. Mechor, D. E. Bauman, T. C. Jenkins and D. V. Nydam. 2018. Short communication: Field study to investigate the associations between herd-level risk factors for milk fat depression and bulk tank milk fat percent in dairy herds feeding monensin. J. Dairy Sci. 101:3118–3125. https://doi.org/10.3168/jds.2017-13932

- Mordenti, A.L., N. Brogna, and A. Formigoni. 2017. Review: The link between feeding dairy cows and Parmigiano-Reggiano cheese production area. Prof. Anim. Sci. 33:520–529. https://doi:10.15232/pas.2016-01602.
- Mordenti, A.L., N. Brogna, F. Merendi, G. Canestrari, M. Dall'Olio, G. Biagi, and A. Formigoni. 2015. Effect of feeding whole soybean and linseed on milk and Parmigiano-Reggiano cheese lipid fraction.. Prog. Nutr. 17:220–230.
- Mullins, C.R., L.K. Mamedova, M.J. Brouk, C.E. Moore, H.B. Green, K.L. Perfield, J.F. Smith, J.P. Harner, and B.J. Bradford. 2012. Effects of monensin on metabolic parameters, feeding behavior, and productivity of transition dairy cows. J. Dairy Sci. 95:1323–1336. https://doi:10.3168/jds.2011-4744.
- Newbold, C.J., R.J. Wallace, and N.D. Walker. 1993. The effect of tetronasin and monensin on fermentation, microbial numbers and the development of ionophore-resistant bacteria in the rumen. J. Appl. Bacteriol. 75:129–134.
- Odongo, N.E., M.M. Or-Rashid, R. Bagg, G. Vessie, P. Dick, E. Kebreab, J. France, and B.W. McBride. 2007. Long-Term Effects of Feeding Monensin on Milk Fatty Acid Composition in Lactating Dairy Cows. J. Dairy Sci. 90:5126–5133. https://doi:10.3168/jds.2007-0242.

- Phipps, R.H., J.I.D. Wilkinson, L.J. Jonker, M. Tarrant, A.K. Jones, and A. Hodge. 2000. Effect of monensin on milk production of Holstein-Friesian dairy cows. J. Dairy Sci. 83:2789–2794.
- Piccioli Cappelli, F., M.G. Maianti, and G. Bertoni. 1996. Effect of Monensin supplementation on milk and metabolic characteristics of dairy cows. Pages 561–562 S.I.S. Vet, Perugia.
- Prandini, A., S. Sigolo, G. Tansini, N. Brogna, and G. Piva. 2007. Different level of conjugated linoleic acid (CLA) in dairy products from Italy. J. Food Compos. Anal. 20:472–479. https://doi:10.1016/j.jfca.2007.03.001.
- Ramanzin, M., L. Bailoni, S. Schiavon, and G. Bittante. 1997. Effect of Monensin on Milk Production and Efficiency of Dairy Cows Fed Two Diets Differing in Forage to Concentrate Ratios1. J. Dairy Sci. 80:1136–1142.
- Reverberi, P., G. Gambini, A. Caroli, A. Pecorari, and M. Nocetti. 2009. Profilo dei siero-innesti per Parmigiano- Reggiano e modalità analitiche di valutazione [Profile and analytical valuation of Parmigiano-Reggiano whey starters]. Sci. E Tec. Latt. Casearia 60:37–42.
- Rico, D.E., A.W. Holloway, and K.J. Harvatine. 2014. Effect of monensin on recovery from diet-induced milk fat depression. J. Dairy Sci. 97:2376–2386.
- Russell, J.B., and H.J. Strobel. 1989. Effect of ionophores on ruminal fermentation. Appl. Environ. Microbiol. 55:1.
- Sandri, S., F. Tosi, M.S. Mariani, P. Vecchia, M. Malacarne, and A. Summer. 2001. Osservazioni sull'andamento delle principali caratteristiche casearie del latte per Parmigiano-Reggiano durante gli anni 1990. Ann Fac Med Vet Univ. Parma 21:235–247.
- Savini, E. 1946. Analisi Del Latte e Dei Latticini. Hoepli, Milan.

- Schmidt-Madsen, P. 1975. Fluoro-opto-electronic cell-counting on milk.. J. Dairy Res. 42:227–239.
- Sheehan, J.J. 2011. Cheese. Avoidance of gas blowing. Academic Press, San Diego, CA.
- Stoffel, W., F. Chu, and J.E.H. Ahrens. 1959. Analysis of Long-Chain Fatty Acids by Gas-Liquid Chromatography. Anal. Chem. 31:307–308.
- Suthar, V.S., J. Canelas-Raposo, A. Deniz, and W. Heuwieser. 2013. Prevalence of subclinical ketosis and relationships with postpartum diseases in European dairy cows. J. Dairy Sci. 96:2925–2938. https://doi:10.3168/jds.2012-6035.
- Tosi, F., S. Sandri, G. Tedeschi, M. Malacarne, and E. Fossa. 2008. Variazioni di composizione e proprietà fisico-chimiche del Parmigiano-Reggiano durante la maturazione e in differenti zone della forma. Sci. E Tec. Latt. Casearia 59:507–528.
- Trevisi, E., F. Piccioli Cappelli, M. Mezzetti, G. Lovotti, and P. Bani. 2015. Effect of the ruminal slow-release of monensin during the transition period of dairy cows on health status, energy metabolism and inflammatory conditions.. Ital. J. Anim. Sci. 14:9.
- UNI, 1998. Dairy products. General directives for the nitrogen determination according to Kjeldahl method. UNI 10760. Ente di Normazione Italiano. Milano, Italy.
- Van der Werf, J.H.J., L.J. Jonker, and J.K. Oldenbroek. 1998. Effect of monensin on milk production by Holstein and Jersey cows. J. Dairy Sci. 81:427–433.

- Van Nevel, C., and D.I. Demeyer. 1995. Lipolysis and Biohydrogenation of Soybean Oil in the Rumen In Vitro: Inhibition by Antimicrobials. J. Dairy Sci. 78:2797–2806. https://doi:10.3168/jds.S0022-0302(95)76910-7.
- Walstra, P., A. Noomen, and T.J. Geurts. 1978. Major cheese groups. Elsevier Applied Science, London, UK.
- Wiggans, G.R., and G.E. Shook. 1987. A lactation measure of somatic cell count. J. Dairy Sci. 70:2666–2672.
- Zannoni, M. 2010. Evolution of the sensory characteristics of Parmigiano–Reggiano cheese to the present day. Food Qual. Prefer. 21:901–905. https://doi:10.1016/j.foodqual.2010.01.004.
- Zannoni, M., and R. Mora. 1993. Evolution of the milk quality program for the Parmigiano-Reggiano cheese. Il Latte, 18:572–581.

Table 1. Ingredients and chemical composition (% DM) of diets fed to lactating cows of Treated[1] and Control groups

Ingredients	% (DM)
Grass hay	17.18
Wheat Straw	3.44
Alfalfa hay	27.49
Corn meal fine	3.44
Sorghum meal fine	18.90
Wheat meal fine	11.34
Wheat Bran	7.56
Protein supplement	0.94
Mineral & vitamin supplement	0.94
Chemical composition	**% (DM)**
DM, %	77.77
Crude Protein	16.11
Starch	25.05
aNDFom[2]	28.91
ADF	23.30
ADL	4.21
Fat	2.19
Ash	9.49

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

[2] aNDFom: alpha-amylase treated NDF, ash corrected.

Table 2. Cheese sensorial descriptors evaluated during a Quantitative Descriptive Analysis test performed by a trained expert Panel on Control and Treated[1] cheese samples at 18±1 months of ripening

Descriptor	
Visual	Color, color homogeneity, number of eyes/break, diameter, visual suitability
Aroma	Total intensity, butter smell, crust smell, vegetables smell, dried fruit smell, negative smells, flavor suitability
Taste	Sweet, salted, bitter, spicy, butter taste, crust taste, dried fruit taste, bouillon taste, nutmeg taste, negative flavors, suitability taste.
Texture	Elasticity, friability, humidity, solubility, granularity, suitability structure.

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

Table 3. Morning bulk tank milk composition and quality of Treated[1] and Control group, analyzed twice a week for a total amount of 35 samples per group

Item	Control	Treated	sem
Fat, %	3.45	3.45	0.02
Casein, %	2.51***	2.44***	0.01
Crude Protein, %	3.30	3.21	0.04
Total lactose, %	4.78	4.79	0.03
Urea, mg/100ml	19.69	20.05	0.32
SCS, points	4.06***	3.40***	0.05
Titrable acidity, °SH/50ml	3.69 ***	3.61***	0.01
pH[2]	6.67	6.67	0.00
LDG[3], r'	17.67	17.27	0.23
TBC[4], *1000 UFC/ml	6.71	5.57	0.56

*** $P<0.001$

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

[2] samples temperature 25°C.

[3] clotting time (min.) evaluated through lactodynamographic analysis.

[4] total bacterial count.

Table 4. Whey starter quality of Treated[1] and Control group, analyzed twice a week for a total amount of 35 samples per group

Item	Control	Treated	sem
Titrable acidity, °SH/50ml	30.43 *	29.44 *	0.23
Fermentative activity 45°C, (Δ°SH/50 mL^{-1})	2.51	2.67	0.08
Fermentative activity 52°C, (Δ°SH/50 mL^{-1})	1.93	1.97	0.05
Fermentative activity 54°C, (Δ°SH/50 mL^{-1})	1.47	1.46	0.03
Lactic Bacteria, *million UFC/ml	660.57	613.43	14.19

* $P<0.05$

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

Table 5. Weight, cheese yield, composition and volatile fatty acids content (acetic, butyric and propionic) of cheese produced by Control and Treated[1] milk, analyzed at 18±1 months of ripening by an accredited laboratory for Parmigiano Reggiano analysis (Artest S.p.A.)

Composition	Control	Treated	sem
Weight 36 hrs, kg[2]	93.71***	90.75***	0.222
Cheese yield 36 hrs, %	8.85***	8.59***	0.018
Weight 18 months, kg[2]	81.98***	79.34***	0.193
Cheese yield 18 months, %	7.72***	7.49***	0.016
Skimmed:whole milk ratio	0.68	0.69	0.014
Moisture, %	30.75	30.85	0.076
Fat, % DM	47.58*	48.86*	0.228
Protein, %DM	45.14	44.61	0.208
NT[3], g/100g of cheese	4.9	4.83	0.023
NS[4], g/100g of cheese	1.5*	1.42*	0.019
NS/NT[5], %	30.69*	29.35*	0.361
Volatile fatty acids, mg/100g of cheese[6]			
Acetic acid	98.87	103	4.627
Butyric acid	37.3***	28.56***	1.499
Propionic acid	0.79	0.94	0.302

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.
[2] Weight of two twin cheese wheels.
[3]NT= Total nitrogen
[4]NS= Water Soluble Nitrogen
[5]=Ripening index
[6] Volatile fatty acids assessed by HPLC analysis
* $P<0.05$
*** $P<0.001$

Table 6. Fatty acid composition (% of fatty acids) of 18±1 months aged cheese produced with Control and Treated[1] milk

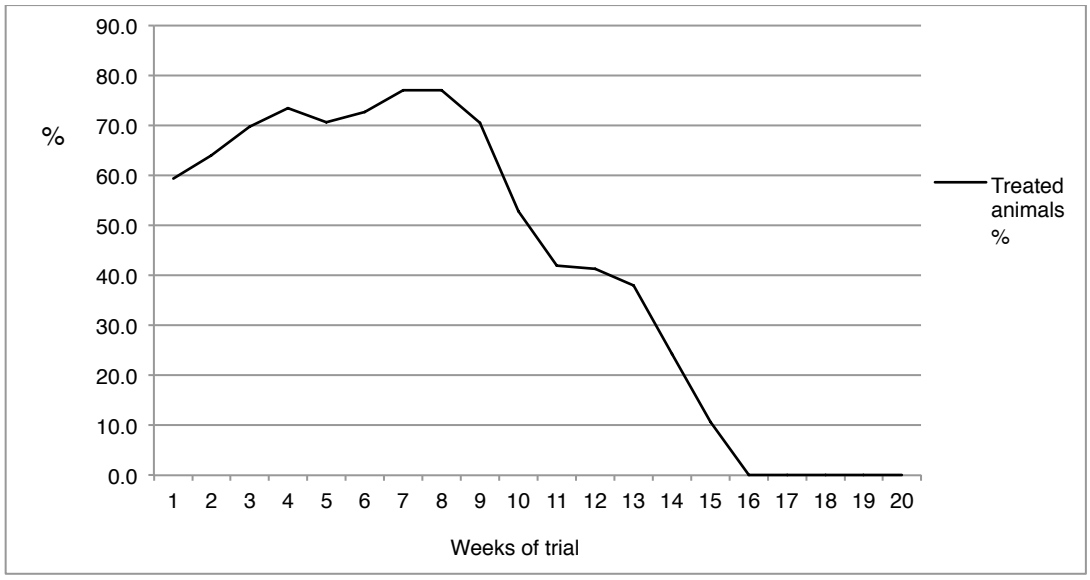
Fatty acid	Control	Treated	sem
C4:0	3.35	3.6	0.291
C6:0	1.51	1.44	0.118
C8:0	1.28	1.19	0.056
C10:0	3.59*	3.31*	0.095
C10:1	0.3**	0.25**	0.009
C12:0	4.23**	3.79**	0.087
C12:1	0.12**	0.1**	0.004
C14:0	12.34*	11.77*	0.164
C14:1	1.15***	1***	0.018
C15:0	1.52	1.45	0.032
C16:0	34.44	34.07	0.288
C16:1	1.47	1.4	0.08
C17:0	0.61*	0.66*	0.015
C18:0	6.84	6.97	0.155
C18:1	20.79***	22.77***	0.316
C18:2	2.14	2.16	0.056
C18:3 n3	0.54	0.5	0.02
C20:0	0.08	0.07	0.007
C20:4 n6	0.14	0.12	0.007
CLA tot	0.35	0.36	0.014
Others[2]	3.21	3.02	0.254

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

[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

[2] Non-identified fatty acids

Figure 1. Percentage of animals in Treated group within 95 days since treatment[1] administration, from the 1st to the 20th week of trial.



[1] Treatment: monensin control release capsule, administered to cows 21 days before predicted calving date.

5. Conclusions

The peripartum is the only moment in a cow life where lots of physiological, environmental and nutritional changes (or stress) come together in a very short period. The combination of such stress frequently results in the failure of homeoretic mechanisms and as a consequence in diseases.

Despite the large amount of studies, transition still remains a big challenge for both researchers and farmers, therefore the main purpose of this thesis was to further investigate the possibility to improve transition dairy cows health and milk quality through different strategies.

The first study highlighted an increase of hematic markers of stress like deydroepiandrosterone (DHEA) and cortisol in cows during overstocking, confirming that this common situation in dairy herds can have important consequences on animal welfare and health. The same cows showed augmented general activity indicating restlessness behaviour of these animals when overstocked, as previously suggested also by other authors. These behavioural effects can have negative consequences on cow energy metabolism, while at the same moment, stress related hormones like cortisol could further increase immune suppression of these animals.

In the second study the possibility to reduce the incidence of disease, while maintaining milk quality, through the use of an immunomodulant feed supplement was confirmed.

Cows that received the supplement from dry-off to 150 days in milk (DIM) showed a better health status, proved by a lower number of total clinical cases, lower incidence of mastitis among Holstein cows and a lower culling rate within the first 60 days of lactation.

Finally, the effect of a treatment used on dry cows for the prevention of ketosis

was investigated on milk and Parmigiano Reggiano quality.

This study showed that this treatment has no negative impact on milk and cheese characteristics, suggesting that its usage to improve metabolic status of transition cows can be safely adopted even in those farms that produce milk for Parmigiano Reggiano production.

Overall, the present studies confirm that factors involved in a successful transition of dairy cows are several and various. This work highlighted the need and the effectiveness of different environmental, managerial and feeding strategies that can be adopted in dairy herds to improve cows health without interfering with the quality of their milk production.