UNIVERSITÀ CATTOLICA del Sacro Cuore

Dottorato per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

cycle XXXI

S.S.D: VET/02; AGR/18; AGR/17

New insights on metabolism and the immune response from dry-off to early lactation

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Academic Year 2017/2018

ABSTRACT

Immune system is made of a variety of cells, molecules and biological processes that interacts to prevent microbial invasions, recognize foreign molecules and eliminate existing sources of cellular injuries to restore tissues to their normal functions once problem has been solved. Innate immunity is the primary defense line against pathogens invasions. Its functioning typically undergoes severe alterations during transition period (**TP**) of dairy cows. An impairment of polymorphonuclear cells (**PMN**) functions related to reactive oxygen metabolites (**ROM**) production, myeloperoxidase (**MPO**) activity, chemotaxis and phagocytosis has been reported in this phase. Bovine PMN have an altered abundance in mRNA transcripts encoding for such functions between -1 and 2 weeks from calving, in comparison to the level found at 4 weeks after calving for the same genes. The exact cause of immune dysfunctions occurring in peripartum has never been clearly identified. Reduced immune competence could arise from the interaction of different factors affected from the typical peripartum trends (i.e. changes in endocrine asset, limitations of maternal immune responses against the allogeneic conceptus, alterations in energy balance and oxidative stress status). Nevertheless, its duration could be modified when peripartal changes exceed the control of homeorhetic and homeostatic mechanisms, leading to the physiological imbalance (**PI**) condition. Such a condition could also trigger the inflammatory-like status. It consists in a prepartal raise of pro-inflammatory cytokines (**PICs**) levels, that is linked to a raise in body temperature at calving, and that typically affects liver metabolism, implying severe losses in hepatic functions and a shift of anabolic priority of the organ in early lactation. The liver produces more α-globulins, known as positive acute phase proteins (**APP**), i.e. haptoglobin, ceruloplasmin and serum amyloid alpha (**SAA**). Conversely, it reduces the synthesis of albumin, retinol binding protein, paraoxonase (**PON**) and lipoproteins, known as negative APP and sequesters minerals, as zinc and iron, from blood flow. Inflammation lead to the activation of PMN, while the reduced immune competence commonly reported in TP has been associated to an opposite effect on leukocytes. Thus, these should be considered as two

distinct phenomena, but they could arise from a common cause with a different magnitude and duration. Nutritional strategies to optimize dairy cow's immunity during TP should be focused on reducing the PI degree related to calving, as this condition could be referred as a common denominator between immune dysfunction and diseases causes. Among such nutritional strategies, the correct management of energy sources to fit with altered requirements should be considered. Furthermore, fatty acids profile of lipid sources administered could also modify immune functions. Finally, the administration of supplementary products exerting antioxidant or anti-inflammatory activities, as well as methyl donors species, could be beneficial for dairy cows immunity in TP. In a wider perspective, although feed additives and nutritional strategy could be effective in mitigate immune alterations, we can conclude that adoption of proper management practices aimed to avoid PI condition in peripartal period of dairy cows could be the most effective strategy to prevent dysfunctions.

Three experiments have been designed to elucidate the linkage between sudden changes occurring in peripartum and immune alterations in dairy cows. Throughout such experiments Holstein dairy cows were housed in tied stalls and monitored regularly for body condition score (**BCS**), body weight (**BW**), dry matter intake (**DMI**), milk yield (**MY**) and rumination time. Blood samples were collected regularly to assess a wide hematochemical profile and to test white blood cell functions through *ex-vivo* challenges. Furthermore, PMN diapedesis has been tested *in-vivo* through a carrageenan-skin test and rumen samples were collected at 30 days from calving (**DFC**).

The first experiment was aimed in investigate the main causes of metabolic changes occurring at dry-off and the contribution of MY in such alterations. In fact, dry-off is related to deep changes in feeding behavior, gastro intestinal adaptations, metabolism and immune parameters in high-yielding cow's career. Indeed, the release of cortisol, signals of systemic inflammation and altered redox balance have been reported immediately after milking interruption, and high MY have a role in aggravating such conditions. In our study, a group of 13 Holstein dairy cows were dried off at 55 days from expected calving day, and regularly monitored from -7 to 34 days from dry-off (**DFD**).

Animals were retrospectively divided in two groups according to their average MY in the last week of lactation, assuming a cut-off of 15 kg*d^{-1}: low MY (6 cows) and high MY (7 cows). Data were submitted to ANOVA using a mixed model for repeated measures including MY at dry-off, time and their interaction as fixed effects. Increased fiber amounts of dry ration reduced DMI and increased rumination time. Leukocytes migration into mammary gland to contribute in the involution phase decreased their abundance in blood at dry-off, and their activity. Such activation of leukocytes at mammary site increased the abundance of nitrogen species in plasma and triggered a systemic inflammation in all the animals, as reflected from increased concentrations of positive and reduced concentrations of negative APPs. Such inflammation impaired liver functions, as suggested from the increased gamma-glutamyl transferase (**GGT**), bilirubin and alkaline phosphatase (**ALP**) concentrations. Both the production of nitrogen species and the systemic inflammatory status contributed in the depletion of antioxidant system in blood (thiol groups -**SHp**-, tocopherol, β-carotene, ferric reducing antioxidant power -**FRAP**- and oxygen reactive antioxidant capacity -**ORAC**-). Animals with higher MY at dry-off showed the worst condition, likely for the deeper metabolic changes they faced at milking interruption, and to the greater amount of mammary parenchyma to be reabsorbed. This study highlights the dry-off as a thorny point to manage dairy cows' health and depose for a relationship between dry-off and immune alteration that typically occurs at calving.

The second experiment was aimed in investigate changes occurring in the immune system prior to ketosis onset to elucidate their role in disease occurrence. Thus, a group of 13 Holstein dairy cows were monitored from -48 to 35 DFC and retrospectively divided into 2 groups basing on their plasma BHB levels: lower (**CTR**; 7 cows) or higher than 1.4 mMol/L (**KET**; 6 cows). Data were submitted to ANOVA using a mixed model for repeated measures including health status, time and their interaction as fixed effects. KET cows had a greater activation of the immune system prior to calving (higher plasma concentrations of PICs, myeloperoxidase and oxidant species, and greater interferon gamma responses to *Mycobacterium avium*) impaired liver functions (higher blood

concentration of GGT) and lower plasma minerals. High plasma NEFA, BHB and glucose levels in KET cows suggest an insulin resistance status and a marked mobilization of body fat occurring during dry period. They were also associated to reduced DMI around calving and worse negative energy balance in early lactation. This caused in turn reduced MY and increased fat mobilization in early lactation. Impairment of liver function and activation of leukocytes during the dry period accentuated the acute phase response in KET cows after calving (greater concentrations of positive APPs and lower concentration of retinol binding protein), further impairing liver function (higher blood concentrations of glutamate-oxaloacetate transaminase -**AST**-**GOT**- and bilirubin). Leukocytes of KET cows had reduced inflammatory functions after an ex vivo stimulation assay (lower production of PICs and greater production of lactate). These alterations on WBC could be driven by the combined action of metabolites related to the mobilization of lipids and of anti-inflammatory actions aimed to prevent over exuberant inflammation. This suggests that prepartal trends of immune parameters be highly related with the likelihood of developing diseases in early lactation.

The third experiment consisted in the administration of Omnigen-AF (**OAF**), an immune stimulant that is effective in increasing leukocytes functions in immunosuppressed animals and in reducing incidence of infectious diseases in early lactating dairy cows. Its mode of action has never been elucidated, and a wider perspective of its metabolic effect could highlight its effectiveness in facing metabolic disorders of transition period also. Thus, a group of 10 Holstein dairy cows were divided into 2 groups: treated group (**TRT**; 5 cows) received 32.5 g of Omnigen-AF® (Phibro Animal Health Corporation) twice a day (65 g d^{-1}) as top-dress on the morning and afternoon feeds, while control group (**CTR**; 5 cows) did not receive any supplementation. From -62 to 42 DFC animals were monitored regularly. Data were submitted to ANOVA using a mixed model for repeated measures including treatment, time and their interaction as fixed effects. Administration of OAF at dry-off did not affect BW, BCS, milk yield, milk and rumen fluid composition, and neither affected blood neutrophils concentrations. Nevertheless, it increased rumination time and improved

the energy metabolism after calving (lower NEFA and BHB concentrations). TRT cows had an increased lymphocytes abundance at blood level, and their leukocytes had greater efficiency in facing biological stressors during the peripartum (lower lactate production and lower glucose consumption after a challenge with bacterial lipopolysaccharides). Despite these positive effects on immune cells, OAF did not affect the positive APPs concentrations after calving. A reduced abundance of albumin, PON and antioxidants also occurred with OAF after calving, suggesting some impairment of hepatic functions to occur. Nevertheless, the lack of any effect on main biomarkers related to liver function (bilirubin) and liver damage (GGT, AST-GOT, ALP) dismisses a real impairment of liver activities to occur with OAF. Positive effects in favoring the recovery of rumen functions, reducing mobilization of body fats after calving suggest OAF to be an effective strategy in preventing metabolic disorders of transition period.

Key words: Immune dysfunctions, peripartum, dairy cows, innate immunity, dry-off, inflammation, metabolic stress, immune response, lipid metabolism, liver function, immune stimulant, metabolic disorders, transition period

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CHAPTER I - IMMUNE DYSFUNCTIONS AND PARTURITION

The immune system of dairy cows: an in-depth analysis on alterations occurring from dry-off to early lactation

ABSTRACT

Immune system is made of a variety of cells, molecules and biological processes that interacts to prevent microbial invasions, recognize foreign molecules and eliminate existing sources of cellular injuries in order to restore tissues to their normal functions once problem has been solved. Innate immunity is the primary defense line against pathogens invasions. Its functioning typically undergoes severe alterations during transition period (**TP**) of dairy cows. An impairment of polymorphonuclear cells (**PMN**) functions related to reactive oxygen metabolites (**ROM**) production, myeloperoxidase (**MPO**) activity, chemotaxis and phagocytosis has been reported in this phase. Bovine PMN have an altered abundance in mRNA transcripts encoding for such functions between -1 and 2 weeks from calving, in comparison to the level found at 4 weeks after calving for the same genes. The exact cause of immune dysfunctions occurring in peripartum has never been clearly identified. Reduced immune competence could arise from the interaction of different factors affected from the typical peripartum trends (i.e. changes in endocrine asset, limitations of maternal immune responses against the allogeneic conceptus, alterations in energy balance and oxidative stress status). Nevertheless, its duration could be modified when peripartal changes exceed the control of homeorhetic and homeostatic mechanisms, leading to the physiological imbalance (**PI**) condition. Such a condition could also trigger the inflammatory-like status. It consists in a prepartal raise of pro-inflammatory cytokines (**PICs**) levels, that is linked to a raise in body temperature at calving, and that typically affects liver metabolism, implying severe losses in hepatic functions and a shift of anabolic priority of the organ in early lactation. The liver produces more α-globulins, known as positive acute phase proteins (**APP**), i.e. haptoglobin, ceruloplasmin and serum amyloid alpha (**SAA**). Conversely, it reduces the synthesis of albumin, retinol binding protein, paraoxonase (**PON**) and lipoproteins, known as negative APP and sequesters minerals, as zinc and iron, from blood flow. Inflammation lead to the

activation of PMN, while the reduced immune competence commonly reported in TP has been associated to an opposite effect on leukocytes. Thus, these should be considered as two distinct phenomena, but they could arise from a common cause with a different magnitude and duration. Nutritional strategies to optimize dairy cow's immunity during TP should be focused on reducing the PI degree related to calving, as this condition could be referred as a common denominator between immune dysfunction and diseases causes. Among such nutritional strategies, the correct management of energy sources to fit with altered requirements should be considered. Furthermore, fatty acids profile of lipid sources administered could also modify immune functions. Finally, the administration of supplementary products exerting antioxidant or anti-inflammatory activities, as well as methyl donors species, could be beneficial for dairy cows immunity in TP. In a wider perspective, although feed additives and nutritional strategy could be effective in mitigate immune alterations, we can conclude that adoption of proper management practices aimed to avoid PI condition in peripartal period of dairy cows could be the most effective strategy to prevent dysfunctions.

Key words: Immune dysfunctions, peripartum, dairy cows, innate immunity

IMMUNE SYSTEM OVERVIEW

Immune system is made of a variety of cells, molecules and biological processes that interacts to prevent microbial invasions, recognize foreign molecules and eliminate existing sources of cellular injuries in order to restore tissues to their normal functions once problem has been solved (Sordillo, 2016). Two main defense lines could be distinguished according to the specificity of their mode of action and on the time course of their activation during a defensive process.

Innate immunity

Innate immunity is the primary line, which provides an immediate and nonspecific defense against any tissue injury and a variety of potential invading pathogens. It acts within seconds from the initial damage, as its components are already present or are activated quickly at the site of pathogen exposure. Its activation results in the inflammatory response, that induces alteration of vascular endothelium, leading to characteristic symptoms in affected tissues. Redness, heat and pain results from increased blood flow caused by enlarged vascular diameter. Swelling or edema results from the exit of fluid and proteins from the blood and their accumulation in tissues, consequently to the separation of tightly joined endothelial cells of blood vessels walls (Ryman et al., 2015). Innate immunity consists in a wide range of physical barriers, receptors, soluble and cellular components (phagocytes).

Physical barriers. Physical barriers as skin, tears and mucus exert a mechanical action against infectious agents and are essential to avoid the pathogens entering the body.

Recognition pathways. Once physical barriers have been compromised, the recognition of damaging agents is required to activate body defenses. Recognition is driven by factors known as pathogen-associated molecular patters (**PAMPs**) that are expressed on the surface, secreted or expressed intracellularly in invading bacteria, and damages associated molecular

patterns (**DAMPs**) that are expressed in hosts cells undergoing a tissue damage or apoptosis condition (Jungi et al., 2011; Kumar et al., 2011; Sordillo, 2016; Trevisi et al., 2016). Examples of PAMPs include lipopeptides of Gram-positive bacteria and lipopolysaccharides (**LPS**) of Gram-negative bacteria, while DAMPs include uric acid and extracellular ATP. Recognition occurs through a complex of proteins acting synergistically to identify the cause of damage: patterns of recognition receptors (**PRRs**) and cluster of differentiations (**CDs**). Once recognition occurred, these proteins trigger an intracellular signaling cascade that directly facilitate antimicrobial activity or activate cellular and soluble components of innate immunity.

PRRs are proteins located on both non-immune and immune cells (ascribed to both innate and adaptive immunity). Main PRRs are summarized in Table 1. Toll-like receptors (**TLRs**) are single membrane spanning proteins that are the most studied PRRs in mammals. Ten different TLRs have been identified in the bovine (McGuire et al., 2006). Family of TLRs includes TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13. C-type lectin receptors (**CLRs**) are membrane proteins involved in the recognition of carbohydrates (i.e. beta-glucans and mannose) through a calcium domain. Nucleotide binding oligomerization domain-like receptors (or Nod-like receptors - **NLRs**) are plasmatic proteins which act as intracellular sensors of PAMPs that enter the cell via phagocytosis or pores (as bacterial peptidoglycans), and DAMPs associated with cell stress. They include class II major histocompatibility complex transactivator (**CIITA** - that is involved in the activation of major histocompatibility complex of class II), IPAF and inhibitor of apoptosis domain-containing protein 1 (**BIRC1**). Retinoic acid-inducible gene (**RIG**)-like receptors (**RLRs**) are helicases involved in the release of pro inflammatory cytokines and type I interferons against RNA and DNA viruses. They include RIG-I (that binds short 5'-

phosphate-double strained RNA), melanoma differentiation-associated protein 5 (**MDA5** - that binds long double strained RNA) and LGP2 (Takeuchi and Akira, 2010).

CDs are cell-surface molecules (receptors or ligands) that could be used to distinguish between leukocytes classes (immunophenotyping). Main CDs utilized with this aim include CD4 (helper T-cells), CD8 (cytotoxic T-cells) and CD14. They are involved in cell signaling or adhesion of innate immune cells and act synergistically with PRRs in the recognition pathway. As an example, LPS binding to TLR4 in complex with CD14 and LPS-binding protein induce inflammatory pathways as the signaling cascades related to mitogen-activated protein kinase and nuclear factor-κB (**NF-κB**).

Soluble components. Several endogenous defenses could act against invading pathogens once they have been recognized. Some of those are preexisting, while some others are directly induced from the activity of recognition pathways.

Complement is contained in serum and body fluids and consists in a complex of proteins that are synthesized mainly from hepatocytes, but also from monocytes and tissue macrophages (Sordillo, 2016). After its activation through PRR, complement triggers a cascade within its effector molecules, as complement factors C3, C5a and C5b-9 complex, creating pore-forming complexes with a direct antimicrobial action against bacterial cells (Hajishengallis and Lambris, 2010). Complement also have a role in opsonizing bacteria, in the recruitment of phagocytes and in the activation of their phagocytosis and bacterial killing activities (Rainard, 2003).

Cytokines are soluble proteins with a very short half-life that are produced by both immune and nonimmune cells. They could interact with each other synergistically, additively or antagonistically and high affinity receptors for each cytokine are expressed on host cells (Sordillo, 2016). Their effect is driven, other than the quantity and type of cytokine expressed, from the relative expression of receptors on their target cells (Sordillo and Streicher, 2002;

Bannerman, 2009). Cytokines could be divided in three main groups according to their effects on inflammatory processes (Table 2). Pro inflammatory cytokines (**PIC**) are rapidly secreted at the beginning of the infection and are aimed to enhance inflammatory response (Ryman et al., 2015). Their systemic effects include the increase in body temperature and hearth rate and the decrease in feed intake (Dantzer and Kelley, 2007). They include tumor necrosis factor, alpha (**TNFA**), interleukin-1, beta (**IL-1B**), interleukin-2 (**IL-2**), interleukin-8 (**IL-8**), interleukin-12 (**IL-12**), interleukin-17 (**IL-17**), interferon alpha (**IFNA**) and gamma (**IFNG**). Anti-inflammatory cytokines (**AIC**) are typically related to the final phase of infection and they are involved in solving the inflammatory process. They include interleukin-4 (**IL-4**) and interleukin-10 (**IL-10**). Third class of cytokines could exert both pro and anti-inflammatory actions, depending on the infection phase at which they are released (Droogmans et al., 1992). They include interleukin-6 (**IL-6**). Release of cytokines is mainly induced from the signaling cascade related to the activation of NF-κB (Takeuchi and Akira, 2010) that lead to the expression of different patterns of cytokines based on the ligand (PAMP or DAMP) that has been recognized. LPS (PAMP related to Gram negative bacteria) lead to a greater expression of PIC, while lipopeptides (PAMP related to Gram positive bacteria) express a slower cytokines response during the early stages of infection (Bannerman, 2009).

Oxylipids are lipid mediators that are mainly synthesized from the oxidation of polyunsaturated fatty acids (**PUFA**) of cell membranes through processes that could be both non-enzymatic, and triggered by reactive oxygen metabolites (**ROM**), or enzymatic, and triggered by cyclooxygenases (**COX**), lipoxygenases (**LOX**) or cytochrome P-450. Two main classes of oxylipids were classically distinguished based on the type of PUFA they came from. Omega-6 derived oxylipids that originate mainly from arachidonic and linoleic acids and include prostaglandins (**PG**), thromboxane (**TX**), leukotrienes (**LT**) and lipoxins (**LX**). Omega-3 derived oxylipids that originate from eicosapentaenoic acid (**EPA**) and

docosahexaenoic acid (**DHA**) and include protectins and resolvins (Raphael and Sordillo, 2013). These compounds regulate the onset, magnitude and duration of inflammatory response, exerting both a pro or anti-inflammatory action depending on the timing and magnitude of their expression. Historically it was thought that Omega-6 derived oxylipids were pro-inflammatory, while Omega-3 derived oxylipids were anti-inflammatory (Serhan and Chiang, 2009). Nowadays, a more complex relationship between oxylipids and inflammation is emerging, as biosynthetic profiles of oxylipids and their effects have been related to both the availability of diverse PUFA and the timing of their metabolism through various oxidizing pathways (Table 3; Sordillo, 2016).

Phagocytes. Phagocytes are white blood cells (WBC) that exert a common anti-bacterial action via phagocytosis at the infection site (Paape et al., 2002). It consists in the engulfment of bacteria in a phagosome followed by killing and the elimination of wastes with exocytosis. Bacterial killing occurs with the production of hydrolytic enzymes or highly toxic products, such as ROM. These result from respiratory burst, a reaction triggered from a membranal nicotinamide adenine dinucleotide-phosphate (NADPH) oxidase, which reduce oxygen and produce superoxide anion and hydrogen peroxide. Other ROM sources includes the combination of hydrogen peroxide with chloride triggered from the enzyme myeloperoxidase (MPO) to obtain hypochlorite. Phagocytes includes three main cellular classes that could be present in the infection site or recruited from blood flow.

Macrophage are mononucleate WBC located in peripheral tissues and aimed to detect, recognize and kill pathogens. Their activation via PRR induces the production of pro inflammatory oxylipids and cytokines as IL-1B, IL-6 and TNFA, that acts on vascular endothelium to reduce blood velocity and increase the expression of adhesion molecules on endothelial cells, in order to recruit polymorphonuclear cells (**PMN**) to the infection site (Ryman et al., 2015). Macrophages also provide a connection between innate immunity and

specific defenses, as they are involved in the antigen presentation to primary T-cells (Rainard and Riollet, 2006).

Dendritic cells (**DCs**) act as messengers between innate and adaptive immune responses, migrating into lymph nodes from tissues that are in contact with external environment (i.e. skin, nose, lung, stomach and intestine) once activated. Similar to macrophages, they act as antigen presenting cells in lymph nodes, activating lymphocytes and adaptive immune response (Mackensen et al., 2000; Sordillo, 2016). They are involved in the production of cytokines as IL-12, IL-23 and IFNA (**Table 2**).

PMN includes neutrophils and eosinophils. Those cells act as sentinels, monitoring blood flow through chemotaxis and searching for signals of bacterial invasion in peripheral tissues. This activity largely depends on L-selectin (**SELL**), a surface protein that allows PMN slow rolling along the interior vessel walls. Once PIC and oxylipids produced from activated macrophages are detected, they trigger the secretion of IL-8 by epithelial cells (Table 2). This PIC stimulate the production of adhesion molecules that stop PMN rolling along the vessel and allows their migration to peripheral tissues (Suriyasathaporn et al., 1999; Paape et al., 2002). This process is named diapedesis and consists in the movement of PMN between endothelial cells and across the basement membrane of the vessel to infected sites (Maddox et al., 1999; Hodgkinson et al., 2007). Once inflammatory response starts, neutrophils became the main cell type into affected tissues within 30 to 60 minutes from the beginning of extravasation (Summers et al., 2010). Furthermore, IL-8 increase the production of ROM to enhance respiratory burst activity against bacteria (Paape et al., 2003; Forsberg, 2004). PMN use also neutrophil extracellular traps to kill bacteria, consisting in a net of fibers of chromatin and serine proteases that trap and kill bacteria, acting as a physical barrier to avoid their spread through tissues (Sordillo, 2016).

Adaptive immunity

When innate immune system and inflammation are ineffective in solving infection, the presentation of antigens mediates the activation of adaptive immunity. Generation of adaptive immunity is time consuming (could take days for developing), and the efficiency of innate immunity became crucial to face infections in the meantime (Janeway et al., 2005). Innate immunity involves two types of cells: antigen presenting cells and lymphocytes (Sordillo, 2016). The result of their interaction is a response that is specific and diverse for each antigen involved, that could distinguish between self and non-self-antigens and allow to develop a memory component against repeated exposition to the same antigen.

Antigen presenting cells. Adaptive immunity can selectively react to foreign antigens only, without responding to host's own antigens (Sordillo, 2016). This recognition is driven from membrane bound proteins named major histocompatibility complexes (MHCs). Those complexes could trigger an immune response only when combined with antigens on specific cells, in a process known as antigen presentation (Kazansky, 2008). MHC could be differentiated in two main classes. MHC of class I is expressed in all host cells, while MHC of class II is expressed on antigen presenting cells ascribed to innate immunity (as macrophages and DCs) and lymphocytes (as mature B cells).

Lymphocytes. Adaptive immunity consists in mononuclear leucocytes named lymphocytes. Those recognize bacterial antigens through membrane receptors and provides a specific protection through the expression of antibodies (Shafer-Weaver and Sordillo, 1997). Lymphocytes could be divided in T and B-cells that differ in functions and protein products (Table 4).

T-cells are a wide group of lymphocytes that could be further distinguished in two sub-groups. αβ-T-cells includes two classes of lymphocytes, distinguished based on the expression of different CDs on their surface. T-helper cells (**Th**) are differentiated based on the

expression of CD4 (but not CD8) on their surface. They are involved in the production of cytokines, as IL-2 and IFNG (Table 2). These mediators regulates magnitude and duration of cell-mediated immune response, exerting a role in the activation of other lymphocytes, but also affects innate components, acting on macrophages, neutrophils and other cells that participate in immune response (Sordillo and Streicher, 2002). Th lymphocytes could be further differentiated in functional groups that includes Th1, Th2, Th17 and T-regulatory cells (**Treg**). The proportion of these can vary significantly depending on tissue localization (Sordillo, 2016).

T-cytotoxic cells (**Tc**) are differentiated based on the expression of CD8 and negative expression of CD4 on their surface. They are aimed to the recognition and elimination of host cells infected with antigens, based on the alteration of MHC I. Furthermore, they are involved in apoptotic processes in old and damaged host cells, in order to decrease the susceptibility of the organism to infections. Their action could have a scavenger effect on secretory cells in mammary gland (Sordillo, 2016). They also produce cytokines involved in the regulation of the immune response (Aitken et al., 2011). γδ-T-cells has not been fully elucidated in their function, but their percentage is higher in bovine peripheral blood mononuclear cells compared with humans (Baldwin and Telfer, 2015). They are involved in the protection of epithelial surfaces against bacterial diseases and mediate cytotoxicity with variable involvement of MHCs. Their action is based on unique surface molecules (WC1) that are co-receptors for recognition of microbial pathogens and signaling cell activation.

B-cells are lymphocytes that proliferate and differentiate in two sub-groups once activated from antigens (Shafer-Weaver and Sordillo, 1997). The first sub-group, named plasma-cells is involved in the production of antibodies, known as immunoglobulins (**Igs**) that recognize and act against specific microbial virulence factors influencing host defenses against pathogens. Igs are differentiate into several functional isotypes and their abundance and composition

within tissues affect the likelihood of developing new infections. IgA are present at mucosal surfaces and are involved in agglutination, which impede certain pathogens (i.e. viruses) to spread through tissues. Furthermore, they are able to neutralize some bacterial toxins (Sordillo and Streicher, 2002; Aitken et al., 2011). IgG are highly concentrated in serum and have a role as opsonin, increasing phagocytosis in PMN. Two sub-isotypes could be further distinguished: IgG1 and IgG2. IgE are associated with allergic reactions and parasitic infections. IgM is the largest and first produced Ig and exert a role in opsonization, agglutination and complement fixation. IgD is a non-secreted regulatory molecule. The second sub-group, named memory cells, is involved in the heightened state of immune reactivity and clonal expansion of antigen-specific effector cells that occurs when host cells and tissues are re-exposed to the same antigen. This memory response is much faster, stronger, longer lasting and more effective in solving infection compared with primary responses (Sordillo, 2016).

PERIPARTURIENT PERIOD AND IMMUNE DYSFUNCTIONS

In dairy cows, peripartum includes last two months of gestation and first month of lactation. Within peripartum, the three weeks prior to calving and the three weeks following parturition are named transition period (**TP**) and are known as the most challenging phase during life of all the mammals (Drackley, 1999). For dairy cows, such challenge arise from the transition between two phases of life that differ on endocrine and physiological assets and nutritional requirements: the dry period, that includes the time between halting of milk removal (milk stasis) and the subsequent calving, and the early lactation (Van Knegsel et al., 2014).

Peripartum alteration of metabolic functions

Sudden changes in body homeostasis are to a certain extent physiological during the TP. Peripartum is known to be related to altered endocrine asset, as trend of different hormones changes during transition from lactation to dry period and, even more, at calving time. Steroids, as estradiol and progesterone, are known to fluctuate before calving, while protein homeorhetic hormones such as prolactin, growth hormone (**GH**), insulin-like growth factor-1 (**IGF-1**) and insulin are known to fluctuate during the whole TP (Bauman and Bruce Currie, 1980; Tucker, 1985). Such hormonal changes typically accompany drastic alteration in physiological assets and nutrient requirements. Cows entering dry period are in a positive energy balance (**PEB**) condition (Mäntysaari and Mäntysaari, 2015), that is further accentuated by the reduction in energy requirements when milk production is halted. In late dry period, three weeks prior to calving, two-thirds of the development of fetus are completed and its nutrient requirements reach maximal levels, taking the priority in the redistribution of glucose and amino acids on the cow's own needs for body maintenance (Bell, 1995; Dingwell et al., 2001). As soon as milk production starts, mammary gland changes metabolic asset to

achieve the maximum dairy cow's potential for milk yield, milk proteins, fat and lactose, inducing a drastic increase in energy, protein and mineral requirements. Such an increase in requirements could not be satisfied from the increase in feed intake in early lactation, that is slowed down from the reduced rumen volume at the end of dry period. Furthermore, drastic changes in diet composition from a forage-based to a concentrate reach ration could lead to alteration in rumen microbiota and fermentations reducing feed intake. Those conditions makes requirements to exceed nutrients supplied with feed intake, leading to a physiological negative energy balance (**NEB**) that has been quantified in an average value of -12,2 Mcal/d in Holstein dairy cows (Rastani et al., 2001) and to a mineral deficiency, in particular for calcium and magnesium (Mulligan and Doherty, 2008).

Main effect of NEB at the beginning of lactation is the consumption of blood glucose, leading to a sudden mobilization of reserves to face energy requirements with alternative sources. Lipomobilization consists in a reduced lipogenesis and an increased lipolysis ratio resulting in the brake down of triglycerides stored in the adipose tissue. Therefore, hematic levels of non-esterified fatty acids (**NEFA**) and glycerol increase. In normal conditions, these are completely oxidized in liver to acetyl coenzyme A, that enter the Krebs cycle to produce energy. Other than increased amounts of total circulating NEFA, lipomobilization that occurs in early lactation has been related to changes in fatty acids compositions both in blood circulating NEFA and in phospholipidic membranes of cells and circulating lipoproteins (Drackley, 1999). Saturated fatty acids (**SFA**) increases in comparison to mid lactation, and account for 35.7% of total plasmatic long chained-fatty acids after calving. In normal conditions, palmitic (C16:0) and stearic acids (C 18:0) are the main SFA in cell membranes of dairy cows (Contreras et al., 2010). Nevertheless, membranal amounts of C16:0 and C 18:0 decreases during TP in comparison to mid-lactation, as several other SFA including lauric (C12:0) and myristic acids (C14:0) increase (Drackley, 1999). On the other hand, unsaturated

fatty acids (**UFA**) account for 64.3% on total plasmatic long chained-fatty acids at the beginning of lactation. In cell membranes, they mainly includes oleic and linoleic acids, while the amounts of eicosatrienoic acid (C20:3) and EPA (C20:5) are known to decrease in cell membranes in comparison to mid-lactation (Douglas et al., 2006). Contemporaneously to lipomobilization, also a massive amino acids mobilization from muscle tissues occurs to face growing requirements of mammary gland for the synthesis of milk and new parenchymatic tissue (Doepel et al., 2006).

The physiological imbalance conditions

Changes occurring in peripartum could exceed the control of homeorhetic and homeostatic mechanisms, leading to a physiological imbalance condition (**PI**). During PI, physiological parameters could shift from their normal ranges, impairing body functions. PI could occur in cows with a milk yield higher than 25 kg/d at dry-off, as a consequence of the high WBC activity during the early involution phase (Putman et al., 2018). Results of a recent study (Bertulat et al., 2013) showed also a raise of fecal cortisol (measured as 11,17-dioxoandrostane) to occur immediately after dry-off in these animals, as an effect of increased udder pressure at milking interruption. Another cause of PI could be the administration of acidogenic diets during close-up, that could affect the mineral equilibrium in TP, especially for calcium and magnesium (Nightingale et al., 2015). Furthermore, PI in TP could result from stress induced by pen movements, re-socialization, environment, weather, diet and simply parturition, as these phenomena are related to raises in glucocorticoids (cortisol) and catecholamines (epinephrine, norepinephrine, dopamine) concentrations (Higuchi et al., 1994; Do Nascimento et al., 2004; Cooke et al., 2012). Nevertheless, most of PI conditions are related to energy balance, feed intake and lipomobilization in early lactation. As an example, excessive lipid deposition that arise from a prolonged dry period, or severe NEB that arise

from high milk yields, could increase the magnitude and duration of mobilization at the beginning of lactation. Higher NEFA can decrease appetite, as NEFA exert anorexic effects, reducing dry matter intake (**DMI**) in early lactation and further reducing glycaemia (Ingvartsen and Andersen, 2000; Allen et al., 2009). Another cause of reduced DMI could be the occurrence of infectious diseases, as mastitis or uterus infections related to calving, that triggers the release of PIC with anorexic effects (Trevisi et al., 2011a; Jahan et al., 2015). As soon as lipomobilization became severe, the Krebs cycle gets overloaded by NEFA, leading to their partial oxidation and to the diversion of acetyl coenzyme A to the production of ketone bodies as beta-hydroxybutyrate (**BHB**), acetoacetic acid and acetone (Drackley et al., 2006; Ingvartsen, 2006). Furthermore, a larger amount of amino acids is used for gluconeogenesis to provide energy, reducing their availability for other metabolic functions. Glutamine is one of the main amino acids that undergoes a deficiency in early lactation, as up to 74% of its stocks are completely oxidized for energy production (Newsholme et al., 1985, 1999; Calder et al., 1990).

Reduced immune competence

Reduced immune competence typically affects innate immune cells during TP. An impairment of PMN functions related to ROM production, MPO activity, chemotaxis and phagocytosis has been reported in this phase (Cai et al., 1994; Kimura et al., 1999; Mehrzad et al., 2001). Most of these changes are reflected in altered expression of messengers-RNA (**mRNA**) transcripts, even though such alterations have not been fully elucidated. Previous studies found bovine PMN to have an altered abundance in several mRNA transcripts (see below) between -1 and 2 weeks from calving, in comparison to the level found at 4 weeks after calving for the same genes (O'Boyle et al., 2012; Crookenden et al., 2016, 2017).

A down regulation during the whole TP (Table 5) has been reported for genes related to normal cells functions, as for glucose transporters (solute carrier family-2 member 1), proteins involved in cell survival and differentiation (CD117, CD45, CD337, galectin S8 and annexin-A3) and in the regulation of apoptosis (as protein kinase C beta, BCL2 apoptosis regulator and Fas-ligand). Nevertheless, most of mRNA to be down regulated encode genes related to immune functions, as adherence and migration (integrins family), antioxidant systems (superoxide dismutase 1), generation of oxidant species within the respiratory burst (MPO), for the main pro-inflammatory mediators (PIC as IFNG, IL-12, TNFA and IL-23 and enzymes related to the production of pro-inflammatory oxylipids) and regulator proteins for the antigen presentation (X-box binding protein 1). Same studies found this down regulation to be counterbalanced by a greater expression of other genes (Table 6). This occurs exclusively at calving time for genes related to antimicrobial activity (S100 calcium binding protein A9, beta defensins 1 and 10, integrin alpha-X, peroxisome proliferation activated receptor delta and retinoid-X-receptor alpha), anaerobic glycolysis (lactate dehydrogenase A) and pro inflammatory oxylipids production (phospholipase A2). On the other hand, an up regulation of genes related to anti-inflammatory functions (AIC as IL-10 and IL-6 and receptors that inhibits the activity of PIC, as IL-1B receptor type II) and immunomodulation (defensin beta 4A) has been reported during the whole TP, especially after calving. Finally, other genes were reported to have characteristic variations throughout the TP, being both up or down regulated in comparison to their levels at 4 weeks after calving (Table 7). Receptors involved in the production of PIC through NF-κB (receptor-interacting serine/threonine-protein kinase 1 and TLR4), receptors for Igs (CD16a and Fc-fragment of IgE, high affinity I, receptor for gamma polypeptide), PIC inhibition (Interleukin 1 receptor antagonist) and PMN migration (SELL, interleukin-8 receptor beta), as well as proteins involved in the regulation of apoptosis and cell functions (MCL1 apoptosis regulator, BCL2 family member and ATP

binding cassette subfamily A member 1) were up regulated before calving and down-regulated thereafter. For other genes related to cell-to-cell interactions, induction of apoptosis and inflammation (CD90) and involved in PMNs migration (Matrix metallopeptidase 9) an opposite pattern has been reported: they were down regulated before calving and up regulated thereafter.

Despite the patterns of some genes appeared somehow contradictory, and a transient amelioration of some antibacterial functions could be hypothesized at calving time, the general trend of such variations suggests an impairment of viability, survival and sensitivity to pro-inflammatory mediators in PMNs throughout the TP. Furthermore, their anti-bacterial activities related to chemotaxis, phagocytosis and respiratory burst were also impaired, while their sensitivity to oxidative stress status increased due to a reduced efficiency in their antioxidant systems. Thus, a delayed migration of functionally adequate neutrophils and other innate immune factors could trigger a hypo-responsive state that decrease the capacity of innate immune system to face insults (Sordillo, 2016), and such a condition increase the likelihood to develop diseases in TP (Minuti et al., 2015).

The inflammatory-like status

In dairy cows, an inflammatory-like status commonly occurs during peripartum. It consists in a prepartal raise of PIC levels (Jahan et al., 2015), that is linked to a raise in body temperature at calving, and that typically affects liver metabolism, implying severe losses in hepatic functions and a shift of anabolic priority of the organ in early lactation (Cappa et al., 1989; Trevisi et al., 2005; Loor et al., 2007; Bertoni et al., 2008). In particular, the liver produces more α-globulins, known as positive acute phase proteins (**APP**), i.e. haptoglobin, ceruloplasmin and serum amyloid alpha (**SAA**) (Ceciliani et al., 2012). Conversely, it reduces the synthesis of albumin, retinol binding protein, paraoxonase (**PON**) and lipoproteins, known

as negative APP (Bertoni et al., 2008) and sequesters minerals, as zinc and iron, from blood flow (Bertoni and Trevisi, 2013). Plasmatic trends of those parameters are proportional to the severity of inflammation (Castell et al., 1989), but they have different sensitivity to acute phase, as they are released in different time intervals from the onset of the inflammation. Haptoglobin in particular, have a long half-life relative to other APP (i.e. SAA) and reflects instantaneously an inflammatory condition and thus, it has been widely used as a biomarker of such a condition in dairy cows (Huzzey et al., 2011). Effects of inflammation on WBC functions has been investigated by Nightingale et al. (2015), who grouped 240 Holstein transition dairy cows in three classes according to their postpartal haptoglobin level: low (0 to 8.4 µg/mL), moderate (8.5 to 458 µg/mL) and high (459 to 1757 µg/mL). They found animals with highest haptoglobin levels after calving to have the most severe neutropenia in blood that was driven from the recruitment of their PMN in inflamed peripheral tissues. Furthermore, their PMN showed the greatest activated status, as they showed the greatest production of PIC (TNFA), the highest expression of extravasation-related receptors (SELL) and the greatest respiratory burst activity. Nevertheless, T-lymphocytes and adaptive immunity appeared to be impaired in these animals, as suggested from their lower expression of IFNG in comparison with cows that showed low haptoglobin levels after calving (Nightingale et al., 2015). Thus, an inflammation in peripartum seem to activate innate-immune cells impairing functions of adaptive components and thus, impairing the immune system capacity to restore normal functions in tissues. Investigations on the main causes triggering a haptoglobin peak in peripartum suggests the occurrence of a PI condition as the real driving element of inflammatory-like status (Table 8). The worsening of the inflammatory-like status could affect productive performances of dairy cows in early lactation (Trevisi et al., 2012, 2016).

IMMUNE DYSFUNCTION: CAUSES AND EFFECTS

The exact cause of immune dysfunctions occurring in peripartum has never been clearly identified. Reduced immune competence could arise from the interaction of different factors affected from the typical peripartum trends (Trevisi et al., 2011a). Nevertheless, its duration could be modified from the occurrence of the PI, that could also trigger the inflammatory-like status. Inflammation lead to the activation of PMN, while the reduced immune competence commonly reported in TP has been associated to an opposite effect on WBC (Trevisi and Minuti, 2018). Thus, these should be considered as two distinct phenomena, but they could arise from a common cause with a different magnitude and duration.

Endocrine asset

A direct relationship with immune functions has been well documented for most of hormones that are known to fluctuate during peripartum (Table 9). Cortisol is known to reduce PMN functions through specific receptor-ligand interactions that are linked to the NF-κB functioning. Same mode of action on the activation of cyclic adenosine monophosphate response element binding protein has been reported for catecholamines (Burton et al., 1995). Also fluctuations of estrogens and protein hormones affects both innate and adaptive immune systems, impairing functions of PMN and lymphocytes (Davis, 1998; Kelley et al., 2007; Sordillo, 2016). Trends of those hormones could partially account for reduced immune competence occurring in TP, even though their alterations do not overlap with the entire duration of the phenomenon.

Studies on mastectomized pregnant cows showed those animals to face a shorter and less marked immune suppression status at calving in comparison with animals with mammary gland, without any alteration on circulating WBC (Nonnecke et al., 1993; Kimura et al., 1999, 2002). These results suggest a marginal direct contribution of endocrine asset in reducing

immune competence around calving, while the largest effect could result from the contribution of endocrine asset in affecting the NEB triggered from the lactation requirements. Such an interaction has been reported to deeply affect immune functions, contributing both in reduced immune competence and inflammatory-like status (Sordillo, 2016). Reduced leptin levels are known to trigger an insulin resistance status before calving that, together with fluctuation in steroids hormones, reduces prepartal DMI, increasing the NEB at the beginning of lactation (Esposito et al., 2014). NEB conditions are known to reduce the expression of liver GH receptors, leading to an increased GH and reduced IGF-1 blood concentration in early lactation, and such an endocrine asset increases lipolysis and gluconeogenesis (Lucy, 2001). Furthermore, catecholamines provide a direct stimulus for lipomobilization process, and cortisol further increases this phenomenon activating peroxisome proliferation activated receptor, alpha (**PPARA**), that is involved in the regulation of lipid metabolism (Drackley, 1999). Thus, an indirect effect on immune functions (which is discussed in detail in the section related to NEB) should be considered for these hormones (Table 9). Furthermore, the occurrence of an inflammatory-like status could modify the effect of some of these hormones on immune cells. For instance, cortisol levels depend on the synthesis of a specific carrier at liver level, named corticosteroid binding globulin. When an inflammation occurs, and liver syntheses are impaired, reduced production of corticosteroid binding globulin triggers an initial increase in free cortisol, that means more bioactivity. Then, the negative feed-back prevails, and the hypophysis-pituary-adrenal axis secretes less cortisol, decreasing also the depressive effect of this hormone on immune cells (Trevisi et al., 2013a).

Limitations of maternal immune responses against the allogeneic conceptus

Reduced immune competence reported in TP could partially arise from the physiological limitation of maternal immune responses against the allogeneic conceptus during the late gestation period (Esposito et al., 2014). Other than fluctuation in steroids hormones, such a regulation is triggered from the differentiation of regulatory immune cells (i.e. M2 macrophages, γδ T-cells and Treg cells) that can inhibit inflammation and T cells response (Majewski and Hansen, 2002; Padua et al., 2005; Esposito et al., 2014). In particular, M2 macrophages are involved in inhibition of inflammatory conditions, and could increase the risk in developing uterine infections (Esposito et al., 2014).

Alterations in energy balance

Effects of NEB at the beginning of lactation on immune functions are disparate, and could depend on the degree of NEB, on the intensity of lipomobilization and on NEFA profile during this phase. After an inframammary infection with *Streptococcus uberis*, a repression of genes related to inflammatory response and chemotaxis has been reported in PMN of cows under NEB condition (Moyes et al., 2010). In fact, IL-8 signaling pathway, IL-1B receptor-associated kinase-1, mitogen-activated protein kinase-9 and TNF-associated receptor factor-6 were down regulated, while MHC-I was up-regulated. At the same time, a greater expression of mRNA for anti-inflammatory genes, as glucocorticoids receptors, and nuclear factor (erythroid-derived 2)-like 2 -mediated oxidative stress occurred, indicating an impaired capacity of the animal in solving infection, and a greater likelihood in developing further infectious diseases. Nevertheless, the real contribution of energy balance on immune dysfunctions during TP is difficult to assess, as variations in energy balance partially overlaps with other factors, as endocrine changes and health status, during this phase (Table 9). Two main mode of action of NEB in affecting immune functions could be recognized.

Firstly, NEB decreases the availability of essential nutrients for immune cells functions triggering the shift to alternative sources to supply the deficiency. Glucose is the main energy source for immune cells, while glutamine, NEFA and ketones could be used as alternative sources (Calder, 2013). Other than the lower glycaemia, such an effect could be driven from the lower glucose uptake of resting immune cells during TP, that has been related to the lower expression of a family of glucose transporters on their surface (Solute carrier family-2 - Table 5; O'Boyle et al., 2012). Glutamine is a precursor of purines and pyrimidines that is involved in cell division, DNA and RNA synthesis in WBC. Furthermore, it is related to the production of NADPH, that is essential in respiratory burst reaction. The lower efficiency in the utilization of alternative energy sources, the direct toxic effect of certain metabolites at high concentration and the deficiency of essential nutrients could partially explain the reduced immune competence reported with NEB conditions at the beginning of lactation (Table 10), as the impairment of normal functions of phagocytosis, chemotaxis and diapedesis of blood PMN (Stevens et al., 2011). Studies conducted on fed restricted animals in mid-lactation, inducing an early lactation-like NEB condition without the endocrine alteration related to calving (Moyes et al., 2010; Crookenden et al., 2017) revealed an altered expression of transcripts in their PMN that was consistent with a reduced immune competence to occur (Table 11). In fact, most mRNA encoding for defensins, cytokines and eicosanoids were down-regulated, as well as genes related to antigen presentation (MHC-I and human leukocytes antigen - **HLA**), respiratory burst (superoxide dismutase 1) and inflammatory response (TNFA). Most of these genes were further impaired when lipomobilization was increased from a PI condition. In fact, most of genes altered in an underfeeding condition (defensins, IL-10 and eicosanoids) were further altered when NEB occurred in overconditioned cows (BCS > 3.5 out of 5) prior to the NEB condition, suggesting a major role of lipid metabolites in such an alteration (Bernabucci et al., 2005; Crookenden et al.,

2017 - Table 12). Thus, energy balance contributes to immune dysfunctions during peripartum, although transient nutritional deficiencies could not fully explain the degree and duration of alterations. In fact, none of previous experiments that induced an early lactation-like-NEB condition have been able to mimic the extensive immunological changes that normally occurs in TP. A NEB of -6,2 Mcal d^{-1} induced in mid lactating cows (80% of requirements for maintenance) did not affected serum TNFA, WBC count or milk IgG concentrations, and no effect appeared on clinical symptoms following an endotoxin-induced mastitis in comparison to *ad-libitum* fed animals (Perkins et al., 2002). Furthermore, experimentally induced NEB alone did not affected adhesion or antigen presentation in leukocytes (Perkins et al., 2001). A reduced phagocytosis of PMN appeared with a more marked feed restriction (60% of requirements for maintenance for 7 days), even though relatively minimal alteration of other immune functions appeared after a mastitis challenge (Moyes et al., 2009).

Secondly, changes in NEFA composition that occurs during NEB could directly affect immune functions, with different mechanisms. Altered NEFA profile affects the membranes fluidity in WBC (Drackley, 1999), affecting the formation of glycolypoproteins composing lipid raft, that are involved in lymphocytes activation, antibodies production and inflammation (Sordillo, 2016). Changed NEFA profiles in cell membranes could also have a role in altering the production of immune mediators as oxylipids (Table 3; Raphael and Sordillo, 2013) with both pro or anti-inflammatory effects depending on the kind of NEFA. Thus, a possible explanation for different effects sorted on immune functions by NEB induced in early post-partum rather than in mid-lactation could be the different NEFA profile found both in blood and cells membrane during the two different phases. Down regulation of IL-10 and TNFA found in bovine PMN during NEB (Crookenden et al., 2017) is consistent with the effect exerted by PUFA on peroxisome proliferation activated receptor, gamma (**PPARG**)

and TLRs, that affects cytokines production through NF-κB. In human, α-linoleic acid is known to inhibit PPARG, while DHA inhibits both TLR2 and TLR4. However, in bovine PUFA do not appear to affect PPARG activity (Bionaz et al., 2013)**.** Furthermore PUFA are known to impair antigen presentation reducing mRNA expression for HLA in bovine PMN (Gorjão et al., 2006). On the other hand, the inflammatory-like status and the increased production of PIC detected as consequence of NEB (Esposito et al., 2014), could be driven from the increased expression of mRNA encoding for TLR2 and TLR4 sorted by SFA in circulating PMN (Lee et al., 2003, 2004b; Sordillo et al., 2009).

Oxidative stress status

The loss of reduction-oxidation (redox) homeostasis and tissue damaging resulting from the accumulation of excessive oxidants amounts (ROM and reactive nitrogen species) or depletion of antioxidant defenses is referred to as oxidative stress (Sordillo, 2016). This condition has been commonly reported during TP of dairy cows (Bionaz et al., 2007; Celi, 2011; Celi and Gabai, 2015) and its causes could be identified in different metabolic processes that occurs during this phase (Table 13). Most of these processes are directly related to the inflammatory-like status, to the impairment of liver functions and from dietary changes that occurs around calving.

Low to moderate ROM amounts serves as messengers in a wide range of redox regulated signaling pathways, as those related to NF-κB and mitogen activated protein kinase, that are involved in the production of cytokines, oxylipids and immunoregulatory factors (Finkel, 2011; Brown and Griendling, 2015). Nevertheless, oxidative stress status that occurs in TP could cause damages to DNA, proteins and lipids and could contribute to dysfunctional immune responses in this phase (Sordillo et al., 2009). In fact, abnormal ROM levels could trigger the lipid peroxide chain reaction in cell membranes and immune cells are highly

susceptible to this process, due to the high PUFA contents of their membrane. Lipid peroxide reaction consists in the acquisition of electrons from lipids by hydroxyl radicals, which generates lipid-peroxy radicals triggering an autolytic chain reaction within plasma membrane, removing further electrons from adjacent fatty acids. Damages includes altered cellular functions and signal transduction, which could exacerbate the inflammatory response and contribute in dysfunction of the vascular endothelium that occurs during TP (Lacetera et al., 2005; Sordillo et al., 2007, 2009). Furthermore, proteins are highly susceptible to the oxidative stress as they serve as catalysts mediators in cells, and thus the effect sorted by oxidation on one molecule is greater than stoichiometric (Dalle-Donne et al., 2005). Such effect includes alteration of protein functions as receptors, enzymes, transporters or structural elements, and could also directly contribute in provoking immune responses, as oxidized proteins can generate new antigens (Halliwell and Whiteman, 2004). The generation of oxidant species within the activity of immune cells is related to the production of characteristic oxidation products in proteins. In example, neutrophils releases hypochlorous acid through the MPO activity, and this metabolite is involved in the generation of di-tyrosil residues from serum albumin (Bordignon et al., 2014).

Immune dysfunction and diseases in transition period

The occurrence of diseases is known to raise during the TP, and their effect could be severe in impairing productive performances in the following lactation (Pinedo et al., 2010). In fact, TP diseases tend to occur in complexes with each other rather than as isolated events (Sordillo, 2016), and the occurrence of one of those diseases (named as primary) will increase significantly the risk ratio in developing others (named as secondary), up to the culling of the animal (Curtis et al., 1985; Ingvartsen, 2006). Relationship between diseases of TP and immune dysfunction is complex and has not been fully elucidated. A distinction between

metabolic and infective disorders is necessary. Examples of metabolic diseases related to TP are fatty liver, milk fever, retained placenta (**RP**), metritis, ketosis, left displacement of abomasum (**LDA**) and lameness (Kelton et al., 1998; Ingvartsen, 2006). Relationships between these disorders and the occurrence of an immune dysfunction in dairy cows is not clearly understood. As an example, fatty liver is known to contribute in immune dysfunctions impairing the ability of the liver to detoxify endotoxin, and thereby rending the cow extremely sensitive to endotoxic shock and death (Andersen et al., 1996). As most of metabolic diseases occurs at the beginning of lactation, while immune dysfunction seems to begin earlier, is possible to retain PI as the main cause of metabolic disorders. Nevertheless, most of the metabolic disorders are known to further impair PI conditions, triggering the production of PIC with anorexic power and the inflammatory-like status. Such a condition reduces DMI and increases the duration of NEB, increasing the risk ratio for secondary diseases, either metabolic or infectious (Table 14).

Differently from metabolic disorder, increased incidence of infective disease at the beginning of lactation could be clearly interpreted as a direct effect of impaired immune competence at calving (Ingvartsen, 2006). In fact, previous studies found that cows that developed endometritis and metritis had a reduced prepartal phagocytosis in leukocytes (Kim et al., 2005), a reduced glycogen concentration in circulating neutrophils at calving (Galvão et al., 2010) and a reduced TNFA expression in monocytes after a stimulation with *E. coli* (Galvão et al., 2012). Furthermore, reduced chemotaxis, delayed migration, reduced antimicrobial activity and ROM production detected in PMNs during TP are other explaining factors for increased susceptibility to mastitis, endometritis and metritis in transition cows, and could also account for the escalated severity of such diseases registered in comparison to mid lactating animals (Hill, 1981; Cai et al., 1994; Shuster et al., 1996). Further impairment of immune functions has been reported after the development of an infectious disease in TP,

due to the incapacity of immune system to solve the problem, that triggers a reiterated inflammatory condition.

NUTRITIONAL STRATEGIES TO FACE IMMUNE DYSFUNCTION IN TRANSITION PERIOD

Nutritional strategies to optimize dairy cow's immunity during TP should be focused on reducing the PI degree related to calving, as this condition could be referred as a common denominator between immune dysfunction and diseases causes. The provision of a balanced and healthy diet should be considered as a focal point in this respect. In this regard, although optimal dietary concentrations of fermentable carbohydrates fiber, sugar and starch for transition cows are still under definition, NRC provided guidelines for diet formulation that fits most of nutritional requirements of this phase (NRC, 2001).

Variation of energy requirements and sources during peripartum

Dairy cows should enter the dry period with a BCS of 3 out of 5 (ADAS 2006), in order to minimize the amount of lipid stores which could determine an excessive mobilization of NEFA in early lactation, reducing DMI and increasing the duration of NEB condition (Contreras et al., 2004; Esposito et al., 2014). An energy content of 1.25 Mcal kg DM^{-1} has been considered the ideal net energy for lactation (**NE_L**) value to be provided between dry-off and -21 DFC to minimize the BCS gain during dry period. Providing higher energy amounts in this phase has been related to detrimental carryover effects during early lactation. On the other hand, an energy content 1.54-1.62 Mcal kg DM^{-1} has been considered the ideal NE_L value to be provided between -21 DFC and calving day in order to meet the growing requirements of fetus and ensure a cow BCS below the 3.5 out of 5 threshold at calving day.

One week prior to expected calving, an increased amount of not-fiber carbohydrates (**NFC**) or highly digestible neutral detergent fiber (**NDF**) sources should be provided. Such a strategy increases propionate levels, improving gluconeogenesis and bacterial protein synthesis at rumen level. Furthermore, it improves the development of rumen papillae,

ameliorating the VFA absorption in early lactation and minimizing the likelihood to develop rumen acidosis (with subsequent PI conditions) (Rabelo et al., 2003). In early lactation, the limiting factor for feed intake is the chemical effect of metabolites related to the oxidation of fuels. Thus, limiting dietary starch fermentability in this phase should maximize DMI, due to the less rapid production and less marked absorption of propionate (Allen et al., 2009). Conversely, the rumen availability of starch, NFC and fermentable fiber (in rations with adequate physical effective NDF -**peNDF**- amounts) should be incremented since the 2nd-3rd month of lactation only, when the peak of lactation occurs and cows have overcome the NEB condition. In fact, feed intake is limited from gut fill in this phase, and increased propionate productions, driven from the increased fermentable substrate, increase insulin concentration, reducing lipomobilization without affecting DMI (Drackley, 1999). Thus the NEB degree, as well as NEFA and BHB concentrations are reduced. As a consequence, also the liver triglycerides content is decreased, with a positive effect on the incidence of metabolic disorders and reproductive parameters (Gong et al., 2002). Thus, the availability of energy and protein sources for WBC in early lactation raises and the amount of lipomobilization-related metabolites decreases when a proper energy plan is adopted in peripartum diet, suggesting an optimal energy management as a strategy to improve immune competence.

Lipid sources supplementation

Lipid metabolism has been indicated to face important alteration during TP, and fat supplementation could have a main role in affecting immune functions during this phase. Different effects arise from feeding different dietary fat levels and the fatty acids composition used could sort different effects. Feeding a high-fat diet to dairy cows during the dry period has been reported to induce the peroxisomal beta-oxidation process (Drackley, 1999). In comparison to mitochondrial beta-oxidation, which normally occurs, peroxisomal beta-

oxidation is catalyzed by an oxidase (Acyl-CoA-oxidase) that produce hydrogen peroxide rather than reduced NAD, releasing a larger amount of heat and less reduced cofactors (Drackley, 1999). Moreover, peroxisome do not contain any electron chain linked to the ATP production and thus, this process is not regulated from the energy demand of the cell. Consequently, induction of the peroxisomal beta-oxidation has been suggested as a strategy to provide an aid to the mitochondrial pathway during the NEB condition, when a NEFA overflow occurs (Drackley, 1999). Such a strategy has been indicated to reduce the hepatic accumulation of triglycerides at calving, reducing the likelihood to develop fatty liver syndrome and the occurrence of a PI. Furthermore, it could be effective in mitigating negative effects sorted by lipomobilization on immune cells.

Furthermore, the fatty acids composition of lipid sources fed during the TP affect the lipid composition of cell membranes and the NEFA profile at blood level during the lipomobilization process that occurs in early lactation. State the well-known modulatory effect of fatty acids on immune functions (Table 10), an effective strategy to modulate the immune response in TP could be the administration of rumen protected PUFA aimed to shift the fatty acids composition of cell membranes (Table 15).

Omega-3 PUFA, as EPA and DHA, are known to reduce the inflammatory-like status reducing the arachidonic acid amount in cell membranes, that is related to the production of pro-inflammatory oxylipids. Such an effect attenuates the production of PGF2A from endometrium (Lessard et al., 2004; Mattos et al., 2004; Contreras et al., 2012a) and of prostaglandin E2, that is known to reduce IFNG synthesis and lymphocytes proliferation (Trebble et al., 2003; Brassard et al., 2007). Thus, EPA and DHA improve the cell-mediated immune response in early lactation, reducing oxidative stress damages and increasing phagocytosis in PMN, sorting a positive effect on uterine and udder health (Contreras et al.,

2012b; Dirandeh et al., 2013), but also have a positive effect on adaptive immunity (Bertoni et al., 2006; Trevisi et al., 2012).

Omega-6 PUFA includes the conjugated linoleic acid (**CLA**) family, that consists in 28 isomers, even though cis-9 trans-11 and trans-10 cis-12 are the most important. Administration of these PUFA is known to attenuate the inflammatory-like status in early lactation through the modulation of the NF-κB and the inhibition of the LPS-induced inflammatory activity in macrophages. Such an effect ameliorates the acute phase response, ensuring an augmented albumin and cholesterol concentration (both negative APP) in early lactation (Trevisi and Bertoni, 2008). Furthermore, an improvement of neutrophils functions has been reported consequently to the reduced oxidative stress status and to the reduced amount of NEFA and BHB in early lactation. In fact, CLA is known to protect PON, an important antioxidant enzyme, against the oxidative inactivation. It also increases the hepatic secretion of very low-density lipoproteins (**VLDL**) and apolipoprotein-B100 (Cheng et al., 2004). Such proteins are involved in lipid redistribution through tissues, and their increase lead to a lower cellular accumulation of triglycerides containing palmitic acid, increasing DMI, reducing NEB and lipomobilization at the beginning of lactation (Cheng et al., 2004; Silvestre et al., 2011).

Antioxidant elements supplementation

Altered redox homeostasis that occurs in TP is triggered, other than the increased production of oxidant species during inflammation, from the consumption or lack of antioxidant system. An effective strategy to reduce negative effects of oxidative stress status on immunity could be the supplementation of minerals and vitamins involved in antioxidant systems activity during the late gestation and early lactation period (Table 15). Among vitamins, beta-carotene (vitamin A) is known to prevent fatty acid peroxidation chain

reaction, while ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) are known to mitigate oxidative stress acting as radical scavenger and disrupting fatty acid peroxidation chain reaction respectively (Sordillo, 2016).

Trace minerals have critical roles in a variety of physiological process, particularly antioxidant defense, and a deficiency may depress immunity especially in peripartal or transition cows (Spears and Weiss, 2008). In fact, inflammatory-like status that commonly occurs after calving is known to reduce circulating minerals (i.e. zinc, iron and copper) sequestering them in liver (Osorio et al., 2016). Selenium is an active component of thioredoxin reductase and glutathione peroxidase enzymatic complexes, that controls redox signaling and reduce ROM production. Copper is an active component of ceruloplasmin, that exert oxidase activity as peroxyl radical scavenger. Zinc is an active component of metallothionein, which is a cysteine rich radical scavenger. Copper, zinc and manganese are also involved in superoxide dismutase functioning, that converts cytosol superoxide to H_2O_2, while iron allow the catalase action, that converts H_2O_2 to water (Sordillo, 2016).

Methyl donors supplementation

Methyl donors species are involved in lipid metabolism and lipoproteins synthesis. In dairy cows, their availability is limited from the extensive degradation in the rumen (Zhou et al., 2016). At the beginning of lactation the availability of such compounds is suddenly reduced, as furthermore milk from dairy cows is high in methylated compounds, and the levels secreted into milk are maintained even at the cost of depleting liver tissue reserves (Pinotti et al., 2002; Zhou et al., 2016). Thus, their supplementation during late gestation and early lactation phases could ameliorate the utilization of lipid sources, mitigating negative effects of excessive lipomobilization on immune cells (Pinotti et al., 2003). Choline is a quasi-vitamin that is a structural component of phosphatidylcholine, which is contained in all the cell

membranes (Hartwell et al., 2000). Methionine and lysine are two essential amino acids that commonly undergoes a deficiency during NEB conditions. In lipid metabolism, all these compounds are required for the hepatic synthesis of VLDL (Bauchart et al., 1998). Greater VLDL levels ensure a faster redistribution of liver triglycerides, reducing hepatic infiltration of fats. Furthermore, methionine and lysine directly affect mitochondrial beta-oxidation of fatty acids in liver (Esposito et al., 2014). Thus, higher availability of these compounds could improve the lipid utilization during a marked lipomobilization. Furthermore, methyl donors are important sources of the intracellular antioxidants glutathione and taurine (Zhou et al., 2016), and thus their administration in TP could mitigate oxidative stress, ameliorating leukocyte functions and reducing inflammation degree.

Anti-inflammatory products supplementation

Nutritional additives could be used in TP to transform the animal from a pro to an anti-inflammatory phenotype in some extent (Bertoni et al., 2015). Vitamin D3 is known to act on Th cells, inhibiting Th1 sub-type in favor of Th2, and thus down regulating PICs and stimulating AICs production (Bertoni et al., 2015). Plant extract could further provide an aid in modulating over exuberant inflammations around calving. Acetylsalicylic acid is a common drug, formerly derived from plants, that has been successfully used in dairy cows to attenuate their systemic response to inflammations during TP (Bertoni et al., 2013). Its mode of action against inflammation consists in inhibiting COX enzymatic complex, thus reducing the synthesis of pro-inflammatory oxylipids. Providing 15 g/day of acetylsalicylic acid to dairy cows for three-four days after calving reduced the severity of inflammations and the incidence of clinical diseases in early lactation, thus exerting positive effects on their milk yields and fertility (Trevisi and Bertoni, 2008). Other plant extracts, as *Hottuynia cordata* supercritical extract, has been suggested as potential anti-inflammatory drugs. Although none

evaluated its effect on dairy cows in TP, experiments on murine models found an effectiveness in reducing TNFA production and inhibiting pro inflammatory oxylipids synthesis by COX enzymatic complex (Shin et al., 2010; Kim et al., 2012). *Aloe arborescens Mill.* has been successfully utilized in modulating inflammatory status of transition cows (Trevisi et al., 2013b). Administration of 150 g d^{-1} of *Aloe* extract during the 4 weeks around calving did not affect the production of positive APPs, but reduced mobilization of body fats, improving liver metabolism and mitigating the reduction of negative APPs after calving (i.e. albumin, PON, cholesterol, lipoproteins and retinol binding proteins). Finally, the treatment with cytokines during TP has been hypothesized as a potential strategy to reduce the severity of inflammatory phenomena. In an in-vitro model of swine alveolar macrophages, 0.5 UI/mL of IFNA reduced the expression of the TNFA gene (Amadori, 2007), suggesting it as a potential strategy to mitigate inflammatory status during TP of dairy cows. Nevertheless, daily oral administration of either 10 or 0.5 IU/kg BW of IFNA to dairy cows during TP increased the severity of inflammatory phenomena after calving (Trevisi et al., 2009). Such an effect could be driven by lymphocytes in the rumen liquor of dairy cows, that could have counteracted and inverted the antiphlogistic signal of IFNA.

CONCLUSIONS

Mitigate immune dysfunctions occurring in TP of dairy cows is a pivotal goal to prevent the infectious and metabolic diseases in early lactation. Although wide literature background has been developed about this topic, immune dysfunctions in TP remain a challenging topic to investigate. Sudden changes occurring at different levels during this tricky phase of dairy cows life made challenging to identify the real driving mechanism of immune alterations. Nevertheless, a multifactorial contribution could be hypothesized for their appearance. Feed additives and nutritional strategy could be effective in mitigate immune alterations. In a wider perspective, we can conclude that adoption of proper management practices aimed to avoid PI condition in peripartal period of dairy cows could be the most effective strategy to prevent immune alterations.

TABLES

Table 1. Main families of pattern of recognition receptors (PRRs), their classification according to the cell position, their main sites of location within the organism and their target ligands

CATEGORY	FAMILY	DESCRIPTION	LOCATION	TARGET
Membrane PRRs	Toll-like receptors (TLRs)	Single membrane spanning proteins	Macrophages, dendritic cells	PAMPs[1]
	C-type lectin receptors (CLRs)	Proteins (lectins) that recognize carbohydrates with a calcium domain	Macrophages, dendritic cells, lymphatic endothelial cells, carcinomas	PAMPs[1] (carbohydrates)
Cytoplasmic PRRs	Nod-like receptors (NLRs)	Proteins with a triphosphate nucleotides-binding domain	Cytoplasm	PAMPs[1] (peptidoglycans) DAMPs[2] (cell stress)
	RIG[4]-I-like receptors (RLRs)	Helicases	Cytoplasm	DAMPs[2] (5'-phosphate-RNA, dsDNA[3])

[1]Pathogen associated molecular patters.
[2]Damage associated molecular patterns.
[3]Double strained DNA.
[4]Retinoic acid-inducible gene.

Table 2. Main cytokines, their site of production, their role on inflammation and their function on immune system

CYTOKINE	PRODUCTION	EFFECT ON INFLAMMATION AND FUNCTION[1]	REFERENCE
Interleukin-1, beta (IL-1B)	Macrophages, mammary epithelial cells	(+) Stimulate diapedesis in neutrophils	Lopez-Castejon and Brough, 2011; Ryman et al., 2015
Interleukin-2, (IL-2)	T-helper cells	(+) Activation of PMN[4] and lymphocytes	Sordillo and Streicher, 2002
Interleukin-8 (IL-8)	Macrophages, monocytes, epithelial cells	(+) Stimulate chemoattraction and activation (adhesion, diapedesis and ROM[2] production) in PMN[4] and monocytes	Suriyasathaporn et al., 1999; Paape et al., 2000
Interleukin-12 (IL-12)	Dendritic cells	(+) Stimulate activation of T-cells	
Interleukin-17 (IL-17)	T-helper 17 cells	(+) Acting in concert with TNF and IL-1B, it activates signaling cascades that lead to the induction of chemokines. Acting as chemoattractant, chemokines recruit monocytes and neutrophils to the site of inflammation	Sordillo, 2016
Interleukin-23, (IL-23)	Dendritic cells	(+) Upregulation of matrix metalloproteinase 9, increase of angiogenesis, reduction of CD8+[3] T-cell infiltration in tumors	Crookenden et al., 2016; Crookenden et al., 2017
Interferon, alpha (IFNA)	Dendritic cells	(+) Stimulate phagocytosis in macrophage, anti-viral	
Interferon, gamma (IFNG)	T-helper cells	(+) Activation of PMN[4] and lymphocytes, anti-viral	Sordillo and Streicher, 2002
Tumor necrosis factor, alpha (TNFA)	Macrophages	(+) Stimulate diapedesis in PMN[4] (production of IL-8 in PMN[4] and adhesion molecules on vascular endothelial cells), enhances acute phase of inflammation, stimulate the production of antimicrobial peptides	Ryman et al., 2015
Interleukin-4 (IL-4)	Basophils	(-) Induces differentiation of naive helper T cells (Th0 cells) to Th2 cells	Sordillo, 2016
Interleukin-10	Monocytes, type-2 T-helper cells, mast cells,	(-) Pleiotropic effects in	Crookenden et al., 2016; Crookenden

(IL-10)	$CD4^+CD25^+Foxp3^+$ regulatory T-cells and in a certain subset of activated T-cells and B-cells	immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation and antibody production. It can block NF-κB activity and is involved in the regulation of the JAK-STAT signaling pathway	et al., 2017
Interleukin-6, (IL-6)	Macrophages	(+/-) Immune mediator, stimulate the production of antimicrobial peptides, stimulate diapedesis in neutrophils	Crookenden et al., 2016; Crookenden et al., 2017

[1] (+) indicate a pro and (-) an anti-inflammatory effect.
[2]Reactive oxygen metabolites.
[3]Cluster of differentiation-8 positive T-cells.
[4]Polymorphonuclear cells.

Table 3. Main oxylipids classes and species arise from the main polyunsaturated fatty acid (PUFA) through different oxidation pathways, and their effect on inflammatory response

CLASS	PRECURSORS	PATHWAY	OXYLIPID	EFFECT ON INFLAMMATION[1]
Protectin	Omega-3 PUFA (EPA[2] and DHA[3])			(-) Anti-inflammatory
Resolvins	Omega-3 PUFA (EPA[2] and DHA[3])			(-) Anti-inflammatory
Prostaglandin (PX)	Omega-6 PUFA (Arachidonic acid)	Cyclooxygenase (COX)	Prostacyclin (PG-I2)	(+) Vasodilatation, inhibition of platelet aggregation
		Cyclooxygenase (COX2)	PG-E2	(+) Induction of pain and fever
		Cyclooxygenase (COX2)	PG-D2	(-) Inhibition of leukocyte adhesion to endothelial cells, decrease of cytokine expression by blocking NF-κB5, enhancement of LOX activity
		Cyclooxygenase (COX2)	15d-PG-J2	(-) Inhibition of leukocyte adhesion to endothelial cells, decrease of cytokine expression by blocking NF-κB
Thromboxane (TX)	Omega-6 PUFA (Arachidonic acid)	Cyclooxygenase (COX)	Thromboxane-A2 (TX-A2)	(+) Aggregation, vasoconstriction
Lipoxin (LX)	Omega-6 PUFA (Arachidonic acid)	Lipoxygenase (15-LOX + 5-LOX)	Lipoxin-A4 (LX-A4)	(-) Anti-inflammatory proprieties
		Lipoxygenase (15-LOX + 5-LOX)	Lipoxin-B5 (LX-B5)	(-) Anti-inflammatory proprieties
Leukotriene (LT)	Omega-6 PUFA (Arachidonic acid)	Lipoxygenase (5-LOX)	Leukotriene-A4 (LT-A4)	(-) Can be converted by 15-LOX in platelets for LX biosynthesis

[1](+) indicate a pro and (-) an anti-inflammatory effect.

[2]Eicosapentaenoic acid.

[3]Docosahexaenoic acid.

Table 4. Classification of lymphocytes and their main functions within organism

TYPE	GROUP	SUB-GROUP		MARKERS[1]	FUNCTION		
T-cells	αβ-T-cells	T-helper cells (Th)	FUNCTIONAL GROUP Th1 Th2 Th17 T-regulatory cells (Treg)	CD4+/CD8-	Cytokines production (IL-2[2], IFNG[3])		
		T-cytotoxic cells (Tc)		CD4-/CD8+	Elimination of host cells with altered MHC I[4], apoptosis, cytokines production		
	γδ-T-cells	-		-	Protection of epithelial surfaces against bacterial diseases; mediate cytotoxicity through WC1		
B-cells	Plasma-cells	-		-	Production of immunoglobulins (Igs)		
					Ig	LOCATION	FUNCTION
					IgA	Mucosal surfaces	Agglutination, neutralization of bacterial toxins
					IgG (1 and 2)	Serum	Increase phagocytosis in PMN (opsonin)
					IgE		Face parasitic infections
					IgM		Opsonization, agglutination and complement fixation
					IgD		Non-secreted regulatory molecule
	Memory-cells	-		-	Heightened state of immune reactivity and clonal expansion of antigen-specific effector cells that occurs when host cells and tissues are re-exposed to the same antigen		

[1]Immunophenothyping of leucocytes according to the expression of cluster of differentiations 4 and 8 on their surface: (+) indicate positive and (–) indicate negative.
[2]Interleukin-2.
[3]Interferon, gamma.
[4]Major histocompatibility complex of class I.

Table 5. Patterns of mRNA transcripts encoding for genes that are down regulated in bovine PMN during transition period in comparison to 4 weeks after parturition, and their functions (O'Boyle et al., 2012; Crookenden et al., 2016, 2017)

CATEGORY	GENE	FUNCTION	Weeks from calving[1]				
			-1	0	1	2	4
Lectins	Galectin S8 (*LGALS8*)	Development, differentiation, cell-cell adhesion, cell-matrix interaction, growth regulation, apoptosis, RNA splicing	D b	D c	D ab	D ab	a
Receptors	Talin 2 (*TLN2*)	Assembly of actin filaments	a	D b	a	a	a
	Integrin subunit beta-2 (*ITGB2*)	Cell adhesion and signaling	D b	a	D ab	D ab	a
	Integrin subunit alpha M (*ITGAM*)	Neutrophil adherence and phagocytosis	D b	D b	D b	D b	a
	Integrin subunit alpha 4 (*ITGA4*)	α4β1 lymphocyte homing receptor	D b	D c	D b	D b	a
	Integrin subunit alpha L (*ITGAL*)	Cell adhesion and signaling	D b	D b	D ab	D ab	a
Antioxidant	Superoxide dismutase 1 (*SOD1*)	Destruction of superoxide radicals in cytosol	D b	a	a	a	a
Enzymes	Myeloperoxidase (*MPO*)	Antimicrobial (hypochlorite production)	D ab	D bc	D c	D c	a
	Protein kinase C beta (*PRKCB*)	B cell activation, apoptosis, endothelial cell proliferation, intestinal sugar absorption	D b	D ab	D ab	D ab	a
Regulator proteins	X-box binding protein 1 (XBP1)	Regulation of MHC II[2], plasma cell and eosinophil differentiation, angiogenesis, viral replication, endoplasmic reticulum stress response	D a	D b	ab	ab	ab
Transporters	Solute carrier family 2 member 1 (*SLC2A1*)	Glucose transporter	D b	D c	D b	D bc	a
Immune mediators	Spleen associated tyrosine kinase (*SYK*)	Transmit signals from the B-Cell receptor and T-Cell receptor. plays a similar role in transmitting signals from a variety of cell surface receptors (CD74[3], fragment crystallizable receptor and integrins)	D b	D ab	D ab	D ab	a
Cytokines	Interferon, gamma (*IFNG*)	Pro-inflammatory	a	D b	a	a	a
	Interleukin 12A	Pro-inflammatory	D	D	D	a	a

	(*IL12A*)		ab	b	ab		
	Tumor necrosis factor alpha (*TNF*)	Pro-inflammatory	D b	D c	a	a	a
	Interleukin 23 subunit alpha (*IL23A*)	Pro-inflammatory	D b	D c	a	D ab	a
Eicosanoids	Arachidonate 5-lipoxygenase activating protein (*ALOX5AP*)	Leukotrienes synthesis: pro-inflammatory	b	D a	b	b	b
	Arachidonate 5-lipoxygenase (*ALOX5*)	Leukotrienes synthesis: pro-inflammatory	a	D b	a	a	a
Caspase	Caspase 8 (*CASP8*)	Toll like receptor pathway: stimulate apoptosis	a	D b	D ab	D ab	a
Cluster of differentiations	CD117: KIT proto-oncogene receptor tyrosine kinase (*KIT*)	Cell survival, proliferation and differentiation	D b	D c	a	a	a
	CD45: protein tyrosine phosphatase, receptor type C (*PTPRC*)	Cell growth, differentiation, mitotic cycle, oncogenic transformation	D ab	D ab	D ab	D b	a
	CD337: natural cytotoxicity triggering receptor 3 (*NCR3*)	Apoptosis and natural cell death-regulator	D b	D c	D b	D b	a
Receptors	Annexin A3 (*ANXA3*)	Anti-coagulation, regulation of cellular growth and signal transduction pathways, inhibition of phospholipase A2 and cleavage of inositol 1,2-cyclic phosphate to inositol 1-phosphate	a	D b	D ab	D ab	a
	Nuclear receptor corepressor 1 (*NCOR1*)	Inhibition of gene expression	D ab	D b	D ab	D ab	a
Regulator proteins	BCL2 apoptosis regulator (*BCL2*)	Regulation of apoptosis	D b	D c	a	D ab	a
Transmembrane protein	Fas-ligand (*FASLG*)	Apoptosis, regulation of immune system and progression of cancer	D bc	D c	D b	D b	a

[1]D indicates genes that are down-regulated during transition period in comparison to their levels at 4 weeks after parturition; a, b and c indicates the significance of comparisons.
[2]Major histocompatibility complex II.
[3]Cluster of differentiation 74.

Table 6. Patterns of mRNA transcripts encoding for genes that are up regulated in bovine PMN during transition period in comparison to 4 weeks after parturition, and their functions (O'Boyle et al., 2012; Crookenden et al., 2016, 2017)

CATEGORY	GENE	FUNCTION	Weeks from calving[1]				
			-1	0	1	2	4
Cytokines	Interleukin 10 (*IL10*)	Anti-inflammatory	b	U a	b	b	b
	Interleukin 6 (*IL 6*)	Pro- and anti-inflammatory	U bc	U a	U ab	U abc	c
Defensins	Beta-defensin 1 (*DEFB1*)	Antimicrobial (epithelial surfaces), immunomodulatory	b	U a	b	b	b
	Beta-defensin 10 (*DEFB10*)	Antimicrobial, immunomodulatory	b	U a	b	b	b
	S100 calcium binding protein A9 (*S100-A9*)	Respiratory burst (H_2O_2 production)	b	U a	b	b	b
	Defensin, beta 4A (*BNBD4*)	Antimicrobial, immunomodulatory	c	U a	U b	U bc	c
Eicosanoids	Phospholipase A2 group IVA (*PLA2G4A*)	Leukotriene pathway: release arachidonic acid from the membrane	b	U a	b	b	b
Enzymes	Lactate dehydrogenase A (*LDHA*)	Anaerobic glycolysis	b	U a	b	b	b
Matrix metalloproteinases	Matrix metallopeptidase 8 (*MMP8*)	Polymorphonuclear cells migration	b	U a	b	U ab	b
Cluster of differentiations	CD121B: Interleukin 1 receptor, type 2 (*IL1R2*)	Interleukins-1 alpha and beta inhibitor	a	U b	a	a	a
	CD14 molecule (*CD14*)	Toll like receptor pathway: detection of bacterial LPS[2]	U b	U b	U bc	U c	a
	CD44 molecule (*CD44*)	Lymphocyte activation, recirculation and homing, hematopoiesis and tumor metastasis	b	U a	b	b	b
Peptides	Lingual antimicrobial peptide (*LAP*)	Antimicrobial	U bc	U a	U ab	U abc	c
Receptors	Integrin subunit alpha X (*ITGAX*)	Adhesion	b	U a	b	b	b
	Interleukin 2 receptor subunit alpha and beta (*IL2RA* and *IL2RB*)	Allows interleukin-2 functions	U bc	U a	U b	U bc	c
	Chemokine (C-X-C motif) receptor 1	Interleukin-8 membrane receptor: neutrophil	U ab	U a	c	U bc	c

	(*CXCR1*)	chemotaxis and activation					
	Toll-like receptor 2 (*TLR2*)	Membrane receptor involved in pathogen recognition and immunoregulation	U b	U a	U bc	U bc	c
Regulatory genes	MYD88, innate immune signal transduction adaptor (*MYD88*)	Signal transducer in interleukin-1 and toll-like receptor 4 pathways	U b	U a	c	c	c
Ribosomal proteins	Ribosomal protein L23 (*RPL23*)	Protein synthesis	U b	U ab	U b	a	a
Transcription factor	Peroxisome proliferation activated receptor delta (*PPARD*)	Cell inflammatory responses	b	U a	b	b	b
	Peroxisome proliferation activated receptor gamma (*PPARG*)	Cell inflammatory responses	U ab	U a	U ab	b	b
	Retinoid X receptor alpha (*RXRA*)	Endothelial cell inflammatory responses: receptor for retinoic acid	b	U a	b	b	b
	Signal transducer and activator of transcription 3 (*STAT3*)	Immune mediator: mediates cellular responses to interleukins and other growth factors	U a	U a	U b	ab	ab

[1]U indicates genes that are up-regulated during transition period in comparison to their levels at 4 weeks after parturition; a, b and c indicates the significance of comparisons.
[2]Lipopolysaccharides.

Table 7. Patterns of mRNA transcripts encoding for genes that are both up and down-regulated in bovine PMN during transition period in comparison to 4 weeks after parturition, and their functions (O'Boyle et al., 2012; Crookenden et al., 2016, 2017)

CATEGORY	GENE	FUNCTION	Weeks from calving[1]				
			-1	0	1	2	4
Enzymes	Receptor interacting serine/threonine kinase 1 (*RIPK1*)	Apoptosis and necroptosis; participates in NF-κB[2], AKT[3], and JNK[4] functioning	U a	D ab	D c	D bc	b
Cluster of differentiations	CD90: Thy-1 cell surface antigen (*THY1*)	Cell-cell and cell-matrix interactions: neurite outgrowth, nerve regeneration, apoptosis, metastasis, inflammation, and fibrosis	D c	U a	U b	bc	bc
	CD16a: Fc fragment of IgG, low affinity IIIa, receptor (*FCGR3A*)	Cluster of differentiations surface molecules	U a	D c	D c	ab	ab
Matrix metalloproteinases	Matrix metallopeptidase 9 (*MMP9*)	Polymorphonuclear cells migration	D c	U a	U b	U b	bc
Receptors	Interleukin 1 receptor antagonist (*IL1RN*)	Interleukin-1, beta inhibitor	U a	ab	D b	D b	ab
	Selectin L (*SELL*)	Leukocytes migration	U a	U a	D b	D b	ab
	Fc-fragment of IgE, high affinity I, receptor for gamma polypeptide (*FCER1G*)	Immunoglobulin E receptor; involved in allergic reactions	U ab	U a	D c	D bc	abc
	Chemokine (C-X-C motif) receptor 1 (*CXCR1*)	Interleukin-8 membrane receptor: neutrophil	U ab	U a	c	U bc	c
	Chemokine (C-X-C motif) receptor 2 (*CXCR2*)	chemotaxis and activation	U ab	U a	D c	bc	bc
	Toll-like receptor 4 (*TLR4*)	Pathogen recognition and regulation of immune response	U a	ab	D b	ab	ab
Regulator proteins	MCL1 apoptosis regulator, BCL2 family member (*MCL1*)	Regulation of apoptosis: induced myeloid leukemia cell differentiation protein	U ab	U a	D c	D bc	abc
Transporters	ATP binding cassette subfamily A member 1 (*ABCA1*)	Cholesterol efflux: regulation of cellular cholesterol and phospholipid homeostasis	a	U b	D c	D ac	a

[1]D indicates genes that are down-regulated and U those that are up-regulated during transition period in comparison to their levels at 4 weeks after parturition; a, b and c indicates the significance of comparisons.
[2]Nuclear factor κB.
[3]Protein kinase B.
[4]c-Jun N-terminal kinase.

Table 8. Phenomena undergoing a physiological imbalance during peripartum, their possible causes and mechanisms through they are related with a raise in blood haptoglobin concentrations and to a greater inflammatory condition

PHENOMENON	CAUSE	FACTOR STIMULATING HAPTOGLOBIN PRODUCTION	REFERENCE
Udder involution at dry-off	Milk yields higher that 25 kg d^{-1} at dry-off	Leukocytes activities during udder involution	Bertulat et al., 2013
Tissue homeostasis	Infections (metritis, mastitis) and tissue damages related to calving (mechanical damages and altered permeability of rumen epithelia)	Increased production of pro inflammatory cytokines	Vels et al., 2009; Minuti et al., 2015
Mineral equilibrium	Acidogenic close-up diets	Mineral deficiencies during dry period (i.e. Ca and Mg)	Nightingale et al., 2015
Hypothalamus-hypophysis-adrenal axe	Stress related to parturition, pen movements, re-socialization, environment, weather, diet	Glucocorticoids production stimulates haptoglobin release from parenchymal liver cells	Higuchi et al., 1994; Do Nascimento et al., 2004; Cooke et al., 2012
Lipomobilization	Marked negative energy balance condition arise from high milk yields; Excessive lipid deposition during dry-off	Excessive deposition of triglycerides in liver (fatty liver) reduces the production of haptoglobin by parenchymal cells (production is in direct response to lipids infiltration, which acts as harmful stimulus)	Katoh et al., 2002

Table 9. Trends of main hormones during transition period and their effect on immune functions

CLASS	HORMONE	TREND	EFFECT ON IMMUNE SYSTEM[1]	REFERENCES
Glucocorticoids	Cortisol	Increase at dry-off and calving time	Immune competence (D-): down regulation of selectin-L and cluster of differentiation-18 adhesion molecules on PMN[3], impairing their chemotaxis and diapedesis; altered cytokines production through impairment of NF-κB[7] functions Immune competence (I-): reduce feed intake favoring lipomobilization. Induce increased expression of PPARA[2], which leads to coordinate induction of enzymes involved in plasma transport, intracellular trafficking and metabolism of fatty acids	Burton et al., 1995; Drackley, 1999; Drackley et al., 2005; Bertulat et al., 2013
Catecholamines	Epinephrine, norepinephrine, dopamine	Increase at dry-off and calving time	Immune competence (D-): activation of genes encoding for a variety of cytokines through the cAMP-RBP[6] activation Immune competence (I-): provide the primary stimulus for increased mobilization of NEFA[4] from adipose tissue	Drackley, 1999; Padgett and Glaser, 2003
Steroids	Progesterone	High levels during gestation and rapid fall at calving	Immune competence (D-): decrease functional capabilities of lymphocytes and PMN[3], reduce capacity to face bacterial infections	Roth et al., 1982; Drackley et al., 2005; Lamote et al., 2006
	Estrogen	Increase in late pregnancy	Immune competence (I-): decrease feed intake favoring lipomobilization	
Protein hormones	Insulin	Increase in late pregnancy and fall in early lactation	Immune competence (I-): increased insulin resistance of peripheral tissues reduces peripheral glucose increasing negative energy balance condition and lipomobilization	Bell, 1995; Lucy, 2001; Taylor et al., 2004
	Growth hormone (GH)	Increase in early	Immune competence (I-): down regulation of liver	(Lucy, 2001)

	lactation	GH receptor during NEB[5]	
Insulin like growth factor (IGF-1)	Decrease in early lactation	increase GH and decrease IGF-1 blood concentration, increasing lipolysis and gluconeogenesis	
Leptin	Falls in late pregnancy and remain low postpartum	<u>Immune competence (I-):</u> can influence feed intake and contribute to peripheral insulin resistance in peripartum, increasing NEB[5] condition	Esposito et al., 2014

[1](I) and (D) indicate an indirect and direct mode of action respectively; (+) and (-) indicate a positive or negative effect on immune function respectively.
[2]Peroxisome proliferation activated receptor, alpha.
[3]Polymorphonuclear cells.
[4]Not esterified fatty acids.
[5]Negative energy balance.
[6]Cyclic adenosine monophosphate response element binding protein.
[7]Nuclear factor-κB.

Table 10. Main metabolites related to the negative energy balance status, their trend during transition period, their effects on immune cells and their mode of action

METABOLITE, TREND[1]	CELL	EFFECT[2]	MECHANISM	REFERENCE
Glucose, (-) late gestation and after calving	Macrophages, polymorphonuclear cells	Immune competence (-) Decrease of proliferation, differentiation, viability, chemotaxis and phagocytosis	Primary energy source for leukocytes. Low efficiency in alternative energy sources utilization	Barghouthi, Everett, & Speert, 1995; Gamelli, Liu, He, & Hofmann, 1996; Pithon-Curi, De Melo, & Curi, 2004
	Lymphocytes	Immune competence (-) Reduced mitogen-induced activation	Proliferation and secretory activities are glucose dependent	Sordillo, 2016
Not esterified fatty acids (NEFA), (+) after calving	Polymorphonuclear cells	Immune competence (-) Inhibition of phagocytosis and synthesis of DNA	Impairment of cell viability	Lacetera et al., 2004
	Monocytes	Immune competence (-) Decreased diapedesis and secretion of IgM[3], TNFA[4] and IL-6[5]	Reduced expression of mRNA encoding for L-selectin, TNFA[4] and IL-6[5]	Lacetera et al., 2004
	Macrophages	Inflammation (+) Increase respiratory burst and induce apoptosis and necrosis	Increase of ROM production	Scalia et al., 2006
	Lymphocytes	Immune competence (-) Altered proliferation, decreased secretion of IgM[3] and IFNG[6]		Lacetera et al., 2004
Saturated fatty acids (SFA), Palmitic (C16:0) and stearic acids (C18:0) decrease their abundance in cell membranes, while lauric (C12:0) and myristic (C14:0) increase after	White blood cells	Altered lymphocytes activation, antibodies production and inflammation driven from the inhibition of CD14[7] and lipolysaccharides-induced tumor necrosis factor (TNF) production	SFA can covalently modify proteins trough fatty acylation, altering membrane fluidity, influencing how proteins anchor to plasma membrane and affecting lipid	Raphael and Sordillo, 2013

calving			raft formation	
	Polymorphonuclear cells	Inflammation (+) Activation of Toll-like receptors (TLRs) 2 and 4, increased apoptosis and necrosis	Pro-inflammatory. C12:0, C14:0 and C16:0 are PAMP[9] that activate NF-κB[10]	Lee et al., 2003; Lee et al., 2004; Scalia et al., 2006
	Macrophages	Immune competence (-) Decreased secretion of IgM[3], IFNG[6], TNFA and IL-6[5]. Altered ROM[8] production	mediated gene expression, increasing inflammation and respiratory burst activity	
		Immune competence (-) Reduced phagocytosis, diapedesis, antigen presentation and synthesis of DNA	Impaired cell viability for the low efficiency in utilization as energy source	
UFA, Oleic and linoleic acids increase, while eicosatrienoic acid (C20:3) and EPA (C20:5) decrease in cell membranes after calving	Polymorphonuclear cells	Inflammation (+) Increased ROM[8] production, increased phagocytosis	α-linoleic acid interacts with PPARG[11]. EPA and DHA interacts with TLR2, TLR4, PPAR and sterol response element binding protein family of transcription factors. All these genes are involved in NF-κB[10] activation and UFA could act both as pro or anti-inflammatory factors	Lee et al., 2003; Lee et al., 2004; Gorjão et al., 2006; Scalia et al., 2006; Lee et al., 2010
		Inflammation (+) reduced expression of mRNA for IL-10 and IL-8, stimulated expression of IL-1B and ICAM I, altered expression of cyclooxygenase (COX) 2 and TNFA		
	Monocytes	Immune competence (-) Reduced antigen presentation to lymphocytes	EPA and DHA reduce MHC II[12] and ICAM1[13] expression decreasing IFNG[6] production	Lee et al., 2003; Lee et al., 2004; Gorjão et al., 2006; Scalia et al., 2006

	Macrophages	Immune competence (-) Increased phagocytosis, inhibition of TLRs	Anti-inflammatory. EPA and DHA inhibits TLR2 and TLR4 reducing expression of NF-κB[10]	Calder et al., 1990; Lee et al., 2003; Lee et al., 2004
	Lymphocytes	Inflammation (+) Increase the production of IL-10, TNFA and IFNG[6] Increase the production of IL-4 in circulating T-cells	EPA and DHA inhibits the production of prostaglandin E2, that is known to reduce IFNG[6] synthesis and lymphocytes proliferation	Gorjão et al., 2006b; Hughes and Pinder, 2000; Trebble et al., 2003; Brassard et al., 2007
BHB, (+) early lactation	Macrophage, polymorphonuclear cells	Immune competence (-) Decreased chemotaxis, respiratory burst and superoxide anion production	Decrease cell viability as are useless as alternative energy source	Calder et al., 1990; Hoeben et al., 1997; Sartorelli et al., 1999; Suriyasathaporn et al., 1999
	Lymphocytes	Immune competence (-) Decreased blastogenesis, mitogenic responses and IgM[3] production	Decrease cell viability as are useless as alternative energy source	Targowski and Klucinski, 1983; Nonnecke et al., 1992; Takeuchi et al., 2010
Glutamine, (-) late gestation and early lactation	Macrophage, polymorphonuclear cells	Immune competence (-) Reduced production of IL-1B and IL-6, reduced phagocytosis Reduced production of nitric oxide (macrophages) and superoxide anion (polymorphonuclear cells)	Glutamine availability drive those functions (high oxidation efficiency) Glutamine drives the production of NADPH	Wallace and Keast, 1992; Ogle et al., 1994; Yassad et al., 1997
	Lymphocytes	Immune competence (-) Reduced cell division and synthesis of lactate	Glutamine is precursor of purine and pyrimidine: essential in anabolic functions	Newsholme et al., 1985; Newsholme et al., 1999; Pithon-Curi et al., 2004

[1]Trend of main metabolites related to immune cells functions during transition period: (-) indicates metabolites that undergoes a reduction, (+) indicates metabolites that increase during transition period.

[2]Effect of main metabolites related to negative energy balance condition on immune functions: (-) indicate a depressive effect, (+) indicate a stimulating effect.
[3]Immunoglobulins M.
[4]Tumor necrosis factor, alpha.
[5]Interleukin-6.
[6]Interferon, gamma.
[7]Cluster of differentiation 14.
[8]Reactive oxygen metabolites.
[9]Pathogen-associated molecular patterns.
[10]Nuclear factor-κB.
[11]Peroxisome proliferation activated receptor, gamma.
[12]Major histocompatibility complex, II.
[13]Intercellular adhesion molecule.

Table 11. Patterns and functions of mRNA transcripts encoding for genes that are up (U) or down-regulated (D) in bovine PMN with a negative (NEB) or positive (PEB) energy balance, induced feeding mid-lactating cows with 60% and 210% of their energy requirements for maintenance respectively (Crookenden et al., 2017)

CATEGORY	GENE	FUNCTION	NEB	PEB
Antigens	Major histocompatibility complex, class I, A (*HLAA*)	Human leukocytes antigen: antigen presentation	D	U
	Major histocompatibility complex, class II, DR alpha (*HLA-DRA*)	Human leukocytes class II histocompatibility antigen, DR alpha chain: antigen presentation	D	U
Antioxidant	Superoxide dismutase 1 (*SOD1*)	Destroy superoxide radicals in cytosol	D	U
Cytokines	Interleukin-10 (*IL10*)	Anti-inflammatory	D	U
	Interleukin-6 (*IL6*)	Pro- and anti-inflammatory	U	D
	Tumor necrosis factor, alpha (*TNFA*)	Pro-inflammatory	D	U
Defensins	Beta defensin-1 (*DEFB1*)	Resistance of epithelial surfaces to microbial colonization	D	U
	S100 calcium binding protein A9 (*S100A9*)	Respiratory burst (H_2O_2 production)	D	U
Eicosanoids	Arachidonate 5-lipoxygenase activating protein (*ALOX5AP*)	Leukotrienes pathway: leukotrienes synthesis	D	U
	Phospholipase A2 group IVA (*PLA2G4A*)	Leukotriene pathway: release arachidonic acid from the membrane	D	U
Clusters of differentiation	CD121B: interleukin 1 receptor type 2 (*IL1R2*)	Interleukins 1, alpha and beta, inhibitor	D	U
	CD44 molecule (*CD44*)	Lymphocyte activation, recirculation and homing, hematopoiesis and tumor metastasis	U	D
Receptors	Interleukin 1 receptor type 2 (*IL1R2*)	Cytokine receptor	U	D
	Toll-like receptor 2 (*TLR2*)	Membrane receptors for pathogen recognition and regulation of immune responses	U	D
	Toll-like receptor 4 (*TLR4*)		U	D
Ribosomal proteins	Ribosomal protein-L23 (*RPL23*)	Protein synthesis	U	D
Transcription factors	Signal transducer and activator of transcription 3 (*STAT3*)	Immune mediator: mediates cellular responses to interleukins, and other growth factors	U	D

Table 12. Patterns of mRNA transcripts encoding for genes that are up (U) or down-regulated (D) after calving in cows that were over conditioned during dry period (BCS > 3.5), and their functions (Crookenden et al., 2017)

CATEGORY	GENE	FUNCTION	HIGH BCS
Defensins	Beta-defensin 4A (*DEFB4A*)	Antimicrobial, immunomodulatory	D
	Beta-defensin 1 (*DEFB1*)	Antimicrobial (epithelial surfaces), immunomodulatory	D (trend)
	Beta-defensin 10 (*DEFB10*)	Antimicrobial, immunomodulatory	D
	S100 calcium binding protein A9 (*S100A9*)	Respiratory burst (H_2O_2 production)	D
Matrix metalloproteinases	Matrix metallopeptidase 8 (*MMP8*)	Neutrophil collagenase: polymorphonuclear cells migration	D
	Matrix metallopeptidase 9 (*MMP9*)	Neutrophil gelatinase: polymorphonuclear cells migration	D
Cytokines	Interleukin 10 (*IL10*)	Anti-inflammatory	D
	Interleukin 23, alpha (*IL23A*)	Pro inflammatory	U
Eicosanoids	Arachidonate 5-lipoxygenase activating protein (*ALOX5AP*)	Leukotrienes pathway: leukotrienes synthesis	D
Clusters of differentiation	CD14 molecule (*CD14*)	Toll like receptor pathway: detection of bacterial lipopolysaccharides	D
	CD117: KIT proto-oncogene receptor tyrosine kinase (*KIT*)	Cell survival, proliferation and differentiation	U
Transporters	ATP-binding cassette subfamily A member 1 (*ABCA1*)	Transporter involved in cholesterol efflux: major regulator of cellular cholesterol and phospholipid homeostasis	U

Table 13. Main biological process related to calving and involved in the development of the oxidative stress status in dairy cows: their site of occurrence, their biological function and the mechanism by which they contribute in altering redox status

BIOLOGICAL PROCESS	SITE	FUNCTION	MECHANISM	REFERENCE
Cellular respiration	All tissues	Conversion of nutrient to energy aimed to face requirements for milk synthesis and secretion	ROM[1] are formed in mitochondria as byproduct of electron transport chain	Valko et al., 2007
Fatty acid metabolism	Liver		ROM[1] are formed in peroxisome consequently to beta-oxidation	Drackley, 1999; Grum et al., 2002
Respiratory burst	Phagocytes	Kill microbial pathogens during inflammation	ROM[1] are formed in phagocytes from NADPH[2] oxidase system	Babior, 1999
Oxylipid biosynthesis	Cellular membranes	Regulation of inflammatory process and immune response	Lipid peroxide and superoxide anion are byproduct of the oxidative reactions of PUFA[3] triggered by COX[4], LOX[4] or cytochrome P450	Raphael and Sordillo, 2013
Reduced dietary intake	Gut, tissue and blood	Adaptation to physiological imbalance related to calving	Reduced plasma concentrations of serum-derived micronutrients as vitamins and minerals with antioxidant properties	Spears and Weiss, 2008; Sordillo and Mavangira, 2014
Increased utilization of antioxidant systems	Blood and tissues	Facing metabolic stress occurred in transition period		
Lipomobilization and triglycerides deposition	Liver cells	Supply to growing energy demand	Liver damage and dysfunction reduce plasma cholesterol and high-density lipoproteins, which bound paraoxonase, an important antioxidant enzyme, to the blood	Turk et al., 2005
Inflammatory status		Face tissue damages and infections related to calving		

[1]Reactive oxygen metabolites.
[2]Nicotinammide adenine dinucleotide phosphate.
[3]polyunsaturated fatty acids.
[4]Cyclooxygenase.
[5]Lipoxigenase.

Table 14. Time course relationship between main transition period diseases, their effect on physiological imbalance conditions and possible secondary diseases triggered from it

PRIMARY	EFFECT ON PHYSIOLOGICAL IMBALANCE	SECONDARY	REFERENCE
Retained placenta	Raise of PIC[1]; depression of DMI[2]; increase of NEB[3] and lipomobilization	Mastitis	Emanuelson et al., 1993
Ketosis	Ketone bodies production; depression of DMI[2]; increase of NEB[3]; impairment of immune functions	Mastitis, metritis, left displacement of abomasum	Oltenacu and Ekesbo, 1994; Duffield, 2000
Retained placenta	Raise of PIC[1]; depression of DMI[2]; increase of NEB[3] and lipomobilization	Ketosis	Dohoo and Martin, 1984
Milk fever	Depression of DMI[2]; increase of NEB[3] and lipomobilization; decreased smooth muscle function (essential for digestive tract); increased cortisol secretion	Ketosis; left displacement of abomasum; retained placenta	Dohoo and Martin, 1984b; Loor et al., 2013; Esposito et al., 2014
Left displacement of abomasum	Depression of DMI[2]; increase of NEB[3] and lipomobilization	Ketosis	Dohoo and Martin, 1984
Fatty liver	Increased haptoglobin production and inflammation; increased PIC[1] production; depression of DMI[2]; increase of NEB[3] and lipomobilization	Metritis, laminitis, displacement of abomasum, mastitis	Van Winden and Kuiper, 2003; Ametaj et al., 2005
Infectious diseases	Decreased glutamine concentrations due to tissue and immune cells consumption; repression of genes related to inflammatory response and PMN[4] chemotaxis; greater expression of mRNA for anti-inflammatory genes and oxidative stress	Metritis, mastitis, infectious diseases	Holtenius et al., 2004; Moyes et al., 2009

[1]Pro inflammatory cytokines.
[2] Dry matter intake.
[3]Negative energy balance.
[4]Polymorphonuclear cells.

Table 15. Main nutritional supplements aimed to modulate dairy cow's immunity during transition period and their mode of action on immune cells

SUPPLEMENT	EFFECT	MECHANISM	REFERENCE
Omega-3 PUFA (eicosapentaenoic acid - EPA - and docosahexahenoic acid - DHA)	Inflammation (-) Mitigation of proinflammatory response (reduced TNF[1] and IL-6[2] production) of immune cells to high NEFA[3] concentrations (as those observed with high lipomobilization) Immune competence (+) Improved lymphocytes and mononuclear cells function in transition period: improved cell-mediated immune response; increased phagocytosis and decreased oxidative stress damages in PMN[4], leading to improved uterine and udder health in early lactation; modified mononuclear cells/PMN[4] ratio	EPA and DHA decrease the amount of arachidonic acid in cell membranes, shifting the oxylipid profile: increased production of resolvins, protectins and lipoxins (which attenuate the inflammatory process) and attenuated endometrial PGF2A[8] production (pro-inflammatory effect). Decreased expression of adhesion molecules involved in inflammatory interactions between leukocytes and endothelial cells; down regulation of Toll-like receptor 4 reduces the expression of transcription factors and PICs[5]	Lessard et al., 2004; Mattos et al., 2004; Trevisi et al., 2011b; Contreras et al., 2012a; b; Dirandeh et al., 2013; Minuti et al., 2015
Omega-6 PUFA (conjugated linoleic acid - CLA)	Inflammation (-) Increased albumin and cholesterol concentrations in early lactation Immune competence (+) Enhanced neutrophils function in transition period	Modulation of the NF-κB[9] inhibiting the LPS[10]-induced inflammatory activity in macrophages Protection of paraoxonase against oxidative inactivation, reducing oxidative stress status; Increased secretion of VLDL[11] and apolipoprotein B100, decreasing cellular accumulation of triglycerides from palmitic acid, increasing DMI[12], reducing NEB[13] and lipomobilization (lower NEFA[3] and BHB[14] levels in blood)	Cheng et al., 2004; Trevisi and Bertoni, 2008; Silvestre et al., 2011
Vitamin A	Prevention of oxidative stress status	β-carotene prevents fatty acid peroxidation chain reaction	Sordillo, 2016
Vitamin C	Mitigation of oxidative stress status	Ascorbic acid act as a radical scavenger	Sordillo, 2016
Vitamin E	Mitigation of oxidative stress status	α-tocopherol disrupts fatty acid peroxidation chain reaction	Trevisi et al., 2011b; Sordillo, 2016
Vitamin D_3	Inflammation (-)	Inhibits Th1 sub-family of	Bertoni et al.,

	Down-regulation of PICs[5] in favor of AICs[6] production	lymphocytes in favor of Th 2	2015
Selenium	Mitigation and prevention of oxidative stress status	Active component of thioredoxin reductase and glutathione peroxidase enzymatic complexes, that controls redox signaling and reduce ROM[15] production	Sordillo, 2016
Copper	Mitigation and prevention of oxidative stress status	Active component of ceruloplasmin, that exert oxidase activity as peroxyl radical scavenger, and superoxide dismutase, that converts cytosol superoxide to H_2O_2	Osorio et al., 2016; Sordillo, 2016
Zinc	Mitigation and prevention of oxidative stress status	Active component of superoxide dismutase, that converts cytosol superoxide to H_2O_2, and metallothionein, which is a cysteine rich radical scavenger	Sordillo, 2016
Manganese	Mitigation of oxidative stress status	Active component of superoxide dismutase that converts cytosol superoxide to H_2O_2	Osorio et al., 2016; Sordillo, 2016
Iron	Mitigation of oxidative stress status	Active component of catalase, that converts H_2O_2 to water	Sordillo, 2016
Choline	Reduced hepatic triglycerides abundance; mitigation of oxidative stress status	Component of phosphatidylcholine, that is required for the synthesis of VLDL[11] by the liver; important sources of the intracellular antioxidants glutathione and taurine	Esposito et al., 2014; Zhou et al., 2016
Methionine and lysine	Reduced hepatic triglycerides abundance; mitigation of oxidative stress status	They affect mitochondrial beta oxidation of fatty acids in liver and export of triglycerides as VLDL[11]; important sources of the intracellular antioxidants glutathione and taurine	Esposito et al., 2014; Zhou et al., 2016
Acetylsalicylic acid	<u>Inflammation (-):</u> Reduced positive APPs[7] abundance and improved liver synthesis	Inhibition of cyclooxygenase enzymatic complex reducing the synthesis of pro inflammatory oxylipids	Shin et al., 2010; Kim et al., 2012; Grossi et al., 2013
Hottuynia cordata extract	<u>Inflammation (-):</u> Reduced production of TNF[1] and pro		Shin et al., 2010; Kim et al., 2012

	inflammatory oxylipids		
Aloe arborescens Mill. extract	Inflammation (-): Increased negative APPs[7] concentration	Reduced mobilization of body fats and improved liver synthesis	Trevisi et al., 2013b

[1]Tumor necrosis factor.
[2]Interleukin-6.
[3]Not esterified fatty acids.
[4]Polymorhonuclear cells.
[5]Pro-inflammatory cytokines.
[6]Anti-inflammatory cytokines.
[7]Acute phase proteins.
[8]Prostaglandin 2, alpha.
[9]Nuclear factor κB.
[10]Lypopolysaccharides.
[11]Very low-density lipoproteins.
[12]Dry matter intake.
[13]Negative energy balance.
[14]Beta-hydroxybutyrate.
[15]Reactive oxygen metabolites.

CHAPTER II - DRY-OFF AND IMMUNE FUNCTIONS

Innate immune response and metabolic changes at dry-off in high yield dairy cows

ABSTRACT

Dry-off is related to deep changes in feeding behavior, gastro intestinal adaptations, metabolism and immune parameters in high-yielding cow's career. Indeed, the release of cortisol, signals of systemic inflammation and altered redox balance have been reported immediately after milking interruption, and high milk yield (**MY**) have a role in aggravating such conditions. Our study was aimed in investigate the main causes of metabolic changes occurring at dry-off and the contribution of MY in such alterations. A group of 13 Holstein dairy cows were housed in tied stalls and dried off at 55 days from expected calving day. Animals were retrospectively divided in two groups according to their average MY in the last week of lactation, assuming a cut-off of 15 $kg*d^{-1}$: low MY (6 cows) and high MY (7 cows). From -7 to 34 days from dry-off (**DFD**) BCS, BW, DMI and rumination time were measured. Blood samples were collected regularly to assess a wide hematochemical profile and to test white blood cell functions through ex vivo challenges. Data were submitted to ANOVA using a mixed model for repeated measures including MY at dry-off, time and their interaction as fixed effects. Increased fiber amounts of dry ration reduced DMI and increased rumination time. Leukocytes migration into mammary gland to contribute in the involution phase decreased their abundance in blood at dry-off, and their activity. Such activation of leukocytes at mammary site increased the abundance of nitrogen species in plasma and triggered a systemic inflammation in all the animals, as reflected from increased concentrations of positive and reduced concentrations of negative acute phase proteins. Such inflammation impaired liver functions, as suggested from the increased gamma-glutamyl transferase, bilirubin and alkaline phosphatase concentrations. Both the production of nitrogen species and the systemic inflammatory status contributed in the depletion of antioxidant system in blood (thiol groups, tocopherol, β-carotene, ferric reducing antioxidant power and oxygen reactive antioxidant capacity). Animals with higher MY at dry-off showed the worst condition, likely

for the deeper metabolic changes they faced at milking interruption, and to the greater amount of mammary parenchyma to be reabsorbed. This study highlights the dry-off as a thorny point to manage dairy cows' health and depose for a relationship between dry-off and immune alteration that typically occurs at calving.

Key Words. Dry-off, inflammation, immune dysfunction, metabolic stress

INTRODUCTION

The proper dry period of dairy cows includes the time between halting of milk removal (milk stasis) and the subsequent calving. This phase allows the development of the mammary gland and the turnover of the secretory tissue (Oliver and Sordillo, 1988) and its essential to maximize milk yield in the following lactation. At dry-off, milk residuals in the mammary gland induce cellular distention and stimulate the release of autocrine mediators (i.e. feedback inhibitor of lactation protein) inhibiting milk synthesis (Wilde et al., 1998; Capuco and Akers, 1999), while fragments arise from the hydrolysis of caseins acts as putative mediators of mammary gland involution (Shamay et al., 2003).

A sudden dry-off has been widely practiced in all commercial dairy farms until increases in milk yields made challenging to stop milk production in order to achieve the 60 days goal for dry period (Dingwell et al., 2001). Some studies suggested a shortened dry period to face the problem (Van Knegsel et al., 2013), but such solution could not guarantee a fully involution of mammary gland tissues, reducing milk production in the following lactations (Klein and Woodward, 1943; Capuco and Akers, 1999). Thus, dairy farmers started to restrict feed supply and withdraw all grain several days before dry-off, in order to reduce drastically the milk production (Agenäs et al., 2003; Odensten et al., 2005), and to halt milking about 45 to 50 days before expected date of parturition.

At dry-off, the priority in redistribution of energy and protein sources shift from mammary gland to fetus, drowning the cow's own needs for body maintenance (Dingwell et al., 2001). Adaptation to dry ration decreases the surface area of rumen papillae (Dieho et al., 2016), and leads fiber utilizing bacteria to prevail on amylolytic species in rumen fluid. Furthermore, halting of milk removal affects mammary gland both at gross and cellular levels, increasing the susceptibility to infections (Cousins et al., 1980). Finally, the hunger related to feed restriction and the modification of social structure after regrouping could induce

psychological stress in weaker animals (von Keyserlingk et al., 2008). Nevertheless, dry-off did not receive as much attention as other challenging periods, such as transition to calving, and only recent concerns about animal welfare promoted a deeper investigation of dry-off as a cause of metabolic stress (Odensten et al., 2005; Zobel et al., 2015). Consequently, high milk yield at dry-off has been identified as a negative factor for dairy cow's health (Rajala-Schultz et al., 2005). In fact, increased udder pressure that follows milking interruption could induce severe pain and injuries with milk yield higher than 25 $kg*d^{-1}$ at dry-off (Silanikove et al., 2013) triggering a raise of stress-related hormones immediately after dry-off (Bertulat et al., 2013). Glucocorticoids and catecholamines interact with specific receptors on immune cells regulating different pathways (i.e. nuclear factor kappa-light-chain-enhancer of activated B-cells and cyclic AMP response element binding protein). Such interactions modifies the transcription of genes encoding for a variety of cytokines. The resulting dysregulation of immune function could last up to early lactation, with a sufficient magnitude to have health implications (Padgett and Glaser, 2003), and suggesting stress occurring at dry-off to be related with severe immune dysfunction happening during the peripartal period of dairy cows (Trevisi et al., 2010).

Based on above, our objective is to investigate the effect of abrupt dry-off on the metabolic, oxidative and immune status during the whole dry period of cows having high or low milk yield. Our hypothesis is that abrupt dry-off can negatively affect the immune response during the dry period, especially in cows with high milk yields at the end of lactation.

MATERIALS AND METHODS

Experimental design and animal management

The trial was carried out at Università Cattolica del Sacro Cuore research dairy barn (Experiment Station, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 1047/2015-PR). A group of 13 Italian Holsteins dairy cows were raised in individual tied stalls with controlled environmental conditions (room temperature of 20°C, relative humidity of 65%, 14 hours of light) and dried off at 55 days from expected calving day with a deep milking and a treatment with a mammary antibiotic (Mamyzin A; Haupt Pharma Latina S.r.l, Borgo San Michele – Latina, Italy). Before dry-off, cows were milked twice a day, at 4:00 am and pm, and milk yield was recorded. All the cows were individually fed with a component diet. Before dry period, animals received 1 kg of concentrate every 3 kg of produced milk. Since -7 days from dry-off (**DFD**) the concentrate was gradually reduced till the complete elimination at dry-off (Phase 1). After dry-off, animals received only hay till 10 DFD. Subsequently, animals received a hay-based ration with soybean meal and corn silage till the end of the experimental period (Phase 2). Same batches of hays and corn silage were used during the trial. Feeds were collected fortnightly and, after dry matter determination, samples were pooled for subsequent analyses. Feeds and diet composition are shown in table 1.

From -7 to 34 DFD periodical checks were performed and blood samples were collected regularly, according to the time schedule shown in Figure 1 and described in the following sections. In addition, the health status of cows was monitored recording all clinical diseases appeared from -7 DFD till 30 days from subsequent calving. To investigate the effect of milk yield, after dry-off animals were retrospectively divided in two groups according to their

average milk yield in the last week of lactation, assuming a cut-off of 15 kg*d^{-1}: low milk yield (**LM**; 6 cows) and high milk yield (**HM**;7 cows).

Body weight, body condition score, dry matter intake and rumination time

The body weight was measured with a single walking-in scale. The body condition score (**BCS**) was determined from the same operator with a 1 to 5 scale (Agricultural Development and Advisory Service, 1986) and its variation (Δ**BCS**) was calculated as the difference between data at -7 and 34 DFD. The daily dry matter intake was measured weighting the amounts of feed administered and residuals for each cow. Rumination time was registered using the Ruminact system (SCR Europe, Podenzano, PC, Italy) and expressed on a daily base (Figure 1).

Health status

The body temperature was measured daily with a rumen bolus (DVM System TempTrack^{TM}, HerdStrong, LLC, Greeley, CO). Mastitis were diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases, retained placenta when the fetal membranes were not expelled within 24 h after calving, endometritis and metritis according to Sheldon et al. (2006), milk fever, displacement of abomasum and pneumonia by a veterinary diagnosis. Diarrhea was diagnose on visual evaluation of feces consistency and color through the fecal score method (Ireland-Perry and Stallings, 1993), assuming diarrheic feces those have a fecal score ≤ 2.

Blood samples collection

Blood samples were harvested trough jugular venipunction in evacuated collection tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) before the morning feeding. Samples were used to perform different assays (Figure 1).

Metabolic profile assessment. For metabolic profile assessment (Figure 1), samples were collected into heparinized tubes and processed as described by Calamari et al. (2016). After collection, samples were centrifuged and packed cells volume (**PCV**) was determined. A clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA) was used to determine the concentration of glucose, not-esterified fatty acids (**NEFA**), beta-hydroxybutyrate (**BHB**), urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, aspartate amino transferase-glutamate oxaloacetate transaminase (**AST-GOT**), gamma glutamyl transferase (**GGT**), alkaline phosphatase (**ALP**), total protein, haptoglobin, ceruloplasmin, albumin, total bilirubin, cholesterol and globulin in accordance with Calamari et al. (2016). Furthermore, reactive oxygen metabolites (**ROMt**), ferric reducing antioxidant power (**FRAP**), nitrate (**NO3**), nitrite (**NO2**) and nitric oxides (**NOx**) were determined according to Jacometo et al. (2015), paraoxonase (**PON**) according to Bionaz et al. (2007), thiol groups (**SHp**) according to Minuti et al. (2014), myeloperoxidase according to Bradley, Priebat, Christensen, & Rothstein (1982) and advanced oxidation protein products (**AOPP**) according to Hanasand et al. (2012). Finally, L-lactic acid (**LLA**) and D-lactic acid (**DLA**) were determined with a commercial kit (K-DLATE, Megazyme Co., Wicklow, Ireland). A multi-detection microplate reader (BioTek Synergy 2, Winooski, VT, USA) and commercial kits for ELISA method were used to determine the concentration of interleukin 1-beta (**IL-1B**; ESS0029; Thermo Scientific, Frederick, MD, USA) and interleukin 6 (**IL-6**; ESS0027; Thermo Scientific, Frederick, MD, USA) according to Jahan et al. (2015) and those of serum amyloid alpha (**SAA**; TP-802, Tridelta D.L., Ireland). Furthermore oxygen reactive antioxidant capacity (**ORAC**) were determined with a fluorometric method according to Jacometo et al. (2015). Retinol, tocopherol and β-carotene were analyzed by reverse-phase HPLC (LC-4000, Jasco Europe, Carpi MO, Italy), as described by Jahan et al. (2015).

White blood cells profile. For the white blood cells (**WBC**) profile (Figure 1), samples were collected with K-EDTA tubes and analyzed with Cell-DYN 3700 (Abbott Diagnostic Division, Santa Clara, CA). A laser optic assay was used to determine the amounts of total WBC, neutrophils, lymphocytes, monocytes, eosinophils and basophils. The neutrophils to lymphocytes ratio was calculated. The amount of red blood cells, hematocrit, mean cell volume, red cell distribution width, number of platelets and mean platelet volume were determined via electrical impedance assay. The amount of hemoglobin, mean cell hemoglobin and mean cell hemoglobin concentration where determined using spectrophotometry assay.

Whole blood stimulation assay. For whole blood stimulation assay (**WBA**, Figure 1), blood samples were collected with heparinized serum tubes and stimulated with 0 (baseline), 0.01 (low dose; **L**) and 5 µg/mL (high dose; **H**) of bacterial lipopolysaccharides (**LPS,** *Escherichia coli* O111:B4; Sigma–Aldrich Company Ltd., UK, Cat. No. L3012), according to Jahan et al. (2015). After WBA, plasma samples were stored at −80ºC for the measurement of glucose, DLA, LLA, IL-1B, IL-6, NOx, NO2 and NO3. Variation of plasma parameters after WBA with L and H doses of LPS were expressed as fold change relative to the baseline.

Interferon gamma release assay. For the interferon gamma (**IFNG**) release assay, whole blood samples were also collected into heparinized tubes (Figure 1). After collection, the tubes were stored in vertical position in a warm bath at a temperature of 38 ºC and transported to the laboratory within 20 min for the stimulation procedure. Whole blood was used in an IFNG release assay for *Mycobacterium avium* (internal method IZSLER, MP 13/011). Briefly, two 1-ml aliquots of each blood sample were distributed in a 24-well plate. One well was supplemented with 100 µl of a 1:10 dilution of *Mycobacterium avium* purified protein derivative (**PPD,** IZS Umbria e Marche, Perugia, Italy) to PBS, and 1 well with 100 µl of sterile PBS as control. The plate was positioned in a heated incubator (Grant Boekel, HIR10 M) set to a temperature of 38 ºC and with a relative humidity of 95% for 24 h. After

incubation, the blood was centrifuged at 8500 × g for 16 min at 4 ºC and plasma was stored at -20 °C until use. Plasma was later thawed and analyzed in a sandwich ELISA assay for bovine IFNG with a couple of monoclonal antibodies, as previously described (Trevisi et al., 2014). Results were evaluated in terms of optical density difference (**ΔOD**) between avian PPD-stimulated and control wells.

Carrageenan skin test

The carrageenan skin test (**CST**) was performed as specified by Jahan et al. (2015) to evaluate peripheral immune responses (Figure 1). The skin thickness was measured using a skinfold caliper (cat# 470119-588, VWR, USA) immediately before carrageenan injection (0 days), then 2 and 9 days after the injection. The total response to each challenge was calculated as the area under the curve of the thickness, measured at day 2 and day 9, subtracting the thickness measured at day 0.

Statistical analysis

Data in the tables are presented as mean and standard error. Before analysis, the normality of distributions was verified for each parameter by reckoning skewness and kurtosis according to the Shapiro test of SAS. Non-normally distributed parameters were normalized through natural logarithms (among plasma parameters the IL-1B, IL-6, LLA, haptoglobin, SAA, NEFA, SHp, AOPP and β-carotene, among WBA the fold changes of DLA, IL-1B, IL-6, NO2, NO3 and NOx and the total response to CST) and back transformed to plot them in the graphs and tables. Prevalence of health problems recorded during the study was evaluated by $\chi 2$ analysis (Freq procedure, SAS Inst. Inc., Cary, NC). Data of body weight, BCS, dry matter intake, rumination time, metabolic profile, WBC profile, WBA and CST were submitted to ANOVA using a mixed model for repeated measures (Mixed procedure, SAS Inst. Inc., Cary, NC) in accordance with Littell et al. (1998). The statistical model included

the fixed effect of milk yield at dry-off (**MD**), time (**t**) and their interaction (**MD*t**). For those parameters that were measured daily (dry matter intake and rumination time) time effect considered the average weekly value, while for other parameters (BW, BCS, metabolic profile, WBC profile, WBA and CST) it considered single DFD. The time was considered as a repeated measure within cow. For WBA, also the dose (**D**; L and H) and the full interaction effect (**MD*t*D**) were considered. The analysis was carried out using three covariance structures: autoregressive order, compound symmetry, and spatial power. These were ranked according to their Akaike information criterion, with the one having the lowest Akaike information criterion being eventually chosen (Littell et al., 1998). Pairwise comparisons were done using the least significant difference test. For t effect, pairwise comparisons were done between data collected before dry-off (-7 DFD for body weight, BCS, metabolic profile, WBC profile, WBA and CST; -2 weeks from dry-off for dry matter intake and rumination time) and subsequent observations. Data of ΔBCS and IFNG release assay were analyzed by a one-way ANOVA (GLM procedure, SAS Inst. Inc., Cary, NC), considering only the fixed effect of MD. Post-hoc comparisons were discussed when the P-value for main effect was ≤ 0.05. Main effects at $P \leq 0.10$ are discussed in the context of tendencies. Relevant trends not supported from statistical differences are discussed as numerical.

RESULTS

Body weight, body condition score, dry matter intake, rumination time and health status

Body weight (Supplemental file 1) increased after dry-off ($t < 0.01$), resulting higher at 34 DFD in comparison to -7 DFD ($P < 0.05$), while BCS decreased after dry-off in HM cows only (MD*t = 0.04). Dry matter intake decreased (Figure 2.a) and rumination time increased (Figure 2.b) during the whole experiment and was not affected by dry-off. Dry matter intake was numerically higher in HM than LM cows (MD < 0.01) and rumination time was higher in HM than LM cows at -2 weeks from dry-off (MD*t = 0.03 and $P < 0.01$).

No clinical diseases were recorded during the whole experimental period, and no MD effect was detected on the incidence of diseases in the first month of the following lactation (Supplementary file 2).

Metabolic profile

PCV (Figure 3.a) peaked one week after dry-off and decreased thereafter ($t < 0.01$; $P < 0.01$ at 7 DFD). No MD nor MD*t effect appeared.

Energy, protein and mineral metabolism biomarkers. Among energy metabolism biomarkers, glucose did not show any effect (Supplementary file 3.a). NEFA (Figure 3.b) increased immediately after dry-off and decreased thereafter, while BHB (Figure 3.c) decreased after dry-off, reached the nadir at 7 DFD and increased thereafter ($t < 0.01$ for both parameters). LLA (Figure 3.d) increased in the first week after dry-off ($t < 0.01$) and was higher in HM than LM cows at -7 DFD (MD*t = 0.02 and $P < 0.01$). DLA (Figure 3.e) decreased during the trial ($t = 0.02$) but was not affected by MD.

Among protein metabolism parameters, creatinine (Figure 3.f) increased in the first week after dry-off, while urea decreased in the first week after dry-off and then gradually increased

(t < 0.01 in both parameters). Creatinine tended to be higher in HM than LM cows (MD = 0.08).

Among mineral metabolism parameters, Ca (Figure 3.h), P (Figure 4.a) and Zn (Figure 4.d) peaked at 2 DFD and then gradually decreased (t < 0.01). Ca resulted higher in HM than LM cows (MD = 0.03). Zn tended to be lower in HM than LM cows after dry-off (MD*t = 0.03; *P* < 0.1 at 2 and 7 DFD). Mg (Figure 3.i) and Cl (Figure 4.c) had the lowest values at 2 DFD and rose thereafter (t < 0.01). Mg and K (Figure 4.d) resulted higher in HM than LM cows (MD = 0.06 and < 0.01 respectively). Na concentration didn't show any effect (Supplementary file 3.b).

Liver function biomarkers. Among liver function biomarkers, AST-GOT didn't show any effect (Supplementary file 3.c). GGT (Figure 4.e) and ALP (Figure 4.g) increased in the first week after dry-off and decreased thereafter (t < 0.01). Bilirubin (Figure 4.f) peaked at 2 DFD and then gradually decreased (t < 0.01) resulting numerically higher in HM than LM cows during the first week after dry-off (MD*t = 0.02).

Inflammation biomarkers. Total protein (Figure 4.h) and globulin (Figure 4.i) peaked at 2 DFD and then gradually decreased (t < 0.01 and = 0.02 respectively). No effect appeared on myeloperoxidase (Supplementary file 1.d).

Among positive acute phase proteins (**APP**), haptoglobin (Figure 5.a) resulted higher in HM than LM cows (MD = 0.04). Ceruloplasmin (Figure 5.b) and SAA (Figure 5.c) peaked at 2 DFD and decreased gradually (ceruloplasmin; t = 0.07) or slightly (SAA; t < 0.01) thereafter.

Among negative APP, albumin (Figure 5.d) and cholesterol (Figure 5.g) gradually decreased after dry-off till the end of the experimental period (t < 0.01). Retinol (Figure 5.e) reached a nadir at 7 DFD and gradually increased thereafter (t < 0.01). PON (Figure 5.f) peaked at 7 DFD and slightly decreased thereafter (t < 0.01).

Among cytokines, IL-1B (Figure 5.h) resulted numerically higher in HM than LM cows from 7 DFD to the end of the experimental period (MD*t < 0.08). No effect appeared on IL-6 concentration (Supplementary file 1.e).

Oxidative stress biomarkers. Among oxidative stress biomarkers, thiol groups (Figure 5.i) and FRAP reached a nadir at 2 DFD, while tocopherol and β-carotene decreased during the whole experimental period. HM cows had higher concentrations of β-carotene (MD*t < 0.01; *P* < 0.1 at -7 and *P* < 0.05 at 27 DFD) and tendentially higher concentrations of ORAC (Figure 6.d; MD = 0.06) than LM cows. AOPP tended to decrease after dry-off (t = 0.09) and resulted numerically higher in HM than LM cows (MD*t = 0.09). No effect appeared on ROMt (Supplementary file 1.f). Nitrite (Figure 6.f), nitrate (Figure 6.g) and nitric oxides (Figure 6.h) increased after dry-off (t = 0.04 for NO2 and t < 0.01 for NO3 and NOx). Nitrite concentrations was higher in HM than LM cows (MD = 0.03).

White blood cells profile

Among WBC profile (Table 2), total WBC, neutrophils and monocytes decreased after dry-off and reached the nadir at 7 DFD (t = 0.03; < 0.01 and < 0.01 respectively). Eosinophils, red blood cells, hemoglobin and hematocrit peaked at 7 DFD (t = 0.04; = 0.02; < 0.01 and = 0.02 respectively). Platelets resulted numerically higher in HM than LM cows at 34 DFD (MD*t = 0.08). Mean platelet volume was lower in HM than LM cows (MD = 0.06). No effect appeared on other parameters (data not shown).

Whole blood stimulation assay

Fold changes of cytokines increased after WBA (Table 3), showing the greatest effect at the highest LPS dose (D < 0.01). Response to LPS increased after dry-off for the whole experimental period for IL-1B (t < 0.01; *P* < 0.01 at 7 and 34 DFD), while peaked at 7 DFD for IL-6 (t = 0.06; *P* < 0.1 at 7 DFD).

Fold change of DLA was not affected by LPS treatment while those of glucose tended to decrease and LLA increased by increased LPS dose (D = 0.06 and 0.03 respectively; Table 4). Response of LLA to LPS decreased after dry-off and reached the nadir at 7 DFD (t = 0.05).

Fold change of NO3 was not overall affected by WBA, while those of NOx tended to increase by increased LPS dose (D = 0.08) with a higher response in LM cows before dry-off (MD*t = 0.04; Table 5). Response of NO2 decreased after dry-off (t = 0.02) and reached the nadir at 7 DFD, independently from the LPS dose (Table 6).

Interferon gamma release assay and carrageenan skin test

Response to IFNG release assay (Supplementary file 4.a) and CST (Supplementary file 4.b) did not show any effect.

DISCUSSION

Dry-off dramatically affects metabolism and immune system of dairy cows

Approaching to dry-off is related to physiological and nutritional changes that deeply affect nutrients requirements and feeding behavior in dairy cows (Dingwell et al., 2001). These changes could justify the likelihood to develop a metabolic stress condition, even though the magnitude and duration of variations induced from dry-off in biomarkers related to wellbeing has been poorly investigated. Our results suggest that adaptation to high fiber content of dry ration had reduce dry matter intake and increased rumination time at dry-off. In late dry period, two-thirds of the development of fetus are completed (Dingwell et al., 2001). This could explain the increase of body weight and the further reduction of dry matter intake observed, as fetal growth could be implied in the reduction of the rumen volume. A mobilization of lipid sources occurred at two DFD, as suggested by increase in NEFA levels. The light magnitude of such a phenomenon is suggested from the lack of any effect on glucose and BHB, indicating that glycaemia was not modified, and that liver has been able to fully oxidize the amount of NEFA received. Previous studies found similar patterns of energy metabolism biomarkers at dry-off paired with the decrease in insulin concentration (Odensten et al., 2005; Putman et al., 2018). It seems reasonable to assume that two processes could concur in such a mobilization of lipid sources. 1) Mobilization could be triggered by the release of adrenaline at dry-off, consequently to the stress induced by milk stasis in the mammary gland and changes in feeding routine. In fact, adrenaline is known to provide the primary stimulus for the mobilization of NEFA from adipose tissue during a stressing event (Drackley, 1999; Padgett and Glaser, 2003). 2) Mobilization could occur during the last week of lactation consequently to the hormonal response to the withdrawn of concentrates while milk production was still maintained. Our experimental design does not allow us to speculate

on the exact cause of lipid mobilization. In fact, samples were collected at -7 DFD and then at 2 DFD, while an additional sample at time 0, just before dry-off, should be collected to distinguish between metabolic changes occurred due to the milking interruption from those occurred as a feed restriction effect. Whatever should be the driving mechanism of the mobilization process, the reduction of NEFA levels observed at 7 DFD suggests: 1) a mitigation of stressing conditions to occur after dry-off and 2) a reduction of energy requirements to occur with milking interruption, as also reflected from the decrease in dry matter intake observed during this phase.

Changes in diet composition and feeding behavior could partially account for the increase of lactate and for the reduction of BHB and urea levels found in plasma after dry-off. In fact, fluctuations of rumen pH occurring during adaptation to dry ration inhibits bacterial utilization of lactate increasing its efflux to blood (Counotte and Prins, 1981). On the other hand, a half of hematic BHB is directly related to the ruminal production of butyric acid (Church, 1979), while plasmatic urea is paired with its concentration in rumen fluid (Marini and Van Amburgh, 2003; Odensten et al., 2005), and both parameters are known to decrease in rumen during dry period consequently to the higher fiber content of the ration and the lower feed intake of the animals in comparison to lactation. Furthermore, blood urea partially arise from amino acids deamination (Broderick and Clayton, 1997) and its lower level reflects also a lower utilization of amino acids in gluconeogenetic processes in order to face the energy deficit after dry-off. This interpretation, together with the increased creatinine level after dry-off, suggests the interruption of milk synthesis to have reduced protein requirements, increasing the amount of amino acids addressed to anabolic processes. Indeed, creatinine is the product of the metabolism of one of the main molecules for the storage of energy in the muscle (i.e., phosphocreatine), and it is thus a direct indicator of muscular body mass (Hayden et al., 1992).

Effects of dry-off on mineral metabolism are mainly related to the interrupted milking routine. The presence of higher levels of Ca immediately after dry-off is consistent with previous results (Putman et al., 2018). Such an increase could arise from the contribution of two main processes: 1) the sudden interruption of the mammary gland uptake from the hematic circulating pool of calcium and 2) the increased udder pressure arise from the stasis of milk residuals, that is known to weak the tight junctions between epithelial cells increasing paracellular transport of calcium in blood (Aslam and Tucker, 1998). Both processes had a transient effect during milk stasis phase only, as the decreased calcium concentration observed from two DFD up to the end of the experimental period suggests the exhaustion of milk residuals in the mammary gland and the recovery of homeostasis in its hematic pool. Lower concentrations of both Ca and Mg found in late dry period in comparison to late lactation where in agreement with their strong direct relationship with milk production. A similar relationship, even though indirect, has been reported for phosphorus. In fact, blood concentration of this mineral is directly regulated from calcium-regulating hormones, and thus depend on Ca concentrations more than milk yield (Cavestany et al., 2005).

Early involution of mammary gland that occurs at the beginning of dry period implies an important contribution of leukocytes in the reabsorption of mammary tissues (Putman et al., 2018). In our study, reduction of WBC, neutrophils and monocytes populations in blood observed after dry-off are consistent with results of Putman et al., (2018) and are related to the migration of those cells to the mammary gland during acute involution (Atabai et al., 2007). The increased concentration of eosinophils is also consistent with results of Putman et al., (2018), even though the interpretation is less certain. State that eosinophils are related to allergies and parasites (Gouon-Evans et al., 2000), it has been hypothesized that it may represent a subclinical hypersensitivity to the milk residuals in mammary gland after milking interruption (Putman et al., 2018). Such changes in WBC populations in blood is also paired

with their augmented sensitivity to biological stressors, as reflected from our results of WBA test with LPS. In fact, increased production of interleukins and NO2 after dry-off reflect a greater production of metabolites related to inflammation and of oxidant species. These results are consistent with an important leukocytes activity in the mammary gland at dry-off, that could be seeing as a main cause of the systemic inflammatory status observed in the same phase, and reflected from trends of plasma parameters (Castell et al., 1989). Metabolic inflammation typically affects liver metabolism, implying severe losses in hepatic functions and a shift of anabolic priority of the organ (Bertoni et al., 2008). In particular, the liver produces more α-globulins, known as positive APP, i.e. haptoglobin, ceruloplasmin and SAA (Ceciliani et al., 2012). Conversely, it reduces the synthesis of albumin, retinol binding protein, PON and lipoproteins, known as negative APP (Bertoni et al., 2008) and sequesters minerals, as zinc and iron, from blood flow (Bertoni and Trevisi, 2013). In our trial, increase in GGT, ALP and bilirubin concentrations found during the first week after dry-off reflects a reduced capacity of the liver to perform normal metabolic and clearance functions (Rodriguez-Jimenez et al., 2018). In fact, GGT and ALP are enzymes involved in AA metabolism and dephosphorilation of compounds respectively, that increase in blood mostly due to liver damages, while bilirubin results from degradation of red blood cells, and its clearance depends on liver enzymes functioning (van den Top et al., 1996). Furthermore, raise of globulins and total proteins observed during the first week after dry-off could be driven from the α-globulins fraction, as they are paired to the increase in ceruloplasmin and SAA (Crisman et al., 2008). These trends are consistent, together with the drop of retinol found at two DFD, with an acute inflammation occurred at dry-off, despite the lack of a time effect on haptoglobin concentrations and the decrease in albumin, PON and cholesterol at the end of the experimental period only. In fact, haptoglobin is known to peak instantaneously during acute phase, while negative APP could require a longer time to reflect inflammatory

conditions (Bertoni and Trevisi, 2013; Minuti et al., 2015), and their incapacity to detect the inflammation in our experiment could depend on the different sensitivity to acute phase of these APP. Inflammatory events are often linked to a body fat mobilization (Kushibiki et al., 2003), that could partially explain the peak of NEFA observed after dry-off. Furthermore, increase in nitrate and nitrite concentrations found in blood after dry-off is consistent with results of Putman et al., (2018), who related it to the altered redox status during early involution of the mammary gland. On the other hand, the increase of nitric oxide could be related to WBC activities at mammary level, as it is mainly produced by activated macrophages as a cytotoxic agent (Coleman, 2001). Accumulation of such nitrogen species is known to trigger oxidative damages on macromolecules, as nitric oxide exerts a direct oxidative action, while nitrite is converted in nitrating agent for lipids and proteins by MPO during the neutrophils activation (Dedon and Tannenbaum, 2004). Body effort to contain such oxidative damages could account for the depletion of antioxidant systems after dry-off, explaining the decrease in hematic SHp, tocopherol and β-carotene levels. In fact, SHp (and specifically glutathione) allows the reduction of a wide spectrum of hydroperoxides participating in the glutathione peroxidase enzymatic complex functioning (Sordillo and Aitken, 2009), tocopherol is involved in the reduction of the chain propagation and amplification of lipid peroxidation process, while β-carotene indirectly participate in the protection against oxidative stress maintaining other antioxidant molecules in the reduced form (Ghiselli et al., 1995). Consumption of such antioxidant systems in containing oxidative damages, together with the dysregulation they normally undergoes during a systemic inflammations (Celi, 2011), could account also for a wider alteration in the redox status of the body, that is reflected from the decrease in the general indicators of antioxidant capacity after dry-off (i.e. FRAP and ORAC). In fact, FRAP represent a measurement of antioxidant power provided by bilirubin, uric-acid, vitamins C and E and proteins (Benzie and Strain, 1996),

while ORAC is a quantification of antioxidant power provided by water-soluble antioxidants, albumin and uric-acid against oxygen radicals (Cao and Prior, 1998) and concentrations of most of the compounds included in such complex indicators (i.e. albumin, proteins) decreased after dry-off due to the inflammatory status.

The reduction in AOPP found at 27 and 34 DFD suggests a decreased activity of leukocytes in mammary gland during dry period. In fact, AOPP represent a synthetic marker of protein oxidation exerted by hypochlorous acid, that is produced by MPO during neutrophils activation (Celi and Gabai, 2015), and its reduction suggest the interruption of leukocytes contribution in mammary remodeling. Even though Zn levels raised immediately after dry-off, only the interruption of leukocytes activities in mammary gland allowed the organism to face the systemic inflammation occurred at milking interruption. In fact, the increased blood concentration of retinol and the decreased concentrations of positive APP and enzymatic efficiency indicators (ALP and bilirubin) indicate a fully recovery of homeostasis in liver metabolism to occur at 27 DFD only. On the other hand, the altered redox balance did not seem to ameliorate, as the production of nitrogen species and the consumption of antioxidant systems (SHp, FRAP, tocopherol, and β-carotene) continued up to 34 DFD. At least for β-carotene and tocopherol, a contribution of the increased vitamins requirements in growing fetus could be hypothesized in such a reduction.

High milk yield at dry-off is related to greater stress in dry period

The risk related to dry-off a cow with a milk yield higher than 25 $kg*d^{-1}$ has been deeply investigated previously (Dingwell et al., 2001; Rajala-Schultz et al., 2005), and the relationship with the development of diseases such as mastitis is well known. In this respect, the adoption of an average milk yield of 15 $kg*d^{-1}$ in our study demonstrate that "safety threshold" to dry-off a cow could be much lower, as hematic biomarkers allowed the

detection of important alteration in the body homeostasis to occur in animals having the highest milk yield at dry-off. The higher dry matter intake and the greater rumination time found in HM animals depose for a greater rumen volume and a more efficient utilization of feedstuff to face the higher milk production at dry-off. This is also in agreement with the higher creatinine concentration found in blood of HM cows, which suggests a greater muscle tissue abundance in those animals (Hayden et al., 1992). Furthermore, linkage of Ca and Mg with milk production (Dingwell et al., 2001) explains the higher concentration of these minerals found in HM cows. In fact, higher mineral requirements related to production in those animals could lead to higher circulating minerals concentrations as soon as cows were dried-off. Those differences demonstrate that HM cows faced deeper changes during the metabolic adaptation to dry-off, reflecting a greater risk to develop metabolic stress in those animals. HM cows showed greater lactate concentrations before dry-off and higher concentrations of haptoglobin and IL-1B after dry-off. This could indicate that inflammation appeared earlier and that acute phase had a longer duration in these animals (Bertoni et al., 2008; Osorio et al., 2014). Furthermore, the latter raise of Zn and the greater concentrations of bilirubin (even though numerical) and NO2 found after dry-off suggest that these animals required a longer time to recover homeostasis (Bertoni and Trevisi, 2013). The greater degree of inflammation is consistent with a longer mammary tissue remodeling phase in high yielding cows, probably related to the greater amount of parenchymatic tissue to be reabsorbed (Arslan et al., 2013; Putman et al., 2018). In HM cows, higher blood concentrations of β-carotene and ORAC found both before and after dry-off suggested a greater antioxidant power. Furthermore, lower fold variation of NOx found at WBA before dry-off suggested a less marked oxidant production in their WBC. Nevertheless, negative consequences of the severe inflammation occurred at dry-off were reflected on the tendency for a greater AOPP level found at 27 and 34 DFD, indicating HM animals to develop a more

marked oxidative stress in late dry period. State the relationship between stress, inflammation and metabolic disorders (Biswas and Lopez-Collazo, 2009; Van Knegsel et al., 2014) high milk yield at dry-off could be a risk factor for the development of diseases, and a significant effect is well known for mastitis (Grummer et al., 2000). The investigation on this relationship was behind the aim of our study, as the low number of animals enrolled do not allow us to speculate about that. Thus, similar diseases incidence found in the first month of lactation among MD groups in our experiment requires care in the interpretation.

CONCLUSIONS

Dry-off has been revealed as a challenging phase in high-yielding cow's career, related to deep changes in feeding behavior, metabolism and immune parameters. An inflammation occurred at dry-off in all the animals, probably because of leukocytes contribution in the involution phase of mammary gland, impairing liver function and altering redox balance during the early dry period. Animals with higher milk yield at dry-off showed the worst condition, and this could probably be related to the deeper metabolic changes they faced at dry-off consequently to milking interruption, and to the greater amount of mammary parenchyma to be reabsorbed. This study highlights the dry-off as a thorny point to manage dairy cows' health and could depose for a relationship between dry-off and immune alteration that typically occurs around calving time. In order to demonstrate this, a study where cows are followed during the subsequent lactation for all the parameters measured should be performed.

TABLES AND FIGURES

Table 1. Composition and characteristics of the experimental diets fed during the 2 experimental phases. Between dry-off day (-55 days from expected calving) and 10 days from dry-off (DFD) cows received only grass hay

	Phase 1	Phase 2
DFD	-7; 0	10; 34
Diet, % DM		
Item		
Corn silage	28.5	18.6
Alfalfa hay	16.4	-
Grass hay	23.4	71.4
Concentrate (Dry period)	-	10.0
Concentrate (Lactation period)	31.7	-
Concentrate composition, %DM		
Corn flour	-	
Barley flour	-	
Sorghum grain expanded	-	
Soybean meal	90.5	
Soybean dry rolled	-	
Sunflower meal	-	
Corn gluten feed	-	
Beet pulp	-	
Wheat bran	-	
Beet molasse slops	-	
Potato protein	-	
Hydrogenat palm oil	-	
Limestone	-	
Dicalcium phosphate	-	
Sodium bicarbonate	-	
Magnesium oxide	2.2	
Sodium Chloride	1.4	
Supplement[3]	5.9	
Chemical composition		
$NE_{L,}$ Mcal/kg of DM	1.59	
Crude protein, % DM	14.9	
Starch + sugar, % DM	23.7	
Ether extract, % DM	3.80	
NDF, % DM	39.4	
MP[2], %CP	9.80	
RUP[2], %CP	4.64	

[1]Supplements were composited to provide 150000 UI of vitamin A, 10000 IU of vitamin D, 200 mg of vitamin E, 100 mg of vitamin K, 100 mg of vitamin H1 50 mg of vitamin B1, 0.5 mg of vitamin B12, 500 mg of vitamin PP, 4000 mg of choline, 350 mg of Mn, 800 mg of Zn, 40 mg of Cu, 20 mg of I, 1 mg of Co, 1 mg Se.

[2]Estimate using NRC 2001.

Table 2. White blood cells profile in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off

Item, Unit	MD[1]	Days from dry-off			SE[2]	P-value		
		-7	7	34		MD[1]	t[3]	MD*t[4]
White blood cells, $K*\mu L^{-1}$	LM	6.84	6.52	6.54	0.73	0.99	0.03	0.21
	HM	6.91	6.11*	6.92	0.79			
Neutrophils, $K*\mu L^{-1}$	LM	3.45	3.00	3.32	0.30	0.65	<0.01	0.79
	HM	3.73	3.05**	3.50	0.33			
Monocytes, $K*\mu L^{-1}$	LM	0.55	0.41	0.55	0.04	0.88	<0.01	0.78
	HM	0.58	0.43**	0.53	0.04			
Eosinophils, $K*\mu L^{-1}$	LM	0.19	0.45	0.23	0.11	0.32	0.04	0.82
	HM	0.03	0.31*	0.15	0.12			
Red blood cells, $K*\mu L^{-1}$	LM	6.59	6.68	6.36	0.18	0.34	0.02	0.65
	HM	6.26	6.59	6.10	0.20			
Hemoglobin, $g*dL^{-1}$	LM	10.7	11.2	10.7	0.38	0.39	<0.01	0.85
	HM	10.3	10.8**	10.1	0.41			
Hematocrit, %	LM	31.3	32.1	31.1	1.23	0.57	0.02	0.38
	HM	30.2	32.2*	29.3	1.33			
Platelets, $K*\mu L^{-1}$	LM	325.7	318.4	279.1	48.6	0.71	0.17	0.08
	HM	334.0	285.8	348.5	52.5			
MPV[5], $K*\mu L^{-1}$	LM	7.16	7.10	7.58	0.41	0.06	0.44	0.29
	HM	5.84	6.69	6.24	0.52			

[1] Effect of milk yield at dry-off.
[2]Standard error.
[3]Time effect (comparisons are made with respect to -7 days from dry-off value: * is $P < 0.05$; ** is $P < 0.01$).
[4]Milk yield at dry-off x time interaction effect.
[5]Mean platelet volume.

Table 3. Fold changes of cytokines after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off. Values are expressed with respect to baseline (unstimulated sample)

Item	D[2]	MD[1]	Days from dry-off			SE[3]	Effect	P-Value
			-7	7	34			
IL-1B[7]	L	LM	3.74	7.79	8.59	6.54	MD[1]	0.61
		HM	4.54	9.20	10.84	7.06	t[4]	<0.01
	H	LM	15.66	25.38	25.61	6.54	D[2]	<0.01
		HM	15.96	25.67	35.41	7.06	MD*t[5]	0.89
	Tot	LM	9.70	16.58	17.10	5.45	MD*t*D[6]	0.77
		HM	10.25	17.44 **	23.13 **	5.89		
IL-6	L	LM	1.42	1.73	1.36	0.21	MD[1]	0.51
		HM	1.46	1.56	1.41	0.23	t[4]	0.06
	H	LM	2.21	2.40	2.00	0.21	D[2]	<0.01
		HM	1.73	1.93	1.74	0.23	MD*t[5]	0.75
	Tot	LM	1.81	2.07	1.68	0.18	MD*t*D[6]	0.75
		HM	1.60	1.74 +	1.58	0.19		

[1]Milk yield at dry-off effect.
[2]Dose effect: L is low dose (0.01 µg LPS /mL whole blood); H is high dose (5 µg LPS /mL whole blood); Tot is total effect of LPS stimulation.
[3]Standard error.
[4]Time effect (comparisons are made with respect to -7 days from dry-off value: + is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[5]Milk yield at dry-off x time interaction effect.
[6]Milk yield at dry-off x time x dose interaction effect.
[7]Interleukin-1, beta.

Table 4. Fold changes of glucose and metabolites thereof after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off. Values are expressed with respect to baseline (unstimulated sample)

Item	D[2]	MD[1]	Days from dry-off			SE[3]	Effect	P-Value
			-7	7	34			
Glucose	L	LM	0.95	0.97	0.96	0.022	MD[1]	0.17
		HM	0.98	0.95	0.99	0.023	t[4]	0.72
	H	LM	0.92	0.93	0.91	0.022	D[2]	0.06
		HM	0.94	0.95	0.98	0.023	MD*t[5]	0.25
	Tot	LM	0.94	0.95	0.94	0.016	MD*t*D[6]	0.85
		HM	0.96	0.95	0.98	0.017		
D-lactic acid	L	LM	1.00	1.04	1.05	0.032	MD[1]	0.57
		HM	1.04	1.02	1.03	0.034	t[4]	0.32
	H	LM	1.01	1.02	1.08	0.032	D[2]	0.15
		HM	1.07	1.07	1.08	0.034	MD*t[5]	0.33
	Tot	LM	1.01	1.03	1.07	0.025	MD*t*D[6]	0.79
		HM	1.06	1.04	1.06	0.027		
L-lactic acid	L	LM	1.03	1.01	1.04	0.015	MD[1]	0.62
		HM	1.03	1.03	1.04	0.017	t[4]	0.05
	H	LM	1.05	1.02	1.07	0.015	D[2]	0.03
		HM	1.06	1.05	1.06	0.017	MD*t[5]	0.27
	Tot	LM	1.04	1.02	1.06	0.012	MD*t*D[6]	0.97
		HM	1.05	1.04	1.05	0.013		

[1]Milk yield at dry-off effect.
[2]Dose effect: L is low dose (0.01 µg LPS /mL whole blood); H is high dose (5 µg LPS /mL whole blood); Tot is total effect of LPS stimulation.
[3]Standard error.
[4]Time effect (comparisons are made with respect to -7 days from dry-off value: + is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[5]Milk yield at dry-off x time interaction effect.
[6]Milk yield at dry-off x time x dose interaction effect.

Table 5. Fold changes of nitrite, nitrate and nitric oxides after a whole blood stimulation assay with a low (L) or high (H) dose of bacterial lipopolysaccharides (LPS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off. Values are expressed with respect to baseline (unstimulated sample)

Item	D[2]	MD[1]	Days from dry off			SE[3]	Effect	P-Value
			-7	7	34			
Nitric oxide	L	LM	1.00	1.00	1.00	0.075	MD[1]	0.51
		HM	0.88	1.03	1.02	0.081	t[4]	0.42
	H	LM	1.16	1.16	1.04	0.075	D[2]	0.08
		HM	0.97	1.01	1.09	0.081	MD*t[5]	0.04
	Tot	LM	1.08 *a*	1.08	1.02	0.061	MD*t*D[6]	0.51
		HM	0.93 *b*	1.02	1.06	0.066		
Nitrite	L	LM	0.98	0.98	1.03	0.069	MD[1]	0.60
		HM	0.96	0.88	1.19	0.074	t[4]	0.02
	H	LM	1.00	1.02	1.13	0.069	D[2]	0.81
		HM	1.03	0.90	1.01	0.074	MD*t[5]	0.34
	Tot	LM	0.99	1.00	1.08	0.053	MD*t*D[6]	0.60
		HM	0.99	0.89	1.10 *	0.057		
Nitrate	L	LM	1.00	1.00	1.00	0.126	MD[1]	0.48
		HM	0.87	1.08	0.96	0.136	t[4]	0.47
	H	LM	1.23	1.30	1.07	0.126	D[2]	0.11
		HM	0.96	1.04	1.12	0.136	MD*t[5]	0.11
	Tot	LM	1.11	1.15	1.03	0.098	MD*t*D[6]	0.48
		HM	0.91	1.06	1.04	0.105		

[1]Milk yield at dry-off effect.
[2]Dose effect: L is low dose (0.01 µg LPS /mL whole blood); H is high dose (5 µg LPS /mL whole blood); Tot is total effect of LPS stimulation.
[3]Standard error.
[4]Time effect (comparisons are made with respect to -7 days from dry-off value: + is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[5]Milk yield at dry-off x time interaction effect (*a/b* is $P < 0.1$).
[6]Milk yield at dry-off x time x dose interaction effect.

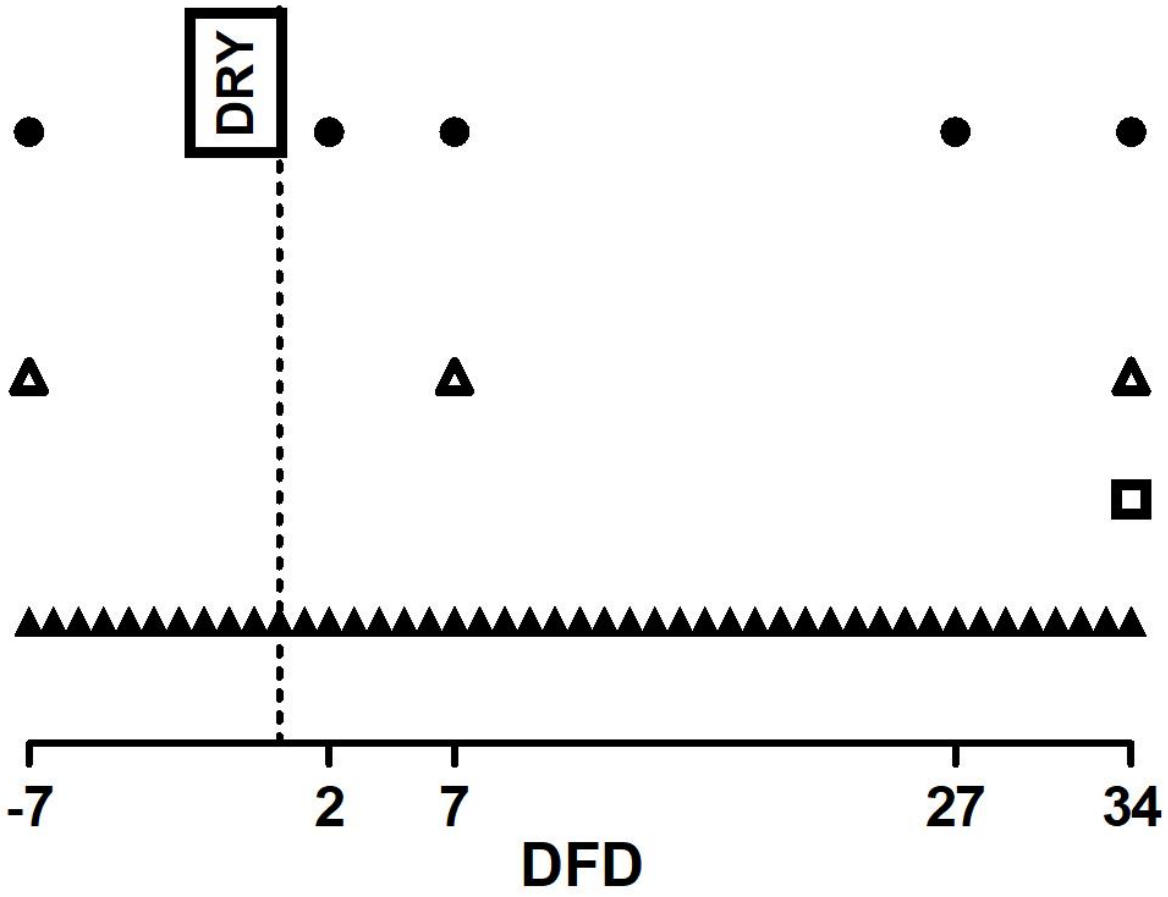


Figure 1. Scheduled time point, expressed as day from dry off (DFD), for hematic metabolic profile (●), body weight and body condition score determination, white blood cells profiling, whole blood stimulation assay and carrageenan skin test performance (△), interferon gamma release assay (□), dry matter intake and rumination time measurement (▲).

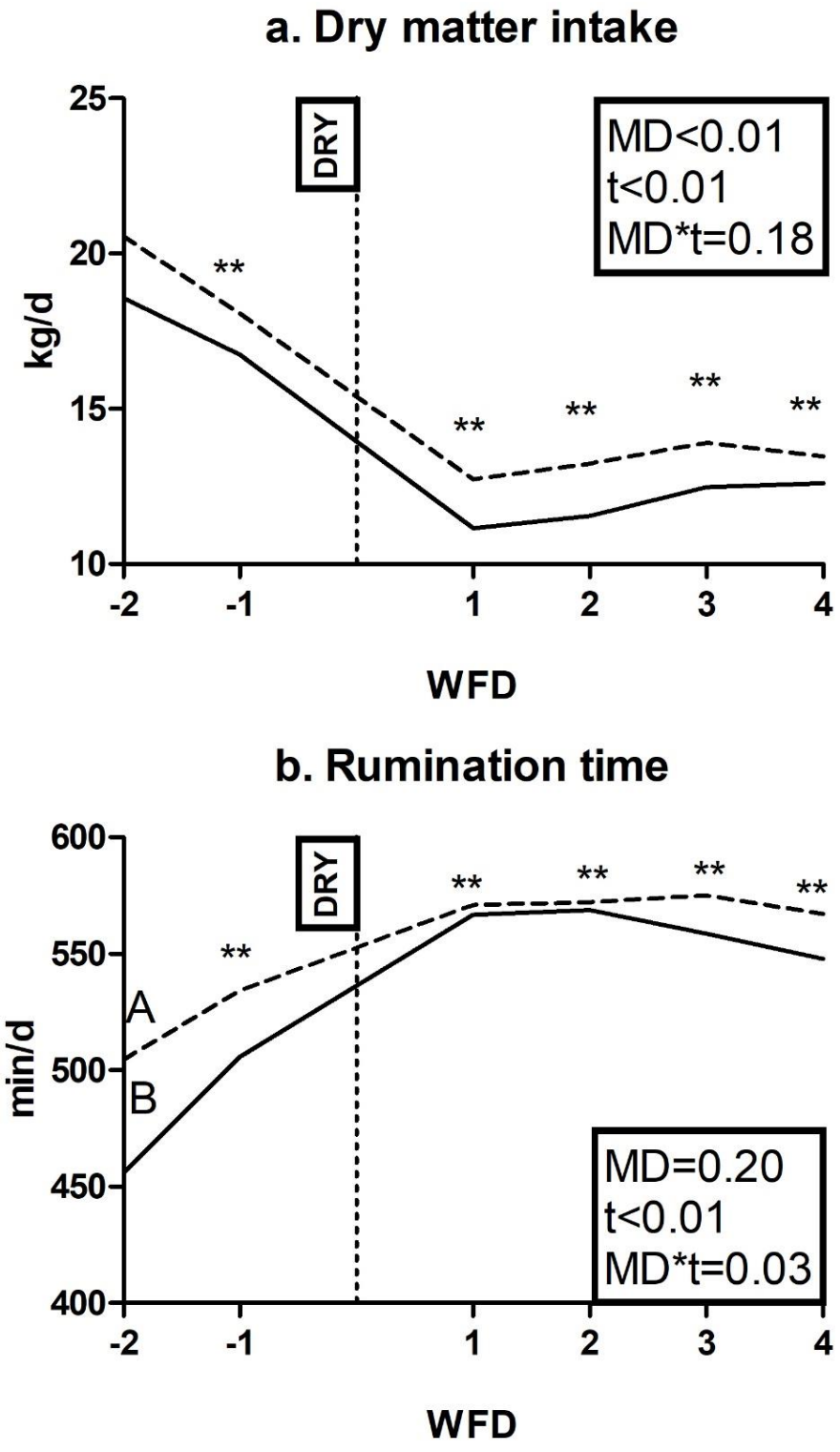


Figure 2. Pattern of dry matter intake (a) and rumination time (b) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 $L*d^{-1}$ (HM; dotted line) in the week prior to dry off. MD is the effect of milk yield at dry off; t is time effect (** is $P < 0.01$); MD*t is the interaction effect (A/B is $P < 0.01$); WFD is weeks from dry off; DRY is dry-off day (-55 days from expected calving).

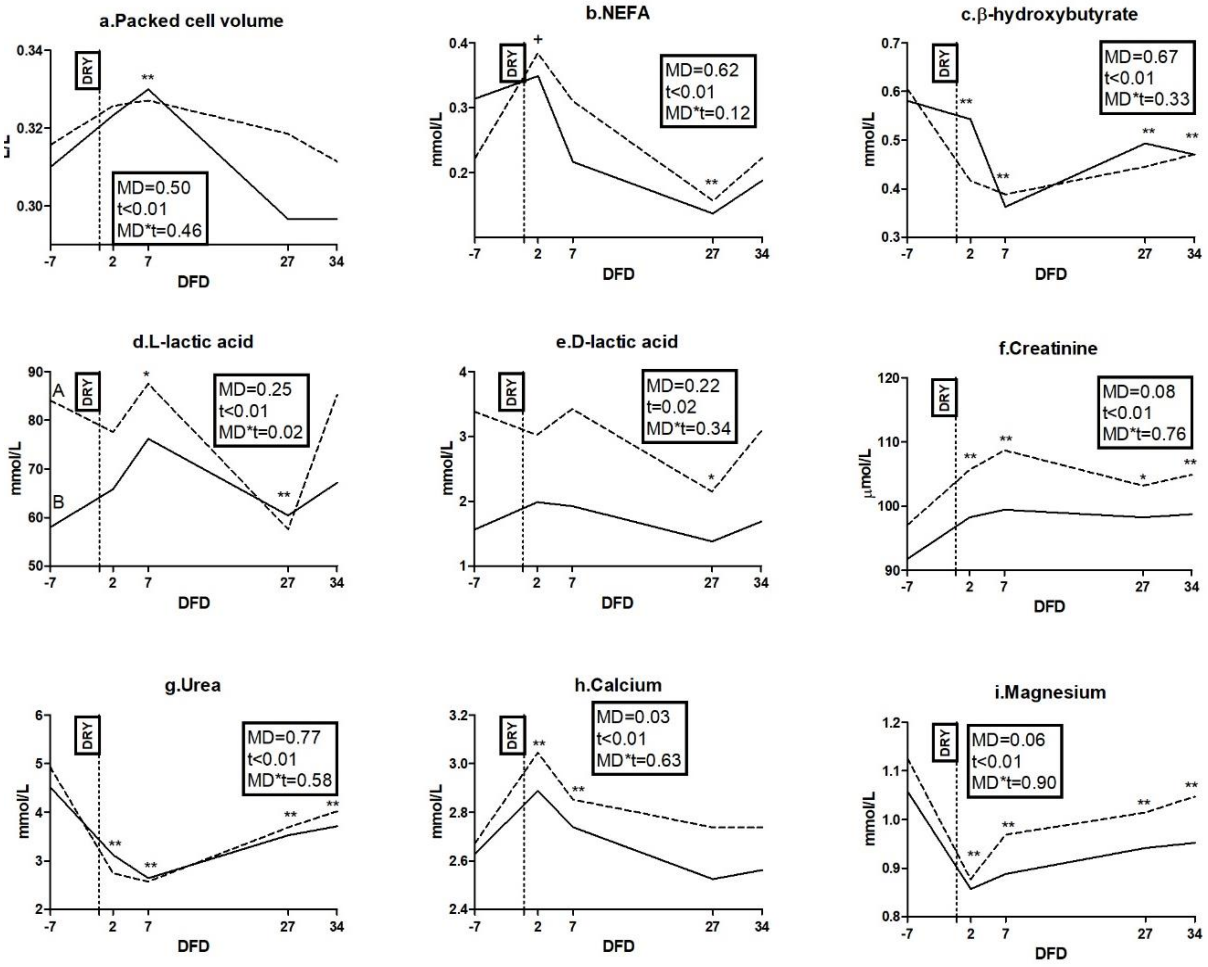


Figure 2.Time course of packed cell volume (a) and plasma concentrations of NEFA (b), β-hydroxybutyrate (c), L-lactic acid (d), D-lactic acid (e), creatinine (f), urea (g), calcium (h) and magnesium (i) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 $L*d^{-1}$ (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); MD*t is the interaction effect (A/B is $P < 0.01$); DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).

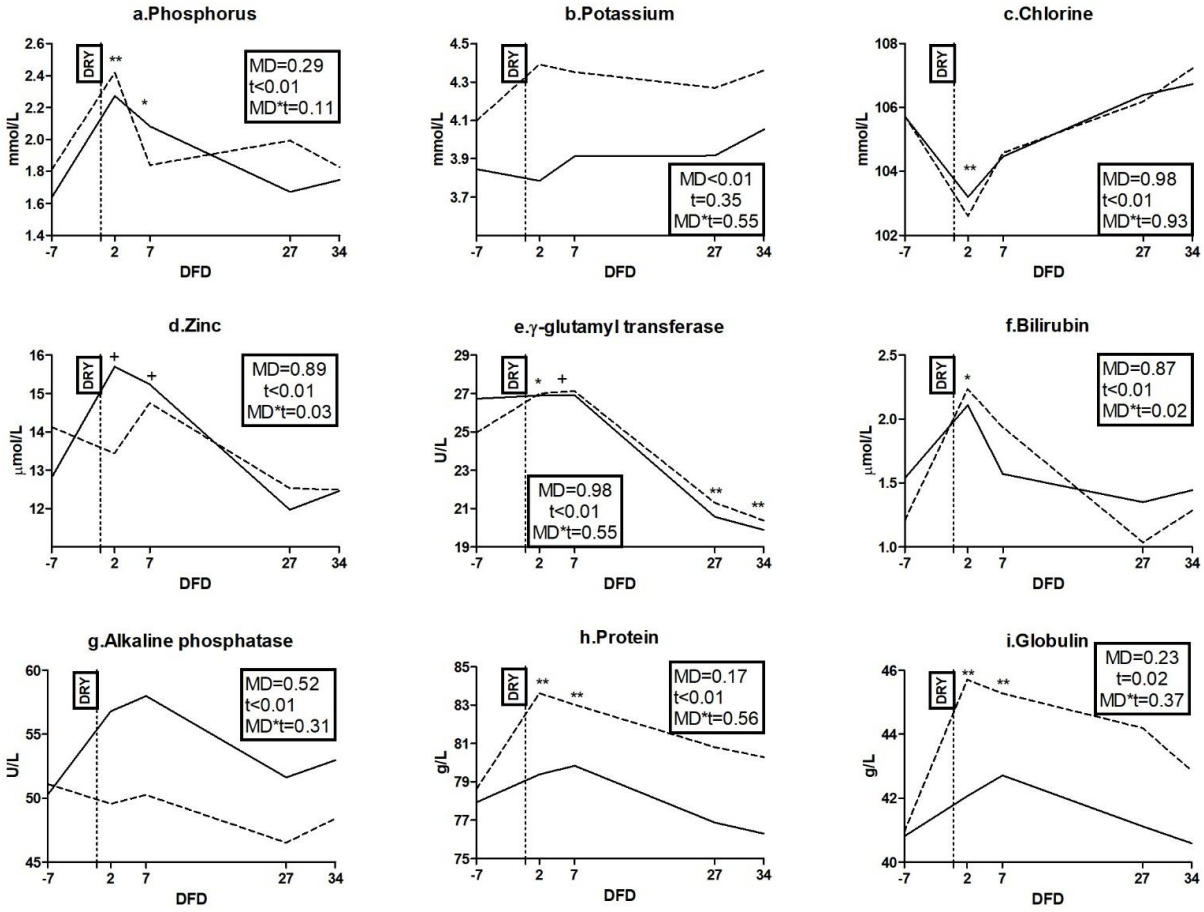


Figure 3. Time course of plasma concentrations of phosphorus (a); potassium (b), chlorine (c), zinc (d), γ-glutamyl transferase (e), bilirubin (f), alkaline phosphatase (g), protein (h) and globulin (i) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 $L*d^{-1}$ (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); MD*t is the interaction effect; DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).

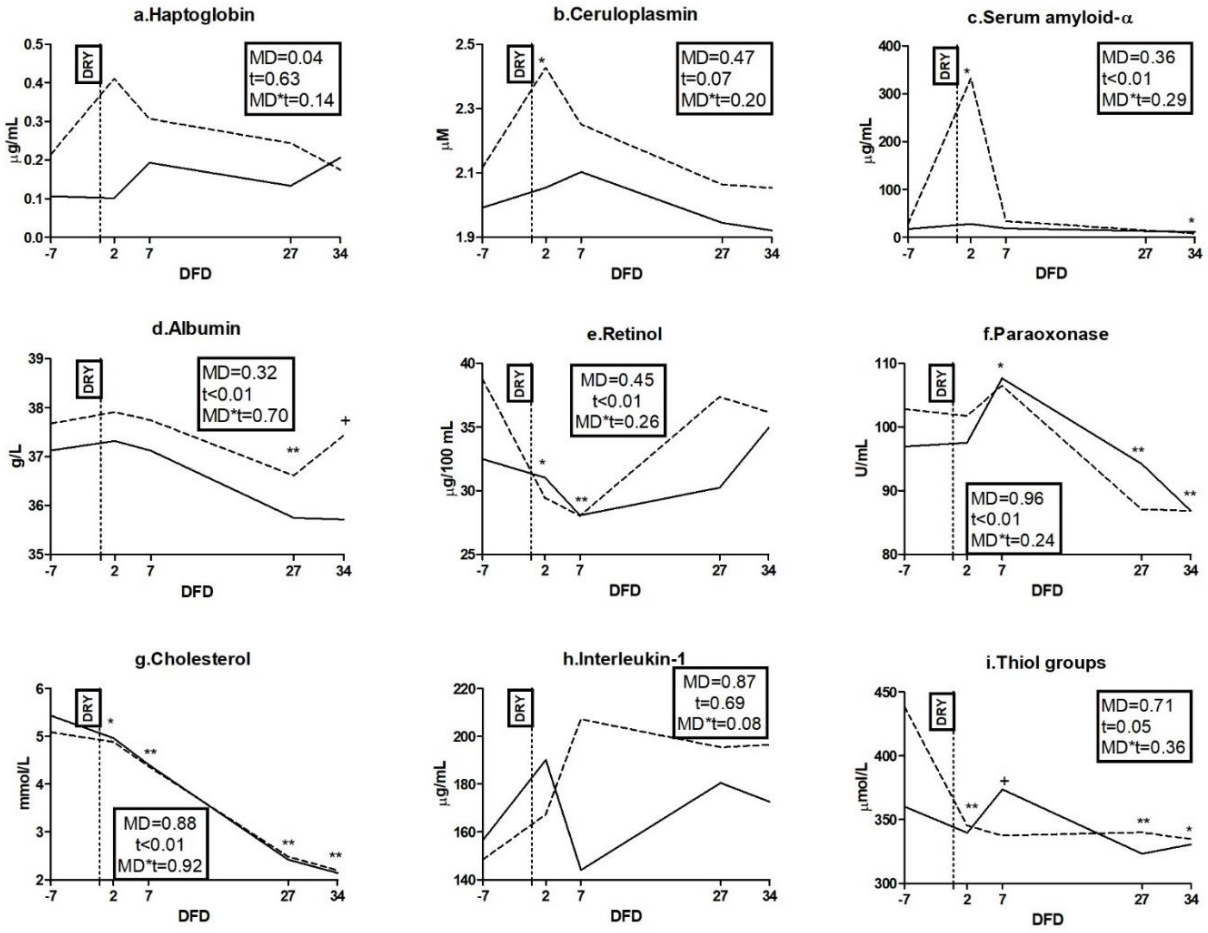


Figure 5. Time course of plasma concentrations of haptoglobin (a); ceruloplasmin (b); serum amyloid-α (c), albumin (d), retinol (e), paraoxonase (f), cholesterol (g), interleukin-1 (h) and thiol groups (i) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 L*d-1 (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); MD*t is the interaction effect; DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).

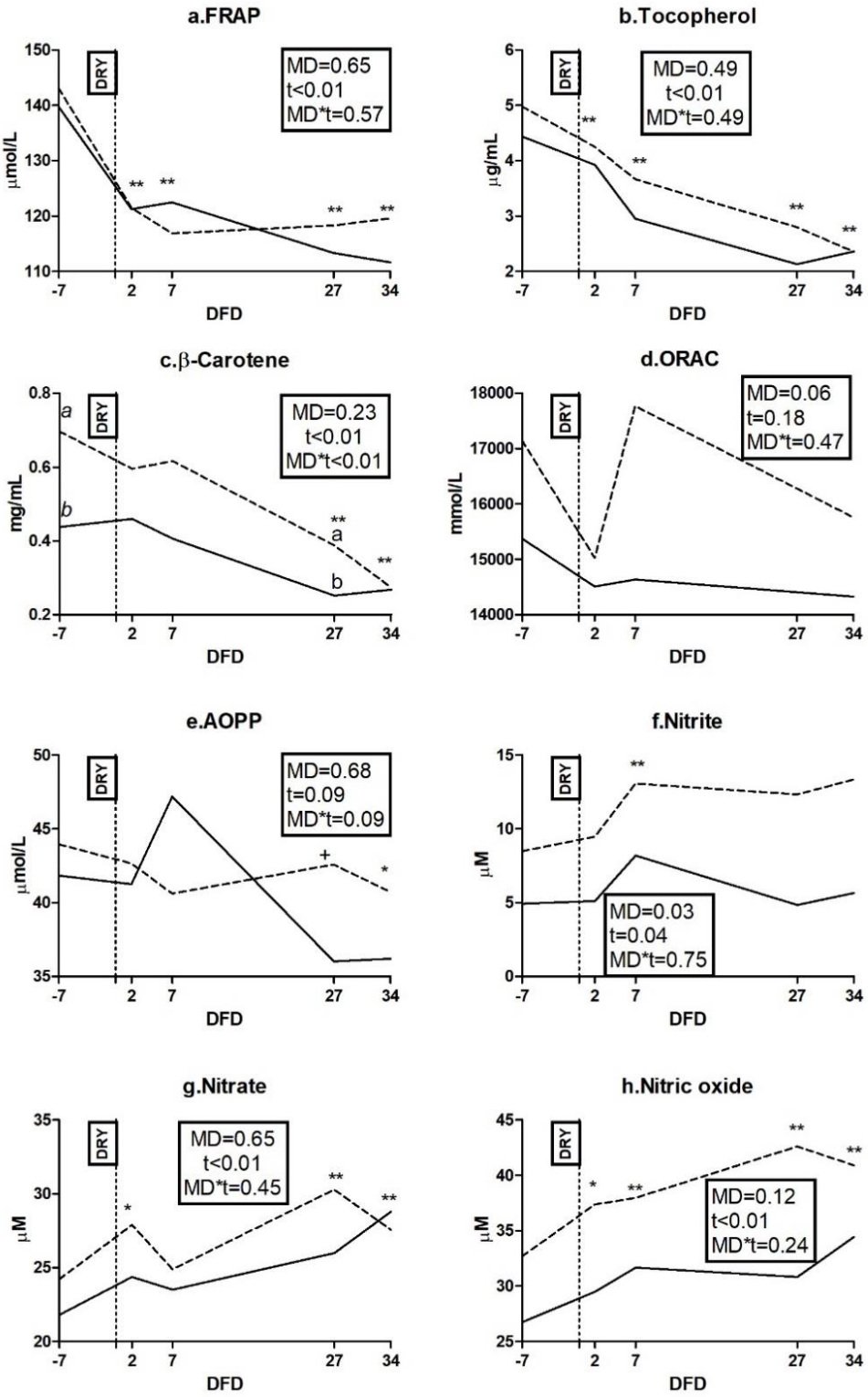


Figure 6. Time course of plasma concentrations of ferric reducing antioxidant power (FRAP; a); tocopherol (b), β-carotene (c), oxygen reactive antioxidant power (ORAC; d), advanced oxidation protein product (AOPP; e), nitrite (f), nitrate (g) and nitric oxide (h) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 L*d-1 (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); MD*t is the interaction effect (a/b is $P < 0.05$; a/b is $P < 0.1$); DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).

SUPPLEMENTARY FILES

Supplementary file 1. Trends of body weight (BW) and body condition score (BCS) in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off

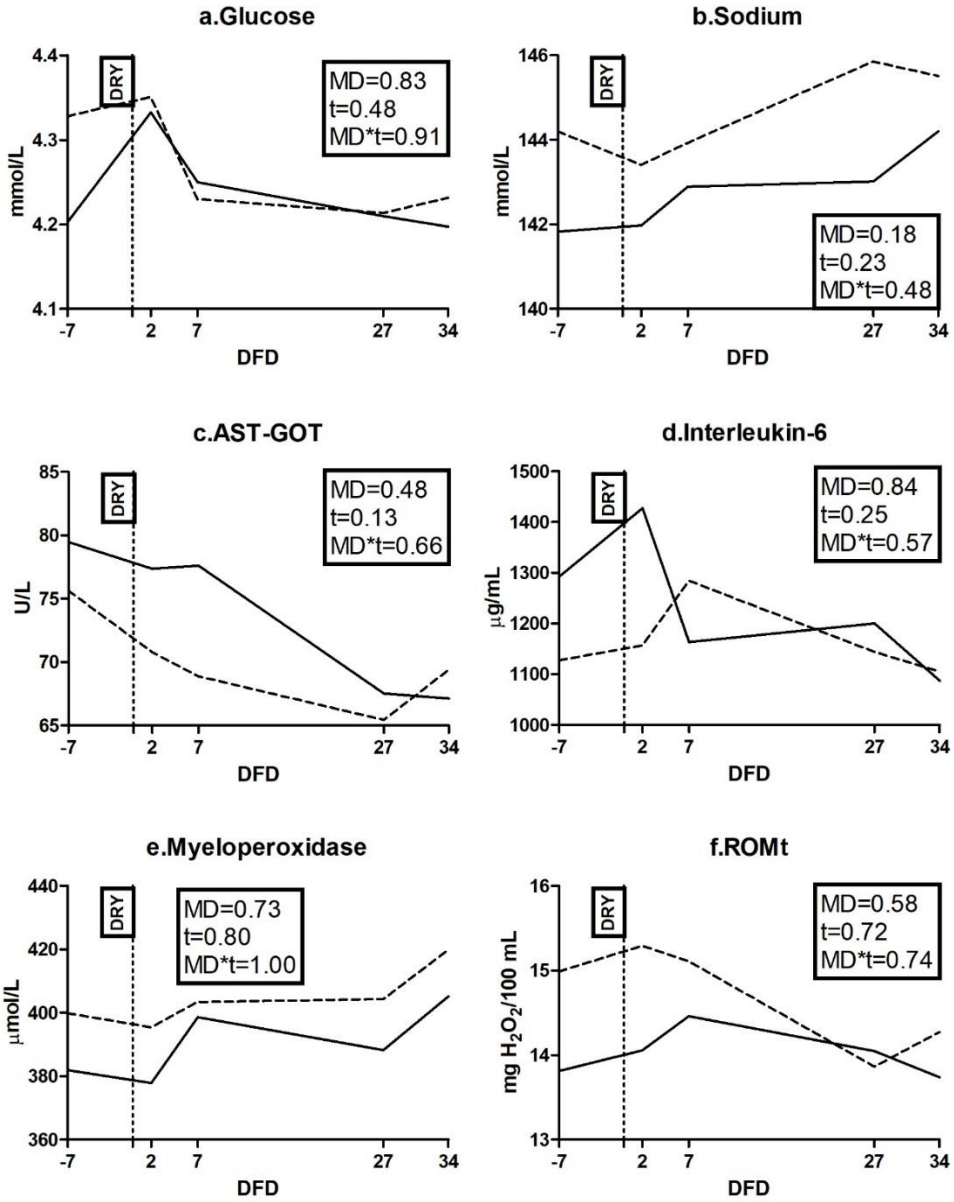
Item, Unit	MD[1]	Days from dry-off			SE[5]	P-Value		
		-7	7	34		MD[1]	t[3]	MD*t[4]
BW, Kg	LM	642.9	653.6	684.1	24.18	0.93	<0.01	0.87
	HM	645.3	653.7	690.5 *	26.11			
BCS	LM	2.5	2.5	2.5	0.099	0.50	0.15	0.04
	HM	2.4	2.4	2.5	0.106			
ΔBCS[2]	LM			0.0	0.051	0.07		
	HM			-0.1	0.055			

[1]Milk yield at dry-off (LM is low milk yield; HM is high milk yield).
[2]Difference between BCS value at -7 and 34 DFD.
[3]Time effect.
[4]Milk yield at dry-off x time interaction effect.
[5]Standard error.

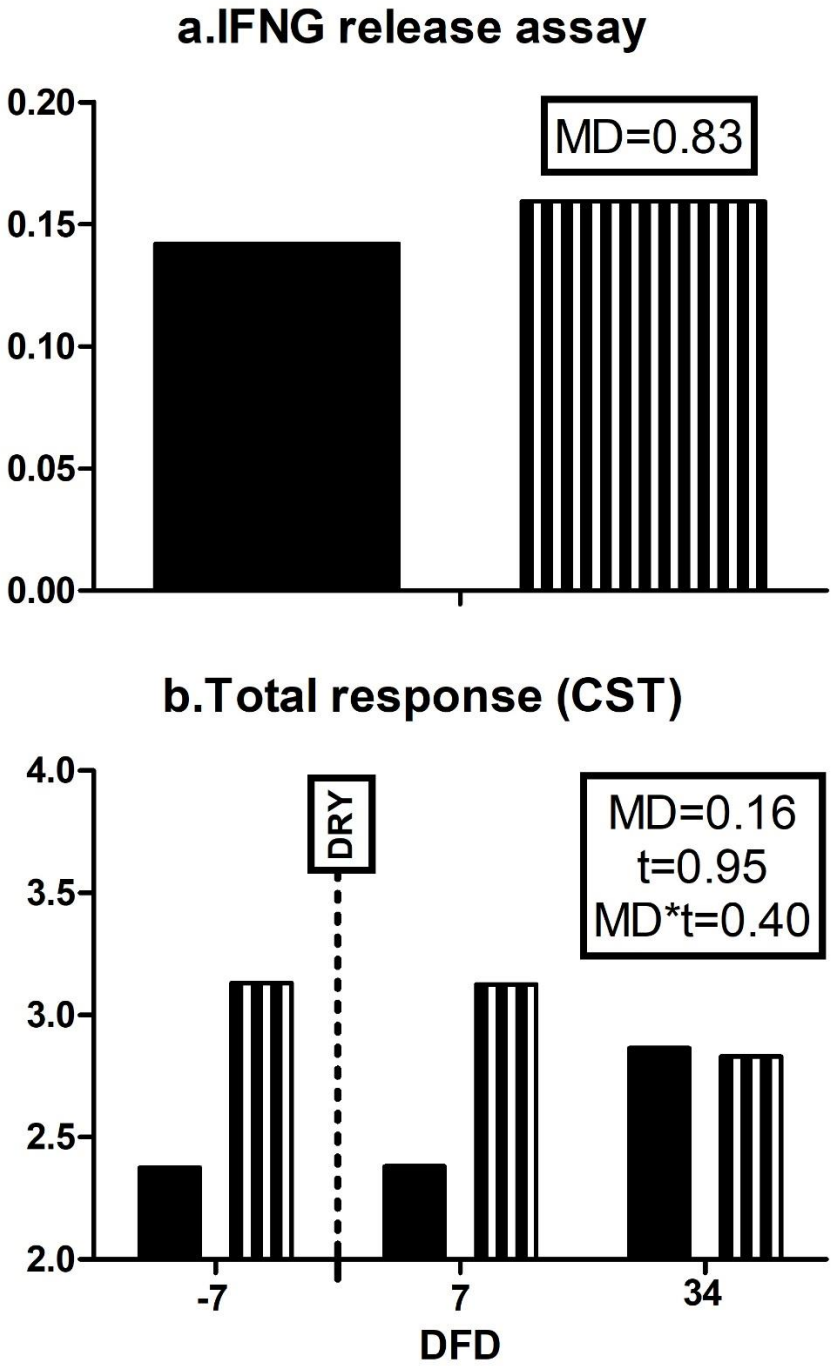
Supplementary file 2. Incidence of diseases observed in the first month of lactation in dairy cows with an average milk production lower (LM) or higher (HM) than 15 $L*d^{-1}$ in the week prior to dry-off

MD[1]	Healthy[2]	Problems occurrence, n								
		RP[3]	ME/ED[4]	MF[5]	DA[6]	MA[7]	PN[8]	DH[9]	KET[10]	Total
LM (7 cows)	3	0	1	0	1	1	1	3	3	4
HM (6 cows)	3	1	0	1	0	1	0	1	3	4
P	0.80	0.26	0.33	0.26	0.33	0.90	0.33	0.31	0.79	0.72

[1]Milk yield at dry-off.
[2]Cows that did not have any clinical diseases in the first month of lactation.
[3]Retained placenta.
[4]Endometritis or metritis.
[5]Milk fever.
[6]Displacement of abomasum.
[7]Mastitis.
[8]Pneumonia.
[9]Diarrhea.
[10]Sub clinical ketosis (blood BHB higher than 1.4 $mmol*L^{-1}$).



Supplementary file 3. Time course of plasma concentrations of glucose (a) sodium (b), aspartate amino transferase-glutamate oxaloacetate transaminase (AST-GOT; c), interleukin-6 (d), myeloperoxidase (e) and total reactive oxygen metabolites (ROMt; f) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 $L*d^{-1}$ (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect; MD*t is the interaction effect; DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).



Supplementary file 4. Optical density difference (ΔOD) between avian PPD-stimulated and control wells in the interferon gamma (IFNG) release assay (a) and total peripheral response after the carrageenan skin test (CST; b) in dairy cows with an average milk production lower (LM; solid line) or higher than 15 $L*d^{-1}$ (HM; dotted line) in the week prior to dry-off. MD is the effect of milk yield at dry-off; t is time effect; MD*t is the interaction effect; DFD is days from dry-off; DRY is dry-off day (-55 days from expected calving).

CHAPTER III - IMMUNE ALTERATIONS BOOST KETOSIS

Altered immune functions in the dry period boost ketosis onset in early lactation

ABSTRACT

Ketosis impairs white blood cells (**WBC**) functions, thus increasing disease prevalence. Our study investigated changes occurring in the immune system prior to disease onset to elucidate their role in ketosis occurrence. A group of 13 Holstein dairy cows were housed in tied stalls and retrospectively divided into 2 groups based on their plasma beta-hydroxybutyrate (**BHB**) levels: lower (**CTR**; 7 cows) or higher than 1.4 mMol/L (**KET**; 6 cows). From -48 to 35 days from calving BCS, BW, DMI, rumination time and milk yield were measured, and blood samples were collected regularly to assess hematochemical profile and to test white blood cell functions by *ex vivo* challenge assays. Data were submitted to ANOVA using a mixed model for repeated measures including health status, time and their interaction as fixed effects.

KET cows had a greater activation of the immune system prior to calving (higher plasma concentrations of pro inflammatory cytokines – **PIC** –, myeloperoxidase and oxidant species, and greater interferon gamma responses to *M. avium*), higher blood concentration of gamma glutamyl transferase and lower plasma minerals. Larger NEFAs, BHB, and glucose in KET vs. CTR was detected during the dry period. The latter may be cause by reduced insulin sensitivity in KET vs. CTR. The effect observed during the dry period was associated to reduced DMI and worse negative energy balance in early lactation and ensued reduced milk yield and increased fat mobilization. KET vs. CTR cows had accentuated acute phase response after calving (greater concentrations of positive acute phase proteins and lower concentration of retinol) impairing liver function (higher blood concentrations of glutamate-oxaloacetate transaminase and bilirubin). WBC of KET vs. CTR cows had reduced response to an *ex vivo* stimulation assay with lower production of PIC and greater production of lactate. These alterations on WBC could be driven by the combined action of metabolites related to

the mobilization of lipids and by the occurrence of a transient unresponsive state against stimulation aimed to prevent over exuberant inflammation.

Key Words. Immune response, dry period, inflammation, liver function

INTRODUCTION

The transition period (**TP**) is the most critical phase for dairy cows due to the dramatic metabolic changes that implies (Drackley, 1999). In some cases the control of the homeorhetic and homeostatic mechanisms is not effective, leading to a physiological imbalance with a substantial increase in the risk of diseases (Ingvartsen and Moyes, 2012).

Ketosis is the most prevalent metabolic disease postpartum and has a significant economic impact in the dairy industry (Duffield, 2000; Youssef and El-Ashker, 2017). It consists in abnormal high levels of circulating ketone bodies that are detectable in blood, milk, and urine. The hematic level of beta-hydroxybutyrate (**BHB**) is the golden standard to define sub clinical (>1400 µmol/L) and clinical (>3000 µmol/L) ketosis respectively (Gordon et al., 2013). The etiology of the disease is related to a prolonged negative energy balance (**NEB**) in early lactation (Herdt, 2000), along with a marked mobilization of non-esterified fatty acids (**NEFA**) from the adipose tissue. Under normal conditions, NEFA are completely oxidized in the liver to produce energy. When mobilization is excessive oxidation is not completed in the liver leading to ketone bodies production and their release into the blood (Ingvartsen, 2006). Classically, pathological levels of ketone bodies are reported during the third and fourth week post calving (Duffield, 2000). Advances in genetics and feeding management have anticipate metabolic challenges closer to calving with higher frequency of pathological peaks of BHB during the first 2 weeks post calving (Herdt et al., 1981; Rodriguez-Jimenez et al., 2018).

Ketosis induces a reduction of feed intake (Kaufman, LeBlanc, McBride, Duffield, & DeVries, 2016) and rumination time (Abuajamieh et al., 2016), reducing milk yields (Duffield, 2000). Blood levels of NEFA, glucose, lactate, positive acute phase proteins (**APP**) and pro inflammatory cytokines (**PIC**) increase during ketosis (Chapinal et al., 2011; Zhang et al., 2016), while negative APP decrease (Rodriguez-Jimenez et al., 2018). Ketosis has also been associated with an impairment of liver functions and oxidative stress conditions

(Rodriguez-Jimenez et al., 2018). Furthermore, ketosis increases the expression of mRNA for anti-inflammatory genes in liver (Akbar et al., 2015) and metabolic changes related to the disease negatively affect white blood cells (**WBC**) functions (Sordillo, 2016). Thus, ketosis onset could impair immune functions and increase the risk of infectious diseases such as metritis and mastitis at the beginning of lactation (Duffield, 2000; Sordillo, 2016).

Prior work detected an association between post-partum ketosis and certain prepartum conditions. Lower DMI and lower standing behavior prepartum was detected in cows with sub-clinical ketosis post-partum compared to a control group (Rodriguez-Jimenez et al., 2018). High blood levels of NEFA (Chapinal et al., 2011) or higher lactate and interleukin-6 (**IL-6**; Zhang et al., 2016) during prepartum was associated with higher risk of ketosis post-partum. All the above conditions are negatively associated with the function of the immune system, including high NEFA (Lacetera et al., 2004); thus, it is possible that a dysfunctional immune system is causative of a higher risk of ketosis post-partum. Therefore, our hypothesis is that cows with clinical ketosis post-partum are characterized by a dysfunctional immune system during the dry period. Owing to the above, our study consisted in a retrospective analysis of blood BHB to detect ketosis in post-partum dairy cows aimed to test the relationship between ketosis onset in early lactation and trends of metabolic and immunological parameters during the dry period, to identify possible alterations reflecting a risky situation for disease occurrence.

MATERIALS AND METHODS

Experimental design and animal management

The trial was carried out at Università Cattolica del Sacro Cuore research dairy barn (Experiment Station, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 1047/2015-PR). A group of 13 Italian Holstein dairy cows were housed in individual tied stalls under controlled environmental conditions (room temperature of 20 °C, relative humidity of 65%, 14 hours of light) from -48 to 35 days from calving (**DFC**). All the cows were milked twice a day at the stand, at 4:00 am and pm. Animals were individually fed a component diet offered with two equal meals of forages at 12 h intervals and 2-8 meals of concentrates supplied by computer feeder. From -48 DFC till -7 DFC animals received a hay-based ration with soybean meal and corn silage (Phase 1). Seven days before calving, 1 kg of lactation concentrate was added to the diet (Phase 2). After calving, 3 kg of alfalfa-dehydrated hay and 2 kg wk^{-1} of corn silage (till a maximum of 20 kg d^{-1}) were added to the diet. Grass hay was gradually reduced to 2-2.5 kg d^{-1} and concentrate was increased by 0.5 kg d^{-1} to satisfy the requirement of 1 kg per 3 kg of produced milk (Phases 3 and 4). The same batches of hay and corn silage were used during the trial. Feeds were collected fortnightly and, after dry matter determination, samples were pooled for subsequent analyses. Feeds and diet composition are shown in Table 1. Periodical checks were performed, and blood samples were collected regularly during the trial, according to the time schedule shown in Figure 1 and described in the following sections.

Body weight, body condition score, dry matter intake, rumination time and milk yield

The body weight (**BW**) was measured with a single walking-in scale. The body condition score (**BCS**) was determined from the same operator with a 1 to 4 scale (Agricultural

Development and Advisory Service, 1986) and his variation (Δ**BCS**) was calculated as the difference between data at calving day and 45 DFC. The individual DMI was measured by weighing the amounts of feed administered and residuals for each distribution. Rumination time was registered using the Ruminact system (SCR Europe, Podenzano, PC, Italy). Milk yield was weighed after each milking. Daily values of DMI, rumination time and milk yield were expressed as average weekly value.

Health status

Health conditions of cows were monitored daily and all veterinary interventions that occurred from -48 to 35 DFC were recorded. The body temperature was measured daily with a rumen bolus (DVM System TempTrack^{TM}, HerdStrong, LLC, Greeley, CO). Mastitis was diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases, retained placenta when the fetal membranes were not expelled within 24 h after calving, endometritis and metritis according to Sheldon et al. (2006), and milk fever, displacement of abomasum and pneumonia by inspection of a veterinary practitioner. Diarrhea was diagnosed by visual evaluation of feces consistency and color according to the fecal score method (Ireland-Perry and Stallings, 1993), assuming diarrheic feces those have a fecal score ≤ 2.

Blood samples collection

Blood samples were harvested trough jugular venipuncture in evacuated collection tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) before the morning feeding. Samples were used to perform different assays (Figure 1).

Metabolic profile assessment. For metabolic profile assessment (Figure 1), samples were collected into heparinized tubes and processed as described by Calamari et al. (2016). A clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA) was

used to determine the concentration of glucose, NEFA, BHB, urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, aspartate amino transferase-glutamate oxaloacetate transaminase (**AST-GOT**), gamma glutamyl transferase (**GGT**), alkaline phosphatase (**ALP**), total protein, haptoglobin, ceruloplasmin, albumin, total bilirubin, cholesterol and globulin in accordance with Calamari et al. (2016). Furthermore, reactive oxygen metabolites (**ROMt**), ferric reducing antioxidant power (**FRAP**), nitrate (**NO3**), nitrite (**NO2**) and nitric oxides (**NOx**) were determined according to Jacometo et al. (2015), paraoxonase (**PON**) according to Bionaz et al. (2007), thiol groups (**SHp**) according to Minuti et al., (2014), myeloperoxidase according to Bradley et al., (1982) and advanced oxidation protein products (**AOPP**) according to Hanasand et al. (2012). Finally, L-lactic acid (**LLA**) and D-lactic acid (**DLA**) were determined with a commercial kit (K-DLATE, Megazyme Co., Wicklow, Ireland). A multi-detection microplate reader (BioTek Synergy 2, Winooski, VT, USA) and commercial kits for ELISA method were used to determine the concentration of interleukin-1, beta (**IL-1B**; ESS0029; Thermo Scientific, Frederick, MD, USA) and IL-6 (ESS0027; Thermo Scientific, Frederick, MD, USA) according to Jahan et al. (2015) and those of serum amyloid alpha (**SAA**; TP-802, Tridelta D.L., Ireland). Furthermore oxygen reactive antioxidant capacity (**ORAC**) were determined with a fluorometric method according to Jacometo et al. (2015). Retinol, tocopherol and beta-carotene were analyzed by reverse-phase HPLC (LC-4000, Jasco Europe, Carpi MO, Italy), as described by Jahan et al. (2015).

White blood cells profile. For the WBC profile (Figure 1), samples were collected with K-EDTA tubes and processed with a clinical auto-analyzer (Cell-DYN 3700, Abbott Diagnostic Division, Santa Clara, CA). A laser optic assay was used to determine the amounts of total WBC, neutrophils, monocytes and basophils. The amount of mean cell volume and number of platelets were determined via electrical impedance assay. The amount of hemoglobin and mean cell hemoglobin where determined using spectrophotometry assay.

Whole blood stimulation assay. For whole blood stimulation assay (**WBA**, Figure 1), blood samples were collected with heparinized serum tubes and stimulated with 0 (baseline), 0.01 (low dose; **L**) and 5 µg/mL (high dose; **H**) of bacterial lipopolysaccharides (**LPS,** *Escherichia coli* O111:B4; Sigma–Aldrich Company Ltd., UK, Cat. No. L3012), according to Jahan et al. (2015). After WBA, plasma samples were stored at −80 ºC for the measurement of glucose, DLA, LLA, IL-1B, IL-6, NOx, NO2 and NO3. Variation of plasma parameters after WBA with L and H doses of LPS were expressed as fold change relative to the baseline.

Interferon gamma release assay. For the interferon gamma (**IFNG**) release assay, whole blood samples were also collected into heparinized tubes (Figure 1). After collection, the tubes were stored in vertical position in a warm bath at a temperature of 38 ºC and transported to the laboratory within 20 min for the stimulation procedure. Whole blood was used in an IFNG release assay for *Mycobacterium avium* (internal method IZSLER, MP 13/011). Briefly, two 1-ml aliquots of each blood sample were distributed in a 24-well tissue culture microtiter plate. One well was supplemented with 100 µl of a 1:10 dilution of *Mycobacterium avium* purified protein derivative (**PPD,** IZS Umbria e Marche, Perugia, Italy, 0.5 mg/mL) in PBS while 1 well was supplemented with 100 µl of sterile PBS as control. The plate was positioned in a heated incubator (Grant Boekel, HIR10 M) set to a temperature of 38 ºC and with a relative humidity of 95% for 24 h. After incubation, the blood was centrifuged at 8500 × g for 16 min at 4 ºC and plasma was stored at -20 °C until use. Plasma was later thawed and analyzed with a couple of monoclonal antibodies according to the method described by (Trevisi et al., 2014), adapted to fit with a commercial ELISA kit for bovine IFNG (BOVIGAM, Prionics AG, 8952 Schlieren-Zurich, Switzerland). Results were evaluated in terms of optical density difference (Δ**OD**) between avian PPD-stimulated and control wells.

Milk samples collection and analysis

Milk samples were collected during the morning milking (Figure 1) into 100-ml polypropylene bottles. Butterfat, protein, lactose, casein contents, titratable acidity and coagulation properties [rennet clotting time (**r**) and curd firmness at 30 min ($\mathbf{a_{30}}$)] were measured by using infrared instrumentation (MilkoScan FT 120, Foss Electric, Hillerød, Denmark) according to Calamari et al. (2010) and Chessa et al. (2014). Furthermore, the outputs of fat and protein, and the fat to protein ratio were also calculated. Urea nitrogen was determined on skimmed milk by a spectrophotometric assay, using a urea nitrogen kit (cat# 0018255440, Instrumentation laboratory, Milano, Italy) in association with a clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA). Somatic cell count (**SCC**) were determined using an optical fluorimetric method with an automated cell counter (Fossomatic 180, Foss Electric).

Carrageenan skin test

The carrageenan skin test (**CST**) was performed as specified by Jahan et al. (2015) to evaluate peripheral immune responses (Figure 1). The skin thickness was measured using a skinfold caliper (cat# 470119-588, VWR, USA) immediately before carrageenan injection (0 days), then at 2 and 9 days after the injection. The total response of each challenge was calculated as the area under the curve of the thickness, measured at day 2 and day 9, subtracting the thickness measured at day 0.

Rumen fluid parameters

Rumen fluid samples were collected with an oro-gastric probe (Ruminator, Proofs Products, Guelph, Canada) before the morning feed administration. pH was immediately measured with a pH-meter (GLP 21, Crison Instrument SA, Alella, Barcellona, ESP). A 2-mL aliquot of the supernatant was transferred into tubes with 1 mL of 0.12 M oxalic acid and

immediately frozen at –20 °C for later analysis. Total volatile fatty acids (**VFA**) concentration, molar proportion of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, caproic, iso-caproic and enanthic acids were analyzed as previously described (Minuti et al. 2014). Concentrations of single VFAs were expressed as relative amounts on total VFA concentration. A spectrophotometric clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA) and commercial kits for urea nitrogen (cat# 0018255440, Instrumentation laboratory, Milano, Italy) and lactate (K-DLATE, Megazyme Co., Wicklow, Ireland) were used to assess the concentration of ammonia and those of LLA, DLA and total lactic acid respectively.

Retrospective analyses and animal grouping

Animals were retrospectively divided in 2 groups based on their BHB levels measured in blood between 0 and 28 DFC (Figure 1), assuming a threshold of 1.4 mMol/L as a cut-off (Duffield, 2000). When blood BHB reached the sub clinical threshold animals were considered affected by ketosis (**KET**; 6 cows) and when remained below this threshold animals were defined as control (**CTR**; 7 cows).

Statistical analysis

Data in the tables are presented as mean and standard error. Before analysis, the normality of distributions was verified for each parameter by reckoning skewness and kurtosis according to the Shapiro test of SAS. Non-normally distributed parameters were normalized through natural logarithms (among plasma parameters IL-1B, IL-6, glucose, NEFA, DLA, LLA, Mg, AST-GOT, GGT, haptoglobin, bilirubin, cholesterol, AOPP, tocopherol, beta-carotene, NOx, NO2 and NO3; among WBC profile the amounts of total WBC, neutrophils, neutrophils to lymphocytes ratio, monocytes, eosinophils and basophils; among milk parameters the butterfat, fat output and fat to protein ratio; among rumen fluid parameters the

ammonia; among WBA the fold changes of DLA, IL-1B, IL-6, NOx and NO3 and the total response to CST) or square root transformation (among the plasma parameters the Ca) and back transformed to plot them in tables and graphs.

Prevalence of health problems recorded during the study was evaluated by $\chi 2$ analysis (Freq procedure, SAS Inst. Inc., Cary, NC). Data of BW, BCS, DMI, rumination time, milk yield, metabolic and WBC profile, WBA, milk quality parameters, response to IFNG release assay and CST were submitted to ANOVA using a mixed model for repeated measures (Mixed procedure, SAS Inst. Inc., Cary, NC) in accordance with Littell et al. (1998). The statistical model included the fixed effect of health status (**Hs**; CTR and KET), time (**t**) and their interaction (**Hs*t**). For those parameters that were measured daily (DMI, rumination time and milk yield) time effect considered the average weekly value, while for other parameters (BW, BCS, metabolic and WBC profile, WBA, milk quality parameters, IFNG release assay and CST) time effect considered single DFC. The time was considered as a repeated measure within cow. For WBA, also the dose (**D**; L and H) and the full interaction effect (**Hs*t*D**) were considered. The analysis was carried out using three covariance structures: autoregressive order, compound symmetry, and spatial power. These were ranked according to their Akaike information criterion, with the one having the lowest Akaike information criterion being eventually chosen (Littell et al., 1998). The pair-wise comparison was done using the least significant difference test. Data of rumen fluid parameters were analyzed by a one-way ANOVA (GLM procedure, SAS Inst. Inc., Cary, NC), considering only the fixed effect of Hs.

Post-hoc comparisons were discussed when the P-value for main effect was ≤ 0.05. Main effects at $P \leq 0.10$ are discussed in the context of tendencies.

RESULTS

Health status

The χ^2 analysis on the occurrence of clinical diseases (Table 2) revealed no Hs effect for retained placenta, metritis and endometritis, milk fever, displacement of abomasum, pneumonia and diarrhea, and neither did we find any effect on cows affected by ketosis. The number of mastitis and total disease cases besides ketosis tended to be higher in KET than CTR cows (Hs = 0.09 and 0.06 respectively).

Body weight, body condition score, dry matter intake, rumination time and milk yield

BW, BCS and ΔBCS, resulted numerically higher in KET than CTR group during the whole experimental period, but no effect was detected (Supplementary file 1). DMI, rumination time and milk yield (Figure 2.a-c) resulted lower in KET than CTR cows after calving (Hs*t < 0.01).

Milk quality parameters

Among milk quality and rheological parameters (Table 3), both fat and protein outputs resulted lower in KET than CTR group at 28 DFC (Hs*t = 0.02 and 0.05 respectively). Urea nitrogen had a tendency for Hs*t effect, resulting lower in KET than CTR group at 7 DFC and numerically lower thereafter. No effect was detected on other parameters.

Rumen fluid parameters

Among rumen fluid parameters (Table 4), KET had a tendency for higher molar proportion of butyric and lower molar proportion of valeric acids (Hs = 0.08 and = 0.07 respectively) and had lower molar proportion of isovaleric acid (Hs = 0.05) compared to CTR group. No effect appeared on other parameters.

Metabolic profile

Haematocrit and energy, protein and mineral metabolism biomarkers. Packed cell volume (Supplementary file 2.a) was not affected by health status. Among energy metabolism biomarkers, glucose concentration (Figure 3.a) was higher in KET than CTR group at -48 and -7 DFC, but KET had a more marked drop at 3 DFC and lower concentrations than CTR thereafter (Hs*t < 0.01). Concentration of NEFA and BHB (Figure 3.b-c) was higher in KET than CTR group during the whole experimental period (Hs = 0.01 and < 0.01 respectively), with larger differences observed after parturition (Hs*t = 0.02 for BHB). Concentration of LLA and DLA (Figure 3.d-e) was lower in KET than CTR group before calving, but both had a more pronounced peak at calving in KET group, resulting higher than in CTR thereafter (Hs*t = 0.01). Among protein metabolism biomarkers (Figure 3.f-g), urea concentration was lower in KET than CTR group at 14 DFC (Hs*t < 0.01), while creatinine concentration was overall higher during the examined period (Hs = 0.04). Among mineral metabolism biomarkers, concentration of P, Na, K, and Zn (Figure 3.h-l) was lower in KET than CTR group during the whole experimental period (Hs < 0.01 for P and K; Hs = 0.03 for Na and Zn). Concentration of Cl (Figure 3.k) was higher in KET than CTR group before calving (Hs = 0.02). Concentration of Ca and Mg was not different between the two groups (Supplementary file 2.b-c).

Liver function biomarkers. AST-GOT and total bilirubin (Figure 4.a;.c) was higher in KET than CTR group after calving (Hs*t < 0.01 and = 0.05 respectively). GGT (Figure 4.b) tended to be higher in KET than CTR group both before and after calving (Hs = 0.06; Hs*t = 0.09). ALP concentrations (Figure 4.d) had a tendency for Hs*t effect, resulting numerically higher in KET than CTR cows after calving.

Inflammation biomarkers. Concentration of myeloperoxidase (Figure 4.e) was higher in KET compared to CTR group during the whole experimental period (Hs < 0.01). No effect

was detected for total protein and globulin (Supplementary file 2.d-e). Among positive APP, haptoglobin (Figure 4.f) was higher in KET vs. CTR group starting from the second week after calving (Hs*t = 0.01). Concentration of ceruloplasmin and SAA (Figure 4.g-h) was higher in KET compared to CTR group after calving (Hs = 0.09 and = 0.05 respectively; Hs*t = 0.01 and 0.03 respectively). Among negative APP, retinol (Figure 4.i) was lower in KET than CTR group after calving (Hs*t < 0.01), while PON was higher in KET compared to CTR cows before calving (Hs = 0.06) with differences disappearing after parturition (Figure 5.a). No difference between groups was detected for albumin and cholesterol (Supplementary file 2.g-f). Concentration of IL-1B and IL-6 (Figure 5.b-c) was higher in KET vs. CTR group before calving (Hs*t = 0.03 and 0.05 respectively).

Oxidative stress biomarkers. Concentration of ROMt, SHp, and AOPP (Figure 5.d-f) was higher in KET compared to CTR group (Hs < 0.01) and a tendency for a Hs*t effect appeared for ROMt. NOx and NO3 concentrations (Figure 5.g-h) resulted higher in KET than CTR group (Hs = 0.02 and = 0.01 respectively). Concentration of FRAP, ORAC, tocopherol, beta-carotene and NO_2 was not different between groups (Supplementary file 2.h-l).

White blood cells profile

Results of WBC profile are reported in Table 5. Amount of total WBC and neutrophils was greater in KET compared to CTR group at 28 DFC (Hs*t = 0.03 and 0.02 respectively). The prevalence of monocytes and basophils resulted higher in KET than CTR group during the whole experimental period (Hs = 0.04 and = 0.02 respectively). Hemoglobin concentration was higher in KET than CTR group at -48 DFC (Hs*t = 0.01). Mean cell volume and mean cell hemoglobin were greater in KET than CTR group (Hs < 0.01 for both). The prevalence of platelets resulted greater in KET than CTR group at -21 and -3 DFC (Hs*t < 0.01).

Whole blood stimulation assay

Amount of cytokines produced *in vitro* by WBC increased after WBA (Table 6). Production of IL-1B was overall lower in KET than CTR group between -21 and 7 DFC (Hs and Hs*t < 0.01). Production of IL-6 by WBC was higher in KET than CTR group at 7 and 28 DFC (Hs = 0.04; Hs*t = 0.01).

Glucose uptake by WBC increased after WBA while production of DLA and LLA increased (Table 7). Glucose uptake by WBC was not different between groups, while lactate production resulted greater in KET than CTR group (Hs = 0.01 for LLA; Hs*t < 0.01 for both DLA and LLA). Production of NOx, NO_2, and NO_3 by WBC was not affected by the grouping (Supplementary file 3).

Interferon gamma release assay and carrageenan skin test

The production of IFNG (Figure 6.a) in whole blood in response to PPD was greater in KET than CTR group (Hs < 0.01). Total response to CST (Figure 6.b) was numerically lower in KET than CTR group for the whole experimental period, particularly at - 3 DFC.

DISCUSSION

Alterations of blood parameters in prepartum in post-partum ketotic cows resemble preeclampsia conditions

The higher level of PICs (IL-1B and IL-6) detected in blood of KET vs. CTR cows before calving is indicative of an activation of the immune system during the dry period. This is supported by the parallel increase of the main markers of neutrophils and monocytes activation (myeloperoxidase and AOPP), and the higher concentrations of oxidant species related to their antimicrobial activity (ROMt and NO_3) in the blood of ketotic vs. control cows. Myeloperoxydase converts NO_3 to a nitrating agent (Dedon and Tannenbaum, 2004) and generates hypoclorous acid that could generate ROM within the respiratory burst (Faith et al., 2008) while AOPP are markers of protein oxidation triggered by these metabolites (Celi and Gabai, 2015). The main cause of such activation of immune systems before ketosis is not clear, but trends of immune parameters during dry period are very similar to those observed in women affected by preeclampsia.

According to Acauan Filho et al. (2016), "…preeclampsia is associated with the sequence of inadequate trophoblast invasion resulting in restricted spiral artery remodelling, low placental perfusion, placental ischemia, oxidative stress, and the consequent imbalance in the factors derived from placenta." Although very little is known about the pathophysiology of this disease in women, and none reported such a condition to affect dairy cows, the generalized dysfunction of the maternal endothelium during preeclampsia appears to be part of an exaggerated systemic inflammatory response that involves maternal leukocytes and PICs (Raghupathy, 2013). Raise of PICs and parameters related to leukocytes activation (myeloperoxidase and ROM) has been widely reported in pre-eclamptic women (Mori et al., 2014; Rocha-Penha et al., 2017; Cornelius, 2018) and suggests the development of a pre-

eclamptic-like status during dry period of ketotic cows in our study, as also supported by trends of other plasma parameters. Increased platelet concentration found in our KET cows are consistent with the increased platelet aggregation reported during pre-eclampsia, as a consequence of vascular damages caused by active neutrophils during disease (Raghupathy, 2013).

Higher GGT levels found in our KET animals during dry period have previosly been reported in dairy cows prior to ketosis (Rodriguez-Jimenez et al., 2018) and suggest a liver damage . In fact GGT, as well as AST-GOT, are enzymes involved in hepatic AA metabolism, that increase in blood when a liver damage occurs, and such a condition has been widely reported in pre-eclamptic women (Cornelius, 2018). Such an impaired hepatic metabolism likely accounts for the greater amounts of thiol groups founded in blood of KET cows throughout our study, as higher thiol groups concentrations could be related to the reduced capacity of liver to pick up free glutathione (the most abundant free thiol group in blood stream) and sulphured amino acids from blood (Guzman Barron, 2006).

Higher levels of NOx in blood of KET cows are also consistent with pre-eclampsia, as NOx is a potent endogenous vasodilator that is produced by nitric oxide synthase enzyme in endothelium. Its release relaxes the blood vessels muscle layer, affecting haemodynamic adaptation to pregnancy and play a pivotal role in pre-eclampsia pathogenesis (Acauan Filho et al., 2016).

Preeclampsia-like conditions in KET cows is further reflected by trends of plasma minerals. In fact, lower levels of Na, K and P observed in our KET cows has been reported in woman affected by pre-eclampsia (Ikaraoha et al., 2012; Ebenezer et al., 2017) and could be driven by renal dysfunctions related to disease. Such an explaination is confirmed from the higher creatinine found in blood of KET cows during the entire experimental period, and reported in preeclamptic women (Leeman et al., 2016). Creatinine is related to

phosphocreatine utilization during normal muscular activity, and an increased hematic concentration of this metabolite indicates an impaired capacity of the kidney to remove it (Zoccali et al., 2006).

Lower plasma zinc found in our cows has also been reported in pre-eclamptic women (Jihye et al., 2012). This mineral is known to serve as a secondary antioxidant aiding superoxide dysmutase enzymatic complex functioning, and its lower level reflect a greater consumption to face the oxidative stress status related to pre-eclampsia (Jihye et al., 2012).

Finally, the greater IFNG response of peripheral blood leukocytes of KET cows to the *ex-vivo* stimulation with *M. avium* further supports the development of a preeclampsia-like status. Increased production of IFNG has been reported in decidual lymphocytes and choriodecidual cells of placental trophoblast from pre-eclamptic pregnancies consequently to the reduced expression of IFNG receptor R2 (Raghupathy, 2013). The production of IFNG by T-helper cells type 1 has also been proposed as one of the primary causes of the disease (Banerjee et al., 2005) and could provide a linkage between preeclampsia and ketosis. Although a direct measurement of plasma IFNG is required to confirm such a hypothesis, we can speculate that higher prepartal IFNG production measured *in vitro* in leukocytes from cows having a preeclamptic-like status could probably reflect their greater activity *in vivo*.

Higher IFNG concentrations could play a direct role in ketosis induction after calving. IFNG is known to inhibit the expression of peroxisome proliferation activated receptor (**PPAR**)-gamma (Floyd and Stephens, 2002). PPAR-gamma plays important roles in adipocytes biology. PPAR-gamma is a main player in maintaining insulin sensitivity of tissues, including adipose tissue (Waite et al., 2001; Choi et al., 2018). A link between insulin resistance and risk of ketosis in dairy cows has already been suggested (Youssef and El-Ashker, 2017), consistently with the higher prepartal glucose concentrations detected in KET vs. CTR, although previous studies failed to reveal any alteration of such a parameter prior to

ketosis (Abuajamieh et al., 2016). Our study could also suggest a linkage of insulin resistance with the preeclamptic-like status affecting our KET cows before calving, consistently with the reported hyperglycaemia that affects preeclamptic women (Kaaja, 2011; Sacks et al., 2015). On the other hand, the prepartal increase of NEFA and BHB at non-pathologic levels found in our cows are well known prior to ketosis (Zhang et al., 2016). NEFA concentration at -14 DFC has also been suggested as a predictive biomarker of disease (Chapinal et al., 2011). Increase plasma concentration of NEFA reflects mobilization of lipid sources while BHB is released in blood when liver oxidation is impaired by a fatty acids overload (Herdt, 2000). In this scenario, IFNG-driven PPAR-gamma inhibition could be pivotal to boosting ketosis onset after calving. Decreased lipogenesis and increased lipolysis arise when PPAR-gamma is inhibited (Waite et al., 2001), which may be conductive to a greater mobilization of NEFA for energy metabolism. However, greater mobilization of NEFA in KET vs. CTR did not reflect on any BCS difference in our study, in agreement with previous results (Rodriguez-Jimenez et al., 2018).

Despite many parameters appears to be in line with a preeclampsia model none of the cows enrolled in this study had any diagnosed preeclampsia. Typical symptoms of preeclampia, including rapid weight gain, proteinuria, edema, increased abortion rates, and/or reduced newborn calf weight, were not detected. Thus, we certainly cannot conclude that our cows experience preeclampsia; however, due to the central role of placenta in preeclampsia (Roberts and Escudero, 2012), we can speculate that a subclinical dysfunction of the placenta might have been a co-cause of the observed changes in measured parameters in plasma prepartum with ensuing ketosis postpartum.

Preeclampsia in women is associated also with obesity (Roberts et al., 2011) and several of the changes of measured parameters appears to be also associated with fat cow syndrome (Morrow, 1976). None of the cows in the study would have been considered obese and no

differences in BCS between KET and CTR were detected. In human, a higher production of adipokines have been associated with preeclampsia (Haugen et al., 2005). Disruptive production of adipokines in preeclampsia cases are associated with visceral adipose tissue (Huda et al., 2017). In non-obese dairy cows large variation in visceral adipose tissue is not visible by using BCS (Drackley et al., 2014). Thus, it is possible that ketotic cows in our study had higher production of adipokines by visceral adipose tissue compared to control animals.

Cows with ketosis have impaired feed behavior

Ketosis onset is related to the impairment of feeding behavior, as suggested by the reduced DMI and rumination time found in our study after calving. Low feed intake (Zhang et al., 2016) and an average reduction of rumination time up to 25 min d^{-1} were consistently reported in dairy cows during the week of ketosis diagnosis (Abuajamieh et al., 2016). Recent studies highlighted alteration of these parameters also before calving (Kaufman et al., 2016; Rodriguez-Jimenez et al., 2018). Such an impairment in feeding behaviours in ketotic cows could arise from the strong anorexic effect resulting from combined raise of PICs, NEFA and BHB (Bertoni et al., 2008; Allen et al., 2009) as well as from the insulin resistance-like status occurred before calving (Youssef and El-Ashker, 2017), that could be involved in the reduction of feed intake. Furthermore, it could also be related to the impairment of rumen fermentations occurring with ketosis. Wang et al. (2012) reported a reduction of propionate-forming bacteria (*Megasphaera elsdenii* and *Selenomonas ruminantium*) and an increase in lactate-producing bacteria (*Streptococcus bovis*), reflected in an increased lactate concentration and reduced VFA production in rumen of cows affected by acute ketosis (blood BHB concentration > 3 mMol/L). Unfortunately, rumen samples were collected during the fourth week of lactation in our study, while most animals had BHB peaks during the first and

second week from calving, which makes it difficult to correlate any rumen alteration with ketosis. Furthermore, the light ketosis status that affected our animals (blood BHB concentration > 1.2 mMol/L) could account for the lack of any effect on VFA and lactate.

Reduced DMI and rumination time in KET cows, together with the increased energy demand related to the activation of their immune system during dry period, could had worsened the physiological NEB condition after calving (Soriani et al., 2012; Osorio et al., 2014). This could account for the depression of milk yield, milk fat and protein outputs detected in our study with ketosis, consistent with milk losses up to 353.5 kg cow^{-1} in the whole lactation reported previously (Zhang et al., 2016). Lower glucose in KET vs. CTR cows post-partum can be due to several factors, including a higher utilization of glucose by immune cells but also an impaired glucogenic capacity of the liver (Loor et al., 2007). Lower glucose in blood had triggered a marked shift to other energy sources. This is consistent with the stepwise increase of both NEFA and BHB, that suggests a major role of lipid sources as energy supplier, as widely reported in ketotic cows during early lactation (Duffield, 2000). On the other hand, lower postpartal urea concentration detected in blood of KET cows is in contrast with Rodriguez-Jimenez et al. (2018), and reflects a less marked contribution of muscle tissues in supplying the energy deficit in KET vs. CTR cows, as blood urea partially arise from deamination of AA. Nevertheless, the detection of similar depression on milk urea level suggests the decreased feed intake of KET cows as the main cause in the alteration of this parameter (Bertoni et al., 2008).

Ketosis is associated with inflammatory-like conditions post-partum

A status of inflammatory-like condition appears physiological during TP in dairy cows (Bionaz et al., 2007; Bertoni et al., 2008). During inflammation, the liver shifts its anabolic priorities, and plasmatic trends of APP reflects the severity of the phenomenon (Castell et al.,

1989). Plasma concentration of positive APP such as haptoglobin, ceruloplasmin, and SAA increases (Ceciliani et al., 2012), while concentration of other typical proteins known as negative APP, such as albumin, PON, lipoproteins, and retinol binding protein typically decrease (Bionaz et al., 2007; Trevisi et al., 2012).

The lack of any prepartal difference in positive APP, together with the greater PON concentration of KET vs. CTR cows, suggests a lack of acute phase reaction despites the larger PIC during this phase. Conversely, the higher levels of positive APPs and the lower level of retinol reflect a more marked inflammatory status in KET animals after calving, although no difference were detected for other negative APP. Lower level of plasma zinc could further support the greater inflammatory conditions occurred in KET cows after calving, as this mineral is sequestred by the liver from the blood during an acute phase response (Bertoni and Trevisi, 2013). Furthermore, augmented postpartal concentration of GGT and AST-GOT found in our KET cows reflects results by Rodriguez-Jimenez et al. (2018), indicating liver damages to be aggravated with delivery. This is further confirmed by the greater bilirubin concentration observed in the same period. Bilirubin results from degradation of red blood cells, and its clearance is related to the activity of liver enzymes (Rodriguez-Jimenez et al., 2018).

Attenuated response of white blood cells to LPS and possible immunosuppression in post-partum ketotic cows

WBC had a lower IL-1B production in response to LPS in KET vs. CTR already 21 days before calving that persisted for the first week post-partum, while the production of IL-6 in response to LPS was not different between KET and CTR during the same time frame, but WBC production of IL-6 after LPS was larger in KET vs CTR after ketosis, along with production of lactate. IL-1B is a well-known PIC, while IL-6 is known to have both pro and

anti-inflammatory activities, and the production of lactate has been reported to suppress the inflammasome and the production of PICs (Zhang et al., 2016). Such altered responses in WBC are consistent with their shift on anti-inflammatory response driven by altered gene expression at liver level with ketosis, although the effect found on IL-1B production prior to ketosis suggests another mechanism to contribute to such alteration. As indicated above, ketotic animals in our study had preeclampsia-like conditions; interestingly, preeclampsia status is known to weakened body defences (Cornelius, 2018). Such effects, occurred in early dry period (or even before), could have admit bacterial cells components to enter the organism (Trevisi et al., 2018), challenging immune cells and inducing an endotoxin tolerance condition in them. This is a protective mechanism against over exuberant inflammations developed from WBC after an endotoxin challenge. It consists in a decreased production of PICs and an up-regulation of anti-inflammatory genes, that triggers a transient unresponsive state against further challenges with endotoxins (Biswas and Lopez-Collazo, 2009).

Metabolic asset related to the more severe NEB occurred with ketosis at the beginning of lactation could have further contribute in affecting WBC functions. In fact, a higher concentration of circulating WBC was expected in KET cows in comparison to CTR during the whole experimental period considering the activation of their immune system that occurred during dry period (higher levels of PICs, myeloperoxidase, ROMt, AOPP and NO_3 and greater IFNG production *in vitro*) and the more marked inflammatory condition they had after calving (reflected in positive APPs and liver function biomarkers trends). Nevertheless, increased numbers of circulating neutrophils and monocytes in KET cows were detected in the late post calving period only, when the metabolic status related to ketosis was overcome, suggesting WBC viability to be reduced during disease. Alternative energy sources are less efficiently utilized in WBC compared with glucose (Sordillo, 2016), and decreased proliferation, differentiation and viability has been reported for macrophages and

polymorphonuclear cells (**PMN**) with low glucose availability (Barghouthi, Everett, & Speert, 1995; Gamelli, Liu, He, & Hofmann, 1996). Furthermore, impaired chemotaxis and phagocytosis were also reported in those cells (Pithon-Curi et al., 2004). Although differences were only numerical in our study, this seems to be consistent with the lower sensitivity to CST found in KET cows 3 days prior to parturition, suggesting WBC ability to reach peripheral tissues to be impaired in cows that developed ketosis after calving (Jahan et al., 2015). Furthermore, high concentrations of NEFA and BHB, as those shown by KET cows throughout our study, are directly involved in the impairment of WBC functions. BHB cannot be utilized as energy source in WBC (Targowski and Klucinski, 1983; Hoeben et al., 1997; Sartorelli et al., 1999; Suriyasathaporn et al., 1999), while NEFA increases apoptosis and necrosis in macrophages and PMN through the stimulation of their respiratory burst activity (Scalia et al., 2006) and this could account for the higher ROMt and AOPP councentration found in KET cows in our experiment. Furthermore, NEFA increase the production of PICs and IFNs in lymphocytes (Hughes and Pinder, 2000); thus, higher NEFA concentrations detected in KET cows before calving could partially account for the higher PICs and for a greater response to IFNG by WBC. Finally, the higher level of lactate in blood of ketotic vs. control cows in early lactation could have impaired the immune system. Lactate is known to impair motility, killing capacity and effector functions of monocytes, macrophages, T and B cells (Zhang et al., 2016). Such an impairment could account for the numerically higher incidence of udder diseases found in ketotic cows, that has also been widely reported in previous works (Duffield, 2000). Mastitis was the only peripartum disease that showed a tendency for a Hs effect in our experiment, and both cases were clearly associated to ketosis, as they appeared three weeks after the BHB peak.

CONCLUSION

In our study, occurrence of ketosis was preceded by preeclampsia-like status during the dry period including high oxidative stress, high PICs, high nitrate, possible lower renal function, liver damage, reduced blood minerals concentrations, and activated immune system. An increased production of IFNG by leukocytes *in vitro* was also detected, which may have affected PPAR-gamma expression and, thus insulin sensitivity. The latter might explain the larger plasma concentration of NEFA and BHB observed in KET cows pre-partum. The anorexic power exerted by PICs, NEFA and BHB and the high circulating glucose levels reduces DMI around calving. Furthermore, increased energy requirement related to activation of immune system in dry period could have contributed to worsen the NEB condition in early lactation. Such a condition induced severe milk yield losses and boosted the mobilization of lipid sources. The above conditions appeared to have accrued the larger acute phase response after calving, partly compromising liver function. Our data is indicative of a possible endotoxin tolerance of WBC in KET cows that could account for the reduced production of PICs and increased production of anti-inflammatory mediators, such as lactate, by WBC under LPS stimulation. Thus, our data is indicative of a role of the immune system, maybe associated with a still unclear placental role (or adipose tissue role), during the dry period in promoting ketosis early post-partum. Nevertheless, an in-depth study on alterations in pathways regulating energy metabolism prior to ketosis onset and on the role of the immune system in such alterations is required to fully elucidate the real contribution of IFNG and other cytokines to disease onset, and the linkage between preeclampsia-like status and ketosis.

TABLES AND FIGURES

Table 1. Composition and characteristics of the experimental diets fed during the 5 experimental phases. Between -55 and -40 DFC cows received grass hay, only

Diet, %DM	Phase 1	Phase 2	Phase 3	Phase 4
DFC	-48; -7	-7; 0	1; 30	31; 60
Item				
Corn silage	18.6	23.6	24.0	23.3
Alfalfa hay	-	-	13.7	11.1
Grass hay	71.4	58.0	18.1	11.9
Concentrate (Dry period)	10.0	10.1	5.00	-
Concentrate (Lactation period)	-	8.30	44.2	53.7
Concentrate composition, %DM	Dry period		Lactation period	
Corn flour	-		40.0	
Barley flour	-		1.4	
Sorghum grain expanded	-		-	
Soybean meal	90.5		13.1	
Soybean dry rolled	-		-	
Sunflower meal	-		4.9	
Corn gluten feed	-		-	
Beet pulp	-		16.6	
Wheat bran	-		9.8	
Beet molasse slops	-		2.6	
Potato protein	-		2.2	
Hydrogenat palm oil	-		3.3	
Limestone	-		1.39	
Dicalcium phosphate	-		1.80	
Sodium bicarbonate	-		0.98	
Magnesium oxide	2.2		0.64	
Sodium Chloride	1.4		0.32	
Supplement[1]	5.9		1.07	
Chemical composition				
NE_L, Mcal kg of DM^{-1}	1.45	1.53	1.60	1.63
Crude protein, % DM	13.6	14.5	16.2	17.2
Starch + sugar, %DM	16.8	19.3	26.0	18.3
Ether extract, %DM	1.80	2.40	4.48	5.08
NDF, %DM	49.3	45.5	35.7	32.6
MP[2], %CP	9.10	9.70	10.5	11.1
RUP[2], %CP	4.48	4.77	5.23	5.96

[1]Supplements were composited to provide 150000 UI of vitamin A, 10000 IU of vitamin D, 200 mg of vitamin E, 100 mg of vitamin K, 100 mg of vitamin H1 50 mg of vitamin B1, 0.5 mg of vitamin B12, 500 mg of vitamin PP, 4000 mg of coline, 350 mg of Mn, 800 mg of Zn, 40 mg of Cu, 20 mg of I, 1 mg of Co, 1 mg Se.
[2]Estimate using NRC 2001.

Table 2. Incidence of diseases (besides ketosis) observed between -48 and 35 days from calving (DFC) in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation

Hs[1]	Healthy cows[2]	Problems occurrence, n								
		RP[3]	ME/ED[4]	MF[5]	DA[6]	MA[7]		PN[8]	DH[9]	Total
CTR (7 cows)	5	0	0	0	0	0		0	2	2
Period of manifestation	*DFC*	-	-	-	-	-		-	*14* *18*	-
KET (6 cows)	2	1	1	1	1	2		1	2	9
Period of manifestation	*DFC*	*1*	*9*	*1*	*3*	*34*	*26*	*7*	*31* *15*	-
	DFK[10]	*-2*	*0*	*-2*	*0*	*20*	*23*	*0*	*17* *8*	
P	0.43	0.26	0.26	0.26	0.26	0.09		0.26	0.85	0.06

[1]Health status (CTR is control; KET is ketosis).
[2]Cows that did not have any clinical diseases (besides ketosis) between -48 and 35 DFC.
[3]Retained placenta.
[4]Endometritis or metritis.
[5]Milk fever.
[6]Displacement of abomasum.
[7]Mastitis.
[8]Pneumonia.
[9]Diarrhea.
[10]Days before BHB peak appearance or after its conclusion (0 indicate that disease occurred during the BHB peak).

Table 3. Milk composition, rheological parameters and somatic cell count in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation

Item, Unit	Hs[1]	DFC			SE	P-Value		
		7	14	28		Hs[1]	t[2]	Hs*t[3]
Butterfat, mg 100 mL^{-1}	CTR	4.47	4.27	4.54	0.349	0.67	0.10	0.18
	KET	5.18	4.60	3.96	0.377			
Fat output, g	CTR	0.28	0.39	0.61	0.090	0.54	0.34	0.02
	KET	0.40	0.40	0.27*	0.097			
Total protein, mg 100 mL^{-1}	CTR	3.81	3.34	3.20	0.100	0.29	<0.01	0.89
	KET	3.73	3.21	3.04	0.108			
Protein output, g	CTR	1.14	1.17	1.35	0.086	0.30	0.09	0.05
	KET	1.11	1.08	1.09*	0.093			
Fat/protein ratio, -	CTR	0.15	0.25	0.32	0.076	0.41	0.64	0.32
	KET	0.33	0.34	0.26	0.082			
Lactose, mg 100 mL^{-1}	CTR	4.90	5.14	5.11	0.074	0.13	<0.01	0.64
	KET	4.71	5.09	5.00	0.080			
Caseins, mg 100 mL^{-1}	CTR	2.85	2.54	2.40	0.079	0.19	<0.01	0.84
	KET	2.76	2.39	2.26	0.085			
Titratable acidity, °SH 50 mL^{-1}	CTR	3.79	3.09	3.15	0.131	0.12	<0.01	0.40
	KET	4.11	3.47+	3.31	0.142			
Urea nitrogen, mg dL^{-1}	CTR	35.0	34.4	28.1	2.80	0.11	0.10	0.10
	KET	23.4*	29.4	27.6	3.02			
Coagulation time (r), min	CTR	13.9	18.4	15.0	2.54	0.22	<0.01	0.15
	KET	9.9*	17.8	14.2	2.74			
Curd firmness (a30), mm	CTR	37.8	28.0	35.7	35.7	0.27	0.25	0.43
	KET	39.1	27.8	28.3	28.3			
SCC[4], n mL^{-1}	CTR	194.6	-	42.3	88.0	0.36	0.04	0.87
	KET	121.2	-	56.9	88.0			

[1]Health status (CTR is control; KET is ketosis).
[2]Time.
[3]Health status x time interaction (* is $P < 0.05$; + is $P < 0.1$).
[4]Somatic cells count.

Table 4. Ruminal pH, ammonia concentrations and volatile fatty acids molar proportion, determined at 28 days after calving, in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation

Item	Unit	Hs[1]		Standard error	P-value
		CTR	KET		
pH	-	6.64	6.67	0.10	0.83
Ammonia	mg L^{-1}	4.53	4.32	0.49	0.71
VFA total[2]	mmol L^{-1}	110.1	100.6	5.04	0.19
Acetic acid	mol 100 mol^{-1}	61.7	63.0	1.63	0.57
Propionic acid	mol 100 mol^{-1}	23.3	21.4	1.52	0.38
Butyric acid	mol 100 mol^{-1}	10.7	11.9	0.48	0.08
Isobutyric acid	mol 100 mol^{-1}	0.78	0.78	0.08	1.0
Valeric acid	mol 100 mol^{-1}	1.34	1.14	0.07	0.07
Isovaleric acid	mol 100 mol^{-1}	1.55	1.31	0.08	0.05
Caproic acid	mol 100 mol^{-1}	0.49	0.38	0.08	0.39
Enanthic acid	mol 100 mol^{-1}	0.04	0.02	0.01	0.18
Levitical lactic acid	mmol L^{-1}	32.2	36.7	5.39	0.55
Dextroric lactic acid	mmol L^{-1}	31.6	37.4	5.34	0.44
Total lactic acid	mmol L^{-1}	63.8	74.2	10.7	0.49

[1]Health status (CTR is control; KET is ketosis).
[2]Total volatile fatty acids.

Table 5. White blood cells profile in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation

Item, Unit	Hs[1]	Days from calving					SE[2]	P-value		
		-48	-21	-3	7	28		Hs[1]	t[3]	Hs*t[4]
WBC[5], K μL^{-1}	CTR	5.9	6.3	7.7	6.2	5.2	1.11	0.25	0.02	0.03
	KET	6.9	7.2	8.1	7.3	9.8**	1.20			
Neutrophils, K μL^{-1}	CTR	3.0	3.4	5.0	3.8	2.9	0.28	0.25	<0.01	0.02
	KET	3.1	3.4	4.7	4.2	6.4+	0.31			
Monocytes, K μL^{-1}	CTR	0.41	0.51	0.77	0.57	0.49	0.15	0.04	<0.01	0.39
	KET	0.43	0.58	0.80	0.75	0.90	0.16			
Basophils, K μL^{-1}	CTR	0.06	0.05	0.04	0.07	0.06	0.21	0.02	0.18	0.41
	KET	0.07	0.06	0.10	0.10	0.11	0.23			
Hemoglobin, g dL^{-1}	CTR	10.4	10.3	10.5	10.3	9.5	0.32	0.18	<0.01	0.01
	KET	11.8**	10.6	10.9	11.0	9.8	0.35			
MCV[6], fL	CTR	46.1	46.5	48.2	48.1	47.3	1.14	<0.01	<0.01	0.24
	KET	51.2	50.8	51.8	50.9	49.8	1.24			
MCH[7], pg	CTR	16.0	16.1	16.5	16.6	16.5	0.26	<0.01	0.03	0.57
	KET	17.4	17.5	17.8	17.6	17.5	0.28			
Platelets, K μL^{-1}	CTR	317	251	390	314	435	30.9	0.67	<0.01	<0.01
	KET	288	381**	294*	347	460	33.3			

[1]Health status effect (CTR is control and KET is ketosis).
[2]Standard error.
[3]Time effect.
[4]Health status x time interaction effect (+ is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[5]White blood cells.
[6]Mean cells volume.
[7]Mean cells hemoglobin.

Table 6. Fold changes of cytokines in a whole blood stimulation assay with a low (L) or high dose (H) of bacterial lipopolysaccharides (LPS) in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation. Values are expressed as fold change with respect to baseline

Item	D[2]	Hs[1]	Days from calving					SE[3]	Effect	P-Value
			-48	-21	-3	7	28			
Interleukin-1, beta	L	CTR	11.8	14.5	17.3	18.1	9.0	0.28	Hs[1]	<0.01
		KET	4.5	3.9	7.0	5.6	11.9	0.30	t[4]	0.02
	H	CTR	38.7	46.7	64.8	75.3	31.6	0.28	D[2]	<0.01
		KET	10.1	10.8	17.5	15.8	50.8	0.30	Hs*t[5]	<0.01
	Tot	CTR	25.3	30.6	41.0	46.7	20.3	0.24	Hs*t*D[6]	0.71
		KET	7.3	7.3 **	12.3 **	10.7 **	31.3	0.26		
Interleukin-6	L	CTR	1.55	1.46	1.53	1.63	1.55	0.12	Hs[1]	0.04
		KET	1.76	1.31	1.89	1.96	2.72	0.13	t[4]	<0.01
	H	CTR	2.14	1.74	1.84	2.52	2.18	0.12	D[2]	<0.01
		KET	2.23	2.04	2.30	2.98	4.74	0.13	Hs*t[5]	0.01
	Tot	CTR	1.8	1.6	1.7	2.1	1.9	0.10	Hs*t*D[6]	0.83
		KET	2.0	1.7	2.1	2.5 +	3.7 **	0.11		

[1]Health status (CTR is control and KET is ketosis).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Health status x time interaction (** is $P < 0.01$; + is $P < 0.1$).
[6]Health status x time x dose interaction.

Table 7. Fold changes of glucose and metabolites thereof in a whole blood stimulation assay with a low (L) or high dose (H) of bacterial lipopolysaccharides (LPS) in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation. Values are expressed as fold change with respect to baseline

Item	D[2]	Hs[1]	Days from calving					SE[3]	Effect	P-Value
			-48	-21	-3	7	28			
Glucose	L	CTR	0.97	0.98	0.93	0.97	0.98	0.05	Hs[1]	0.17
		KET	0.95	0.96	0.95	0.92	0.88	0.05	t[4]	0.18
	H	CTR	0.93	0.95	0.90	0.89	0.95	0.05	D[2]	0.02
		KET	0.95	0.94	0.81	0.83	0.79	0.05	Hs*t[5]	0.34
	Tot	CTR	0.95	0.96	0.91	0.93	0.96	0.04	Hs*t*D[6]	0.94
		KET	0.95	0.95	0.88	0.87	0.83	0.04		
D-lactic acid	L	CTR	1.03	1.05	1.10	1.00	0.96	0.03	Hs[2]	0.16
		KET	1.02	1.04	1.05	1.07	1.14	0.03	t[4]	0.10
	H	CTR	1.05	1.06	1.14	1.06	1.06	0.03	D[1]	0.01
		KET	1.04	1.11	1.11	1.10	1.19	0.03	Hs*t[5]	<0.01
	Tot	CTR	1.04	1.05	1.12	1.03	1.01	0.02	Hs*t*D[6]	0.90
		KET	1.03	1.08	1.08	1.09	1.17 **	0.03		
L-lactic acid	L	CTR	1.02	1.04	1.04	1.02	1.00	0.02	Hs[2]	0.01
		KET	1.03	1.04	1.03	1.09	1.11	0.02	t[4]	<0.01
	H	CTR	1.03	1.05	1.06	1.06	1.08	0.02	D[1]	<0.01
		KET	1.04	1.08	1.07	1.11	1.17	0.02	Hs*t[5]	<0.01
	Tot	CTR	1.02	1.05	1.05	1.04	1.04	0.01	Hs*t*D[6]	0.50
		KET	1.03	1.06	1.05	1.10 **	1.14 **	0.01		

[1]Health status (CTR is control and KET is ketosis).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Health status x time interaction (** is $P < 0.01$; * is $P < 0.05$).
[6]Health status x time x dose interaction.

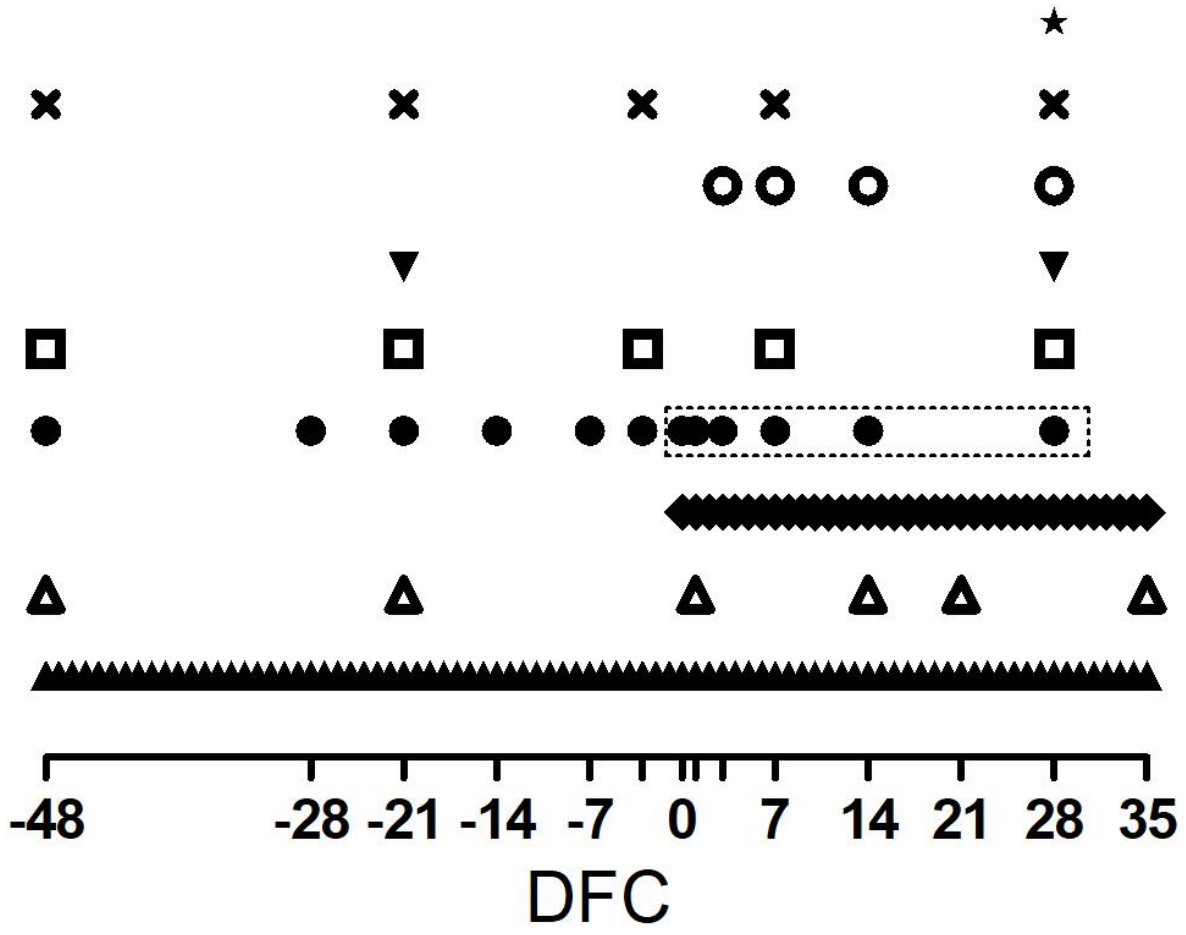


Figure 1. Scheduled time points, expressed as day from calving (DFC), for rumen sample collection (✸), carrageenan skin test performance (✖), milk sample collection (⬭), blood sample collection for the interferon gamma release assay (▼), blood sample collection for white blood cells profile and whole blood stimulation assay (□), blood sample collection for metabolic profile (●; time points surrounded with dotted lines were used in the retrospective analysis of beta-hydroxybutyrate), milk yield measurement (◆), body weight and body condition score determination (△), dry matter intake and rumination time determination (▲).

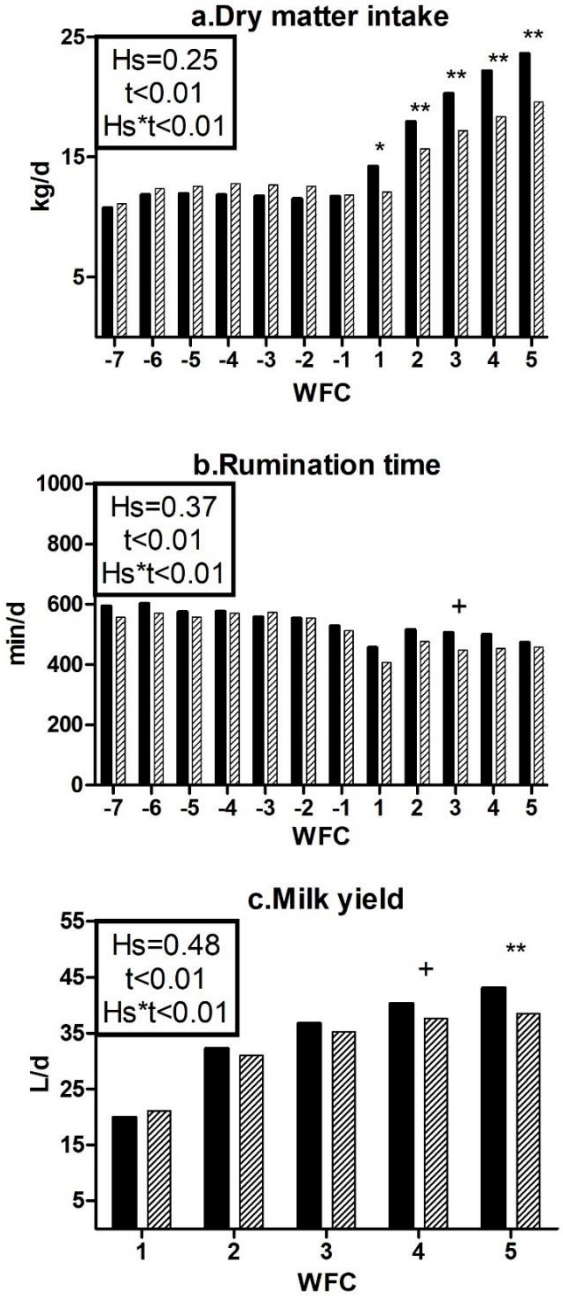


Figure 2. Average week values of dry matter intake (a), rumination time (b) and milk yield (c) in control dairy cows (CTR; solid bars) or cows that showed beta-hydroxybutyrate (BHB) levels higher than 1.4 mmol/L in the first month of lactation (KET; striped bars). ** is $P < 0.01$; * is $P < 0.05$; + is $P < 0.1$; WFC is weeks from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

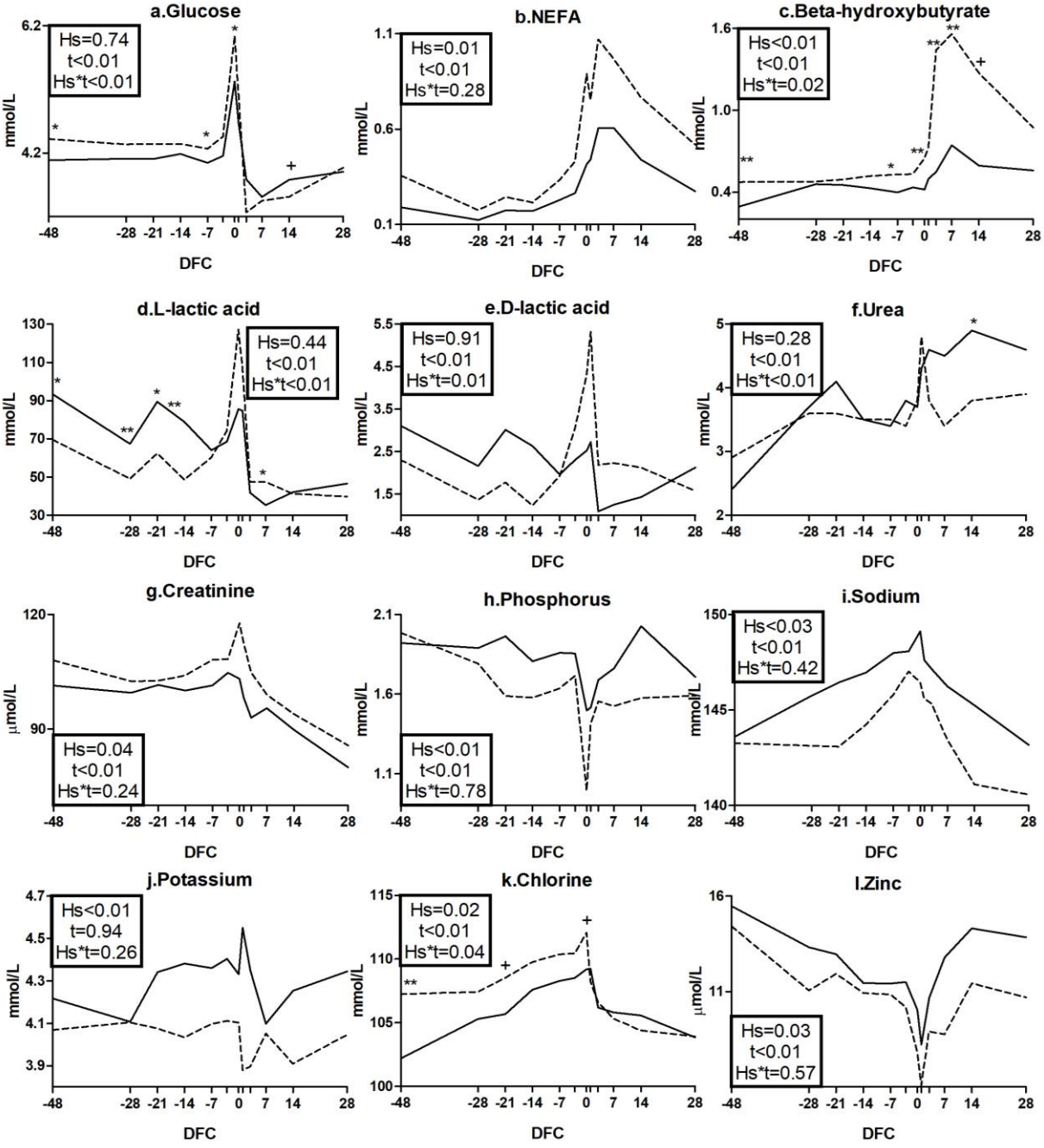


Figure 3. Time course of plasma concentrations of glucose (a), not-esterified fatty acids (NEFA; b), beta-hydroxybutyrate (c), L-lactic acid (d), D-lactic acid (e), urea (f), creatinine (g), phosphorus (h), sodium (i), potassium (j), chlorine (k) and zinc (l) in control dairy cows (CTR; solid line) or cows that showed beta-hydroxybutyrate (BHB) levels higher than 1.4 mmol/L in the first month of lactation (KET; dotted line). ** is $P < 0.01$; * is $P < 0.05$; + is $P < 0.1$; DFC is days from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

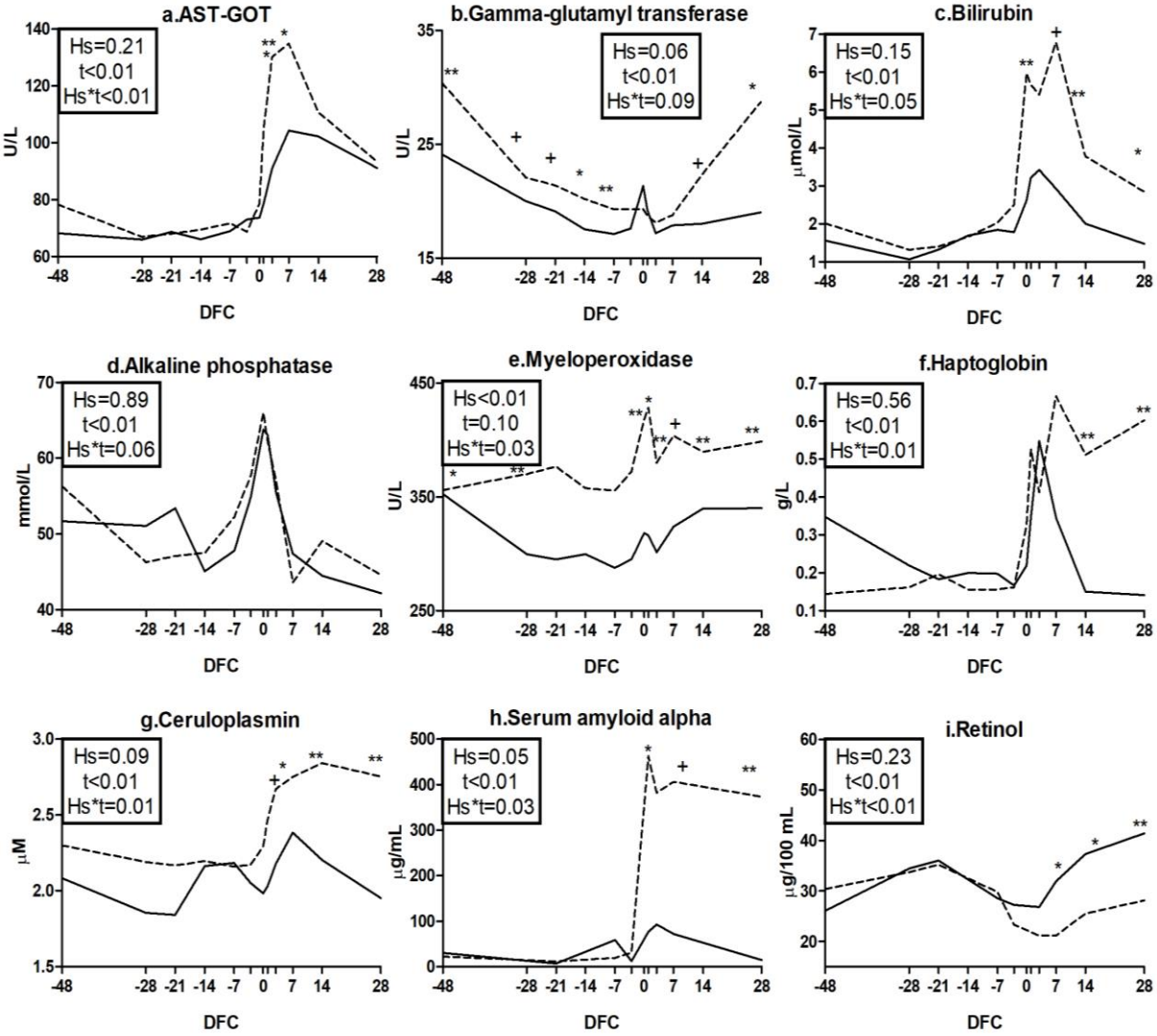


Figure 4. Time course of plasma concentrations of aspartate amino transferase (AST-GOT; a), gamma glutamyl transferase (b), bilirubin (c), alkaline phosphatase (d), myeloperoxidase (e), haptoglobin (f), ceruloplasmin (g), serum amyloid alpha (h) and retinol (i) in control dairy cows (CTR; solid line) or cows showed beta-hydroxybutyrate (BHB) levels higher than 1.4 mmol/L in the first month of lactation (KET; dotted line). ** is $P < 0.01$; * is $P < 0.05$; + is $P < 0.1$; DFC is days from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

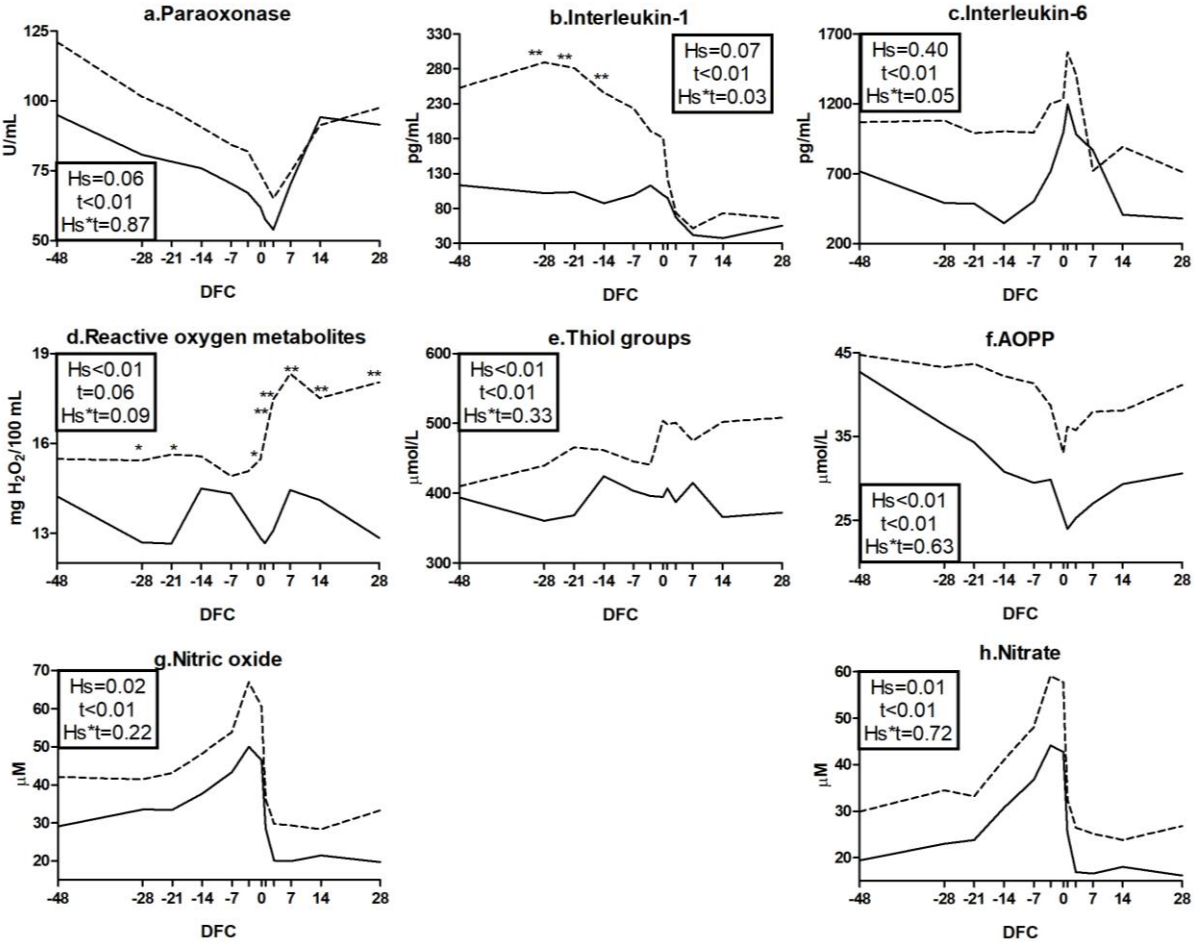


Figure 5. Time course of plasma concentrations of paraoxonase (a), interleukin-1 (b), interleukin-6 (c), total reactive oxygen metabolites (d), thiol groups (e), advanced oxidation protein products (AOPP; f), nitric oxide (g) and nitrate (h) in control dairy cows (CTR; solid line) or cows that showed beta-hydroxybutyrate (BHB) levels higher than 1.4 mmol/L in the first month of lactation (KET; dotted line). ** is $P < 0.01$; * is $P < 0.05$; + is $P < 0.1$; DFC is days from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

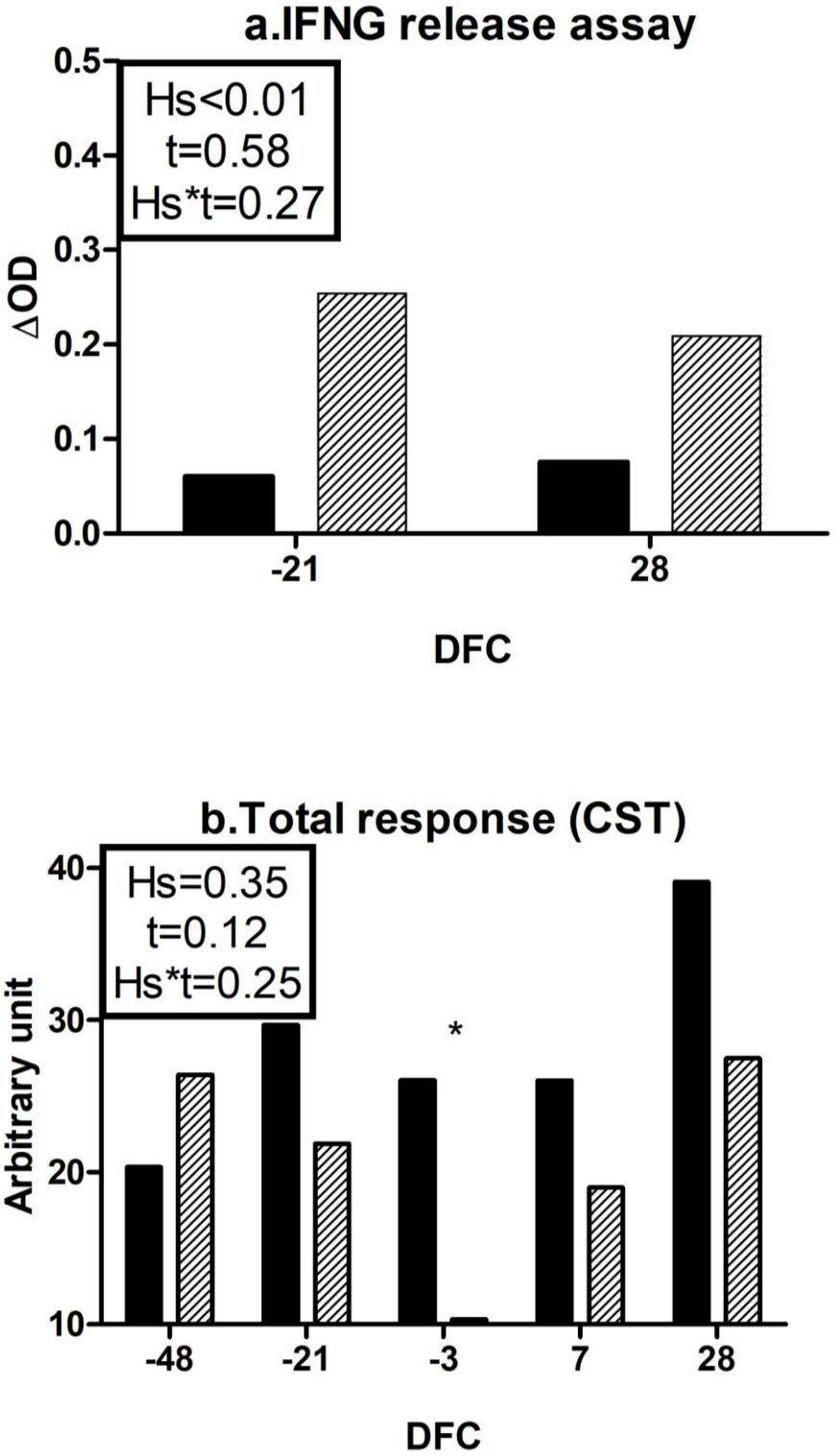


Figure 6. Optical density difference (ΔOD) between avian purified protein derivative-stimulated and control wells in the interferon gamma (IFNG) release assay (a) and total peripheral response after the carrageenan skin test (CST; b) in control dairy cows (CTR; solid bars) or cows that showed beta-hydroxybutyrate (BHB) levels higher than 1.4 mmol/L in the first month of lactation (KET; striped bars). ** is $P < 0.01$; * is $P < 0.05$; DFC is days from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

SUPPLEMENTARY FILE

Supplementary file 1. Trends of body weight (BW) and body condition score (BCS) in control dairy cows (CTR) or cows that showed beta hydroxybutyrate levels higher than 1.4 mmol/L during the transition period (KET)

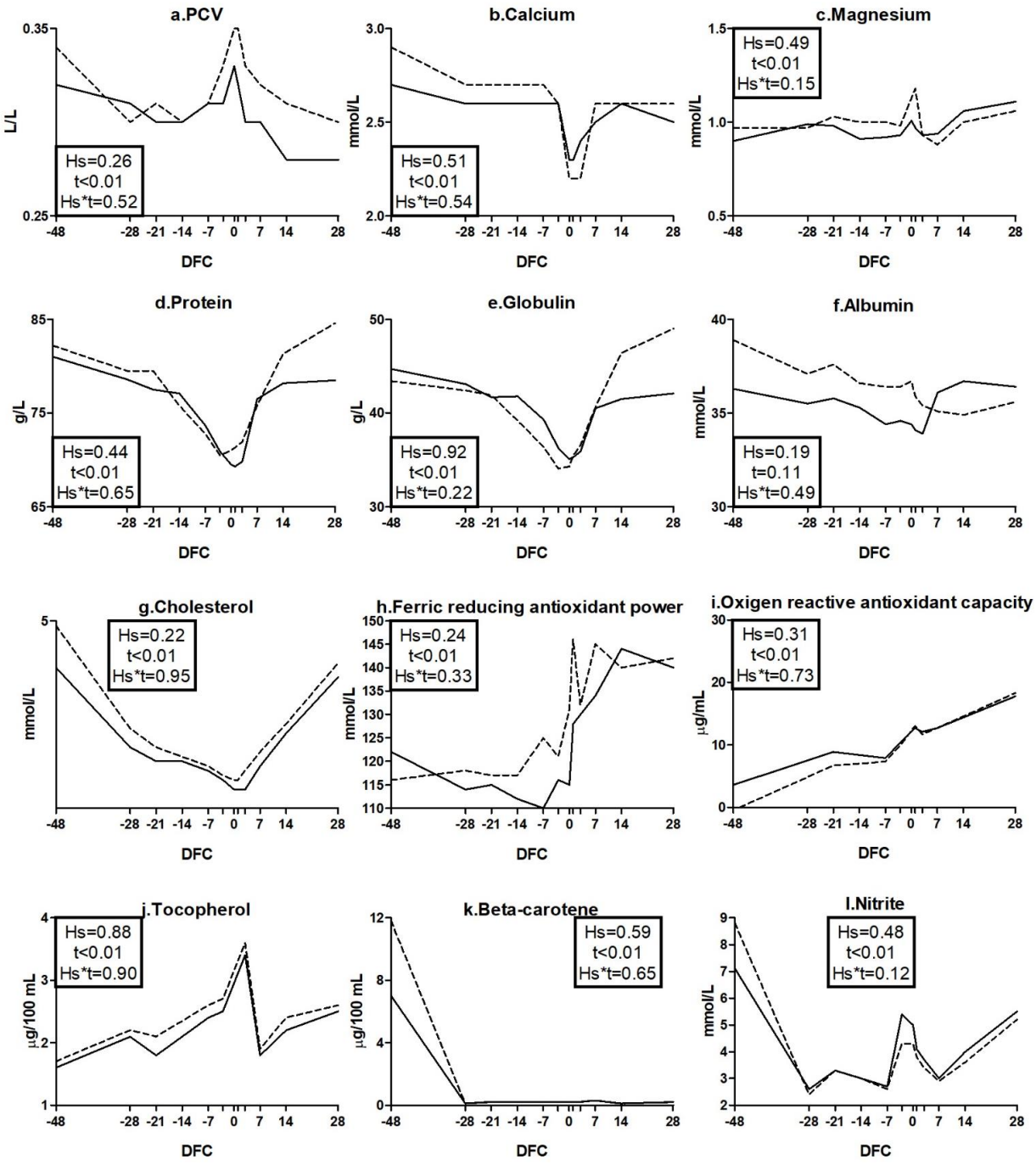
Item, Unit	Hs[1]	Days from calving						Δ[2]	P-Value		
		-45	-21	1	14	21	28		Hs[1]	t[3]	Hs*t[4]
BW, Kg	CTR	628	667	623	577	570	570	-	0.22	<0.01	0.67
	KET	683	711	652	608	593	597	-			
BCS	CTR	2.40	2.43	2.39	2.19	2.09	2.08	0.308	0.31	<0.01	0.99
	KET	2.54	2.57	2.53	2.30	2.20	2.16	0.329			

[1]Health status (CTR is control; KET is ketosis).
[2]Difference between BCS value at 28 and 1 DFC.
[3]Time.
[4]Heath status x time interaction effect.

Supplementary file 3. Fold changes of nitrogen species in a whole blood stimulation assay with a low (L) or high dose (H) of bacterial lipopolysaccharides (LPS) in control dairy cows or cows that showed beta-hydroxybutyrate levels higher than 1.4 mmol/L in the first month of lactation. Values are expressed as fold change with respect to baseline

Item	D[2]	Hs[1]	Days from calving					SE[3]	Effect	P-Value
			-48	-21	-3	7	28			
Nitric oxide	L	CTR	1.03	1.03	1.05	1.00	0.97	0.06	Hs[1]	0.30
		KET	0.99	0.98	1.05	1.07	1.00	0.06	t[4]	0.76
	H	CTR	1.21	1.09	1.06	1.07	1.09	0.06	D[2]	0.11
		KET	0.96	1.03	1.09	1.09	1.03	0.06	Hs*t[5]	0.35
	Tot	CTR	1.12	1.06	1.06	1.03	1.03	0.04	Hs*t*D[6]	0.96
		KET	0.98	1.00	1.07	1.08	1.02	0.04		
Nitrite	L	CTR	0.97	1.02	1.18	0.99	0.99	0.07	Hs[1]	0.13
		KET	0.90	1.20	1.16	0.98	1.10	0.07	t[4]	<0.01
	H	CTR	0.98	1.03	1.14	1.01	0.93	0.07	D[2]	0.79
		KET	0.94	1.12	1.26	0.98	1.16	0.07	Hs*t[5]	0.11
	Tot	CTR	0.98	1.03	1.16	1.00	0.96	0.05	Hs*t*D[6]	0.96
		KET	0.92	1.16	1.21	0.98	1.13	0.05		
Nitrate	L	CTR	1.04	1.04	1.03	1.00	0.96	0.08	Hs[1]	0.13
		KET	1.02	0.91	1.03	1.10	0.97	0.09	t[4]	0.74
	H	CTR	1.40	1.12	1.05	1.09	1.16	0.08	D[2]	0.11
		KET	0.93	1.06	1.05	1.14	0.99	0.09	Hs*t[5]	0.27
	Tot	CTR	1.22	1.08	1.04	1.04	1.06	0.06	Hs*t*D[6]	0.74
		KET	0.98	0.99	1.04	1.12	0.98	0.06		

[1]Health status (CTR is control and KET is ketosis).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Health status x time interaction.
[6]Health status x time x dose interaction.



Supplementary file 2. Pattern of plasmatic concentration of packed cell volume (PCV; a), calcium (b), magnesium (c), protein (d), globulin (e), albumin (f), cholesterol (g), ferric reducing antioxidant power (h), oxygen reactive antioxidant capacity (i), tocopherol (j), beta-carotene (k) and nitrite (l) in control dairy cows (CTR, solid line) or cows that showed beta hydroxybutyrate levels higher than 1.4 mmol/L during the transition period (KET; dotted line). ** is $P < 0.01$; * is $P < 0.05$; + is $P < 0.1$; DFC is days from calving; Hs is health status effect; t is time effect; Hs*t is health status x time interaction effect.

CHAPTER IV - METABOLIC EFFECTS OF AN IMMUNE STIMULANT IN TRANSITION COWS

Administration of an immune stimulant during the transition period improved energy metabolism and rumination without affecting inflammatory conditions

ABSTRACT

Omnigen-AF (**OAF**) is effective in increasing leukocytes functions in immunosuppressed animals and reduces incidence of infectious diseases in early lactating dairy cows. Its mode of action has never been elucidated, and a wider perspective of its metabolic effect could highlight its effectiveness in facing metabolic disorders of transition period also. Thus, a group of 10 Holstein dairy cows were housed in tied stalls and divided into 2 groups: treated group (**TRT**; 5 cows) received 32.5 g of Omnigen-AF® (Phibro Animal Health Corporation) twice a day (65 g d^{-1}) as top-dress on the morning and afternoon feeds, while control group (**CTR**; 5 cows) did not receive any supplementation. From -62 to 42 days from calving BCS, BW, DMI, rumination time and milk yield were measured, and blood samples were collected regularly to assess a wide hematochemical profile and to test white blood cell functions by *ex-vivo* challenge assays. Data were submitted to ANOVA using a mixed model for repeated measures including treatment, time and their interaction as fixed effects.

Administration of OAF at dry-off did not affect BW, BCS, milk yield, milk and rumen fluid composition, and neither affected blood neutrophils concentrations. Nevertheless, it increased rumination time and improved the energy metabolism after calving (lower NEFA and BHB concentrations). TRT cows had an increased lymphocytes abundance at blood level, and their leukocytes had greater efficiency in facing biological stressors during the peripartum (lower lactate production and lower glucose consumption after an LPS challenge). Despite these positive effects on immune cells, OAF did not affect the positive acute phase proteins concentrations after calving. A reduced abundance of albumin, PON and antioxidants also occurred with OAF after calving, suggesting some impairment of hepatic functions to occur. Nevertheless, the lack of any effect on main biomarkers related to liver function (bilirubin) and liver damage (GGT, AST-GOT, ALP) dismisses a real impairment of liver activities to occur with OAF. Positive effects in favoring the recovery of rumen functions, reducing

mobilization of body fats after calving suggest OAF to be an effective strategy in preventing metabolic disorders of transition period.

Key Words. Immune stimulant, metabolic disorders, transition period, immune dysfunctions.

INTRODUCTION

The immune system eliminates sources of tissue injury, restores immune homeostasis and returns tissues to their normal function (Sordillo, 2016). Inflammatory process is the main defense until lymphocytes develop antibodies against antigens (Janeway et al., 2005). During inflammation, bacterial invasions in peripheral tissues stimulates the production of pro-inflammatory cytokines (**PICs**) and chemokines. Interleukin-8 (**IL-8**) enters the blood flow interacting with a surface protein of neutrophilic polymorphonuclear cells (**PMN**) named L-selectin, that allows their slow rolling along the interior vessel walls (Paape et al., 2002). Thus, adhesion molecules and reactive oxygen metabolites (**ROM**) are produced by PMN, promoting their migration to peripheral tissues and enhancing their respiratory burst activity against bacteria (Paape et al., 2003; Forsberg, 2004).

In PMNs of dairy cows, functions related to ROM production, myeloperoxidase activity, chemotaxis and phagocytosis (Cai et al., 1994; Kimura et al., 1999; Mehrzad et al., 2001) are impaired from 2-4 weeks prior through approximately 2 weeks after parturition (Sordillo and Streicher, 2002; Nace et al., 2014; Jahan et al., 2015). Consequently, cows become hyposensitive to antigens, and the incidence of infectious diseases increases (Shuster et al., 1996; Mallard et al., 1998). On the other hand, immune system became unable in solving inflammations (Trevisi et al., 2015; Putman et al., 2018) triggering chronic phenomena (Bionaz et al., 2007; Trevisi et al., 2012; Jahan et al., 2015), while oxidant species produced by active leukocytes deplete antioxidant defenses, triggering an oxidative stress (Celi, 2011; Sordillo, 2016). Those conditions concur in boosting the development of metabolic disorders in transition period (Curtis et al., 1985; Suriyasathaporn et al., 1999).

The immune stimulant Omnigen-AF (**OAF**) improves PMNs functions in immunosuppressed animals (Wang et al., 2007) altering mRNA transcripts and protein expression related to innate immune system (Wang et al., 2009; Ryman et al., 2013). Its mode

of action has not been fully elucidated, but an interaction of yeasts and fungal cells walls (as *Saccharomyces cerevisiae* and *Trichoderma longibrachiatum*) composing OAF with intestinal M-cells mediating the response of lymphoid tissue (Ohno, 2016) could be hypothesized. After its administration in transition dairy cows, OAF down-regulated the expression of genes responsible for leukocytes death (e.g., glucocorticoids modulatory element binding protein-1), inhibiting apoptosis and increasing the circulating leukocytes. mRNA encoding for cytokines (such as receptor alpha for interleukin-4 and myeloid differentiation primary response gene 88) were up-regulated, improving sensitivity of PMN to extracellular signaling and cell-to-cell communication. OAF also enhanced PMN diapedesis through the up-regulation of mRNA encoding for L-selectin (Wang et al., 2004), and augmented phagocytosis and respiratory burst through the improvement of oxidative phosphorylation pathway involved in ROM production (Revelo et al., 2013; Ryman et al., 2013; Nace et al., 2014). Improved PMN functions account for the reduced incidence of mastitis found with OAF (Nace et al., 2014), suggesting its use as a potential strategy to reduce infectious diseases in transition period. However, none of previous studies investigated the effect of OAF on the adverse metabolic conditions of transition period, although elucidate such an effect is crucial to assess its effectiveness in preventing metabolic disorders. Thus, the objective of our study was to assess the effect of supplementing OAF from dry-off to early lactation on metabolism, inflammatory-like conditions, oxidative and immune status in dairy cows. Our hypothesis was that metabolic asset around calving could be improved by the additive, mitigating inflammatory-like status and oxidative stress of treated animals.

MATERIALS AND METHODS

Experimental design and animal management

The trial was carried out at Università Cattolica del Sacro Cuore research dairy barn (Experiment Station, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation (DL n. 26, 04/03/2014) and ethics (Authorization of Italian Health Ministry N 1047/2015-PR). A group of 10 Italian Holsteins dairy cows were housed in individual tied stalls under controlled environmental conditions (room temperature of 20 °C, relative humidity of 65%, 14 hours of light) from -62 to 42 days from calving (**DFC**). At -55 DFC cows were dried off with a deep milking and a treatment with a mammary antibiotic (Mamyzin A; Haupt Pharma Latina S.r.l., Borgo San Michele – Latina, Italy). Before dry-off and after calving, cows were milked twice a day at the stand, at 4:00 am and pm. All the cows were individually fed a component diet offered with two equal meals of forages at 12 h intervals and 2-8 meals of concentrates supplied by computer feeder. Before dry-off, animals received a lactating ration with soybean meal, alfalfa dehydrated hay and corn silage (Phase 1). After dry-off, animals received only hay till -48 DFC. From -47 DFC till -7 DFC animals received a hay-based ration with soybean meal and corn silage (Phase 2). Seven days before the expected day of calving, 1 kg of lactation concentrate was gradually added to the diet (Phase 3). After calving, 3 kg of alfalfa-dehydrated hay and 2 kg $week^{-1}$ of corn silage (till a maximum of 20 kg d^{-1}) were added to the diet. Grass hay was gradually reduced to 2-2.5 kg d^{-1} and concentrate was increased by 0.5 kg d^{-1} to satisfy the requirement of 1 kg per 3 kg of produced milk (Phases 4 and 5). The same batches of hay and corn silage were used during the trial. Feeds were collected fortnightly and, after dry matter determination, samples were pooled for subsequent analyses. Feeds and diet composition are shown in Table 1.

At dry-off, cows were enrolled in 2 homogeneous group. Treated group (**TRT**; 5 cows) received 32.5 g of Omnigen-AF® (Phibro Animal Health Corporation) twice a day (65 g d^{-1}) as top-dress on the morning and afternoon feeds, while control group (**CTR**; 5 cows) did not received any supplementation. The amount of immune stimulant was determined on the average body weight (**BW**) of the animals at dry-off, to ensure the daily assumption of 9 g of product each 100 kg of BW. Between -62 and 42 DFC periodical checks were performed and blood samples were collected regularly, according to the time schedule shown in Figure 1 and described in the following sections.

Body weight, body condition score, dry matter intake, rumination time and milk yield

The body weight (**BW**) was measured with a single walking-in scale. The body condition score (**BCS**) was determined from the same operator with a 1 to 4 scale (Agricultural Development and Advisory Service, 1986) and his variation (Δ**BCS**) was calculated as the difference between data at calving day and 28 DFC. The individual DMI was measured by weighing the amounts of feed administered and residuals for each distribution. Rumination time was registered using the Ruminact system (SCR Europe, Podenzano, PC, Italy). Milk yield was weighed after each milking. Daily values of DMI, rumination time and milk yield were expressed as average weekly value.

Health status

Health conditions of cows were monitored daily and all veterinary interventions that occurred from -62 to 42 DFC were recorded. The body temperature was measured daily with a rumen bolus (DVM System TempTrack^{TM}, HerdStrong, LLC, Greeley, CO). Mastitis was diagnosed by visual evaluation of abnormal milk from each quarter and SCC analysis on suspicious cases, retained placenta when the fetal membranes were not expelled within 24 h after calving, endometritis and metritis according to Sheldon et al. (2006), and milk fever,

displacement of abomasum and pneumonia by inspection of a veterinary practitioner. Diarrhea was diagnosed by visual evaluation of feces consistency and color according to the fecal score method (Ireland-Perry and Stallings, 1993), assuming diarrheic feces those have a fecal score ≤ 2.

Blood samples collection

Blood samples were harvested trough jugular venipuncture in evacuated collection tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) before the morning feeding. Samples were used to perform different assays (Figure 1).

Metabolic profile assessment. Samples were collected into heparinized tubes and processed as described by Calamari et al. (2016). A clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA) was used to determine the concentration of glucose, NEFA, BHB, urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, aspartate amino transferase-glutamate oxaloacetate transaminase (**AST-GOT**), gamma glutamyl transferase (**GGT**), alkaline phosphatase (**ALP**), total protein, haptoglobin, ceruloplasmin, albumin, total bilirubin, cholesterol and globulin in accordance with Calamari et al. (2016). Furthermore, ROM, ferric reducing antioxidant power (**FRAP**), nitrate (**NO3**), nitrite (**NO2**) and nitric oxides (**NOx**) were determined according to Jacometo et al. (2015), PON according to Bionaz et al. (2007), thiol groups (**SHp**) according to Minuti et al., (2014), myeloperoxidase according to Bradley et al. (1982) and advanced oxidation protein products (**AOPP**) according to Hanasand et al. (2012). Finally, L-lactic acid (**LLA**) and D-lactic acid (**DLA**) were determined with a commercial kit (K-DLATE, Megazyme Co., Wicklow, Ireland). A multi-detection microplate reader (BioTek Synergy 2, Winooski, VT, USA) and commercial kits for ELISA method were used to determine the concentration of interleukin-1, beta (**IL-1B**; ESS0029; Thermo Scientific, Frederick, MD, USA) and interleukin-6 (**IL-6**; ESS0027;

Thermo Scientific, Frederick, MD, USA) according to Jahan et al. (2015) and those of serum amyloid alpha (**SAA**; TP-802, Tridelta D.L., Ireland). Furthermore oxygen reactive antioxidant capacity (**ORAC**) was determined with a fluorimetric method according to Jacometo et al. (2015). Retinol, tocopherol and beta-carotene were analyzed by reverse-phase HPLC (LC-4000, Jasco Europe, Carpi MO, Italy), as described by Jahan et al. (2015).

White blood cells profile. Samples were collected in K-EDTA tubes and analyzed with Cell-DYN 3700 (Abbott Diagnostic Division, Santa Clara, CA). A laser optic assay was used to determine the amounts of total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils. The neutrophils to lymphocytes ratio was calculated. The amount of red blood cells, hematocrit, mean cell volume, red cell distribution width, number of platelets and mean platelet volume were determined via electrical impedance assay. The amount of hemoglobin, mean cell hemoglobin and mean cell hemoglobin concentration where determined using spectrophotometry assay.

Whole blood stimulation assay. Samples were collected in heparinized serum tubes and stimulated with 0 (baseline), 0.01 (low dose; **L**) and 5 µg/mL (high dose; **H**) of bacterial lipopolysaccharides (**LPS,** *Escherichia coli* O111:B4; Sigma–Aldrich Company Ltd., UK, Cat. No. L3012), according to Jahan et al. (2015). After whole blood stimulation assay (**WBA**), plasma samples were stored at −80 ºC for the measurement of glucose, DLA, LLA, IL-1B, IL-6, NOx, NO2 and NO3. Variation of plasma parameters after WBA with L and H doses of LPS were expressed as fold change relative to the baseline.

Interferon gamma release assay. For the interferon gamma (**IFNG**) release assay, whole blood samples were also collected into heparinized tubes (Figure 1). After collection, the tubes were stored in vertical position in a warm bath at a temperature of 38 ºC and transported to the laboratory within 20 min for the stimulation procedure. Whole blood was used in an IFNG release assay for *Mycobacterium avium* (internal method IZSLER, MP 13/011).

Briefly, two 1-ml aliquots of each blood sample were distributed in a 24-well tissue culture microtiter plate. One well was supplemented with 100 µl of a 1:10 dilution of *Mycobacterium avium* purified protein derivative (**PPD,** IZS Umbria e Marche, Perugia, Italy, 0.5 mg/mL) to PBS, and 1 well with 100 µl of sterile PBS as control. The plate was positioned in a heated incubator (Grant Boekel, HIR10 M) set to a temperature of 38 ºC and with a relative humidity of 95% for 24 h. After incubation, the blood was centrifuged at 8500 × g for 16 min at 4 ºC and plasma was stored at -20 °C until use. Plasma was later thawed and analyzed in a sandwich ELISA assay for bovine IFNG with a couple of monoclonal antibodies, as previously described (Trevisi et al., 2014). Results were evaluated in terms of optical density difference (Δ**OD**) between avian PPD-stimulated and control wells.

Milk samples collection and analysis

Milk samples were collected during the morning milking (Figure 1) into 100-ml polypropylene bottles. Butterfat, protein, lactose, casein contents, titratable acidity and coagulation properties [rennet clotting time (**r**) and curd firmness at 30 min ($\mathbf{a_{30}}$)] were measured by using infrared instrumentation (MilkoScan FT 120, Foss Electric, Hillerød, Denmark) according to Calamari et al. (2010) and Chessa et al. (2014). Furthermore, the outputs of fat and protein, and the fat to protein ratio were also calculated. Urea nitrogen was determined on skimmed milk by a spectrophotometric assay, using a urea nitrogen kit (cat# 0018255440, Instrumentation laboratory, Milano, Italy) in association with a clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA). Somatic cell count (**SCC**) were determined using an optical fluorimetric method with an automated cell counter (Fossomatic 180, Foss Electric).

Carrageenan skin test

The carrageenan skin test (**CST**) was performed as specified by Jahan et al. (2015) to evaluate peripheral immune responses (Figure 1). The skin thickness was measured using a skinfold caliper (cat# 470119-588, VWR, USA) immediately before carrageenan injection (day 0), then at 2 and 9 days after the injection. The total response of each challenge was calculated as the area under the curve of the thickness, measured at day 2 and day 9, subtracting the thickness measured at day 0.

Rumen fluid parameters

Rumen samples were collected with an orogastric probe (Ruminator, Proofs Products, Guelph, Canada) prior to the morning feed administration (Figure 1). The pH was immediately measured (GLP 21, Crison Instrument SA, Alella, Barcellona, ESP). A 2-mL aliquot of the supernatant was transferred into tubes with 1 mL of 0.12 M oxalic acid and immediately frozen at –20°C for later analysis. Total volatile fatty acids (**VFA**) concentration, molar proportion of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, caproic, iso-caproic and enanthic acids were analyzed as previously described (Minuti et al. 2014). VFA were expressed as relative amounts on total VFA concentration. A spectrophotometric clinical auto-analyzer (ILAB-650, Instrumentation Laboratory, Lexington MA, USA) and commercial kits for urea nitrogen (cat# 0018255440, Instrumentation laboratory, Milano, Italy) and lactate (K-DLATE, Megazyme Co., Wicklow, Ireland) were used to assess the concentration of ammonia and those of LLA, DLA and total lactic acid respectively.

Statistical analysis

Data in the tables are presented as means and standard errors. Before analysis, the normality of distributions was verified for each parameter by reckoning skewness and kurtosis according to the Shapiro test of SAS. Non-normally distributed parameters were normalized

through natural logarithms (among plasma parameters IL-1B, IL-6, LLA, GGT, bilirubin, beta-carotene, NOx, NO2 and NO3; among white blood cells profile eosinophils; among WBA the fold change of IL-1B, IL-6, glucose, DLA, LLA, NO2, NO3 and NOx; the total response to CST; among milk quality parameters fat, fat output, fat/protein ratio, r and SCC) or square root transformation (among plasma parameters the BHB) and back transformed to plot them in the graphs and tables.

Prevalence of health problems recorded during the study was evaluated by $\chi 2$ analysis (Freq procedure, SAS Inst. Inc., Cary, NC). Data of DMI, BW, BCS, rumination time, milk yield, plasma parameters, white blood cells profile, WBA, milk quality parameters, response to IFNG release assay and CST were submitted to ANOVA using a mixed model for repeated measures (Mixed procedure, SAS Inst. Inc., Cary, NC) in accordance with Littell et al. (1998). The statistical model included the fixed effect of treatment (**T**; CTR and TRT), time (**t**), and their interaction (**T*t**). For those parameters that were measured daily (DMI, rumination time and milk yield) time effect considered the average weekly value, while for other parameters (BW, BCS, plasma parameters, white blood cells profile, WBA, milk quality parameters, IFNG release assay and CST) it considered single DFC. The time was considered as a repeated measure within cow. For WBA, also the dose (**D**; L and H) and the full interaction effect (**T*t*D**) were considered. The analysis was carried out using three covariance structures: autoregressive order, compound symmetry, and spatial power. These were ranked according to their Akaike information criterion, with the one having the lowest Akaike information criterion being eventually chosen (Littell et al., 1998). The pair-wise comparison was done using the least significant difference test. Parameters that showed a significant T*t interaction effect before treatment administration were covariate using data collected at -62 DFC (among plasma parameters glucose, IL-1B, IL-6, cholesterol, retinol and tocopherol) as baseline.

Data of rumen fluid parameters were analyzed by a one-way ANOVA (GLM procedure, SAS Inst. Inc., Cary, NC), considering only the fixed effect of T.

Post-hoc comparisons between treatments are discussed when the P-value for main effect was ≤ 0.05. Main effects at $P \leq 0.10$ are discussed in the context of tendencies. Differences between treatments at single time points are discussed at $P \leq 0.10$ for the main interaction effect. Relevant trends not supported from statistical differences are discussed as numerical.

RESULTS

Body weight, body condition score, dry matter intake, rumination time and milk yield

The DMI (Figure 2.a) tended to be lower in TRT than CTR group at 5 weeks from calving ($P < 0.1$). Rumination time (Figure 2.b) resulted higher in TRT than CTR group at 3 and 6 weeks from calving ($P < 0.05$) and tended to be higher at 7 weeks from calving ($P < 0.1$). Body weight, BCS, (Supplementary file 1), and milk yield (Figure 2.c) were not affected by the treatment.

Health status

The χ^2 analysis on the occurrence of clinical diseases (Supplementary file 2) did not found any treatment effect, but the total incidence of diseases resulted lower and the number of cows without any clinical disease resulted greater in TRT than CTR group ($P < 0.01$).

Milk quality and rumen fluid parameters

Among milk quality and rheological parameters (Table 2), lactose tended to be higher in TRT than CTR group at 7 DFC ($P < 0.1$). Other milk parameters were not affected by the treatment, and neither rumen fluid parameters (Table 3).

Metabolic profile

Packed cell volume, energy, protein and mineral metabolism biomarkers. Packed cell volume (Figure 3.a) tended to be lower in TRT than CTR group (T = 0.07). Among energy metabolism biomarkers, TRT cows had tended to have lower concentration of NEFA (T = 0.06; Figure 3.b) and lower concentration of BHB (Figure 3.c) at 14 DFC ($P < 0.01$ for BHB) in comparison to CTR. Furthermore, LLA (Figure 3.d) tended to be lower in TRT than CTR group at -21 and 42 DFC ($P < 0.1$). Glucose (Supplementary file 3.a) and DLA concentrations

(Supplementary file 3.b) were not affected by the treatment. Among protein metabolism biomarkers, creatinine concentration (Figure 3.e) was lower in TRT than CTR group (T < 0.01) with differences between -28 and -3 DFC and from 7 DFC up to the end of the experimental period ($P < 0.05$). No effect was detected on urea concentration (Supplementary file 3.c). Among mineral metabolism biomarkers, Zn concentration (Figure 3.f) was higher in TRT than CTR group at -53 DFC ($P < 0.01$) and tended to be higher at -21 and 42 DFC ($P < 0.1$). No effect was detected on the concentration of other minerals (Supplementary files 3.d-i).

Liver function and inflammation biomarkers. No effect was detected on liver enzyme biomarkers (Supplementary files 4.a-d). Among inflammation biomarkers, cows treated with OAF had lower myeloperoxidase concentration (Figure 3.g) at -21 and -14 DFC ($P < 0.05$) while globulin (Supplementary file 5.a) and total proteins (Supplementary file 5.b) were not affected. None of the positive APP were affected by OAF (Supplementary files 5.e-f). Among negative APP, albumin concentration (Figure 3.h) was lower in TRT than CTR group at -3 and 3 DFC ($P < 0.05$) and tended to be lower at 14 and 28 DFC ($P < 0.1$). Cholesterol concentration (Figure 4.a) tended to be higher in TRT than CTR group at -53 DFC ($P < 0.1$). PON concentration (Figure 4.b) was numerically lower in TRT than CTR group during the entire experimental period. Retinol (Supplementary file 4.h) as well as IL-1B (Figure 4.c) and IL-6 (Figure 4.d) were not affected by OAF.

Oxidative stress biomarkers. Among antioxidant capacity indicators, compared to CTR, TRT had lower concentration of SHp (Figure 4.e) from -3 to 28 DFC ($P < 0.05$) and lower concentration of FRAP (Figure 4.f) at 3 DFC ($P < 0.01$). No effect was detected for tocopherol, beta-carotene, and ORAC concentration (Supplementary files 5.c-e). Among oxidant species, ROM (Figure 4.g) tended to be lower in TRT than CTR group at -28 DFC ($P < 0.1$), while nitric oxygen species were not affected (Supplementary files 5.f-h). AOPP

(Figure 4.h) resulted lower in TRT than CTR group (T = 0.02) at -48, -3, 1 and 3 DFC ($P < 0.05$).

White blood cells profile

Among white blood cells profile (Table 4), the number of lymphocytes resulted numerically higher in TRT than CTR group during the whole experimental period. Mean cell volume and mean cell hemoglobin tended to be lower in TRT than CTR group (T = 0.08). The number of platelet tended to be higher, and the mean platelet volume was lower in TRT than CTR group (T = 0.07 and = 0.01 respectively). In TRT cows, mean platelet volume was lower at -48 and -1 ($P < 0.05$) and tended to be lower at 7 DFC ($P < 0.1$) in comparison to CTR. No differences appeared on total leukocytes, neutrophils, neutrophil to lymphocytes ratio, eosinophils, red blood cells, hemoglobin, mean cell hemoglobin concentration and red blood cell distribution width (data not shown).

Whole blood stimulation assay, interferon gamma release assay and carrageenan skin test

OAF treatment did not affect the cytokines response in WBA (Table 5). Fold change of glucose tended to be higher in TRT than CTR group at -21 DFC ($P < 0.1$) and was higher at 7 DFC ($P < 0.01$). Fold change of DLA tended to be lower ($P < 0.1$) and those of LLA was lower ($P < 0.05$) in TRT than CTR group at 28 DFC (Table 6). The response to LPS stimulation of nitric oxygen metabolites (Table 7), the response to IFNG release assay (Figure 5.a) and the total response to CST (Figure 5.b**)** were not affected by OAF treatment.

DISCUSSION

Administration of OAF did not affect BW, BCS and milk yield in our experiment, confirming previous results (Wang et al., 2007; Ryman et al., 2013). Furthermore, the lack of any differences on milk composition suggests OAF to have no direct effects on metabolic parameters under homeostasis condition. The use of OAF has been suggested as a strategy to reduce the susceptibility to infectious diseases at the beginning of lactation in dairy cows (Wang et al., 2007), as decreased incidences of new cases of mastitis has been reported after its administration (Nace et al., 2014). The investigation of such an effect was beyond the aim of our study, as the small size of our experimental groups did not allow us to speculate on the lower number of total diseases and higher number of healthy cows founded in TRT group. Nevertheless, the occurrence of ketosis in three out five of our CTR cows, together with the lower NEFA and BHB concentration and with the higher rumination time founded in TRT animals after calving, suggest OAF to have improved the capacity of the animals to overcome the severe digestive and metabolic challenges that are widely reported during TP (Trevisi et al., 2011a; Van Knegsel et al., 2014). Two possible mode of action of OAF could account for such effects: 1) OAF could exert a direct effect on energy metabolism, reducing lipomobilization and hepatic ketogenesis, and the higher rumination time founded in TRT animals could thus arise from the reduced incidence of ketosis in comparison to CTR, or 2) yeasts and fungal cells walls composing OAF could have a modulatory effect on rumen fermentations, improving the recovery of rumen motility after parturition and reducing the negative energy balance degree in early lactation, and thus ameliorated energy metabolism founded in TRT cows could be consequential to their more efficient utilization of feedstuffs (Soriani et al., 2012; Calamari et al., 2014). A clear statement on which is the driving mechanism is not possible, as none reported a direct effect of OAF on lipolysis and energy metabolism, while the collection time of our rumen samples makes it difficult to elucidate the

effect of the additive on rumen fermentations after sudden alterations related to calving. In fact, our samples were collected before morning feeding at 28 DFC, way after the occurrence of alterations related to calving and when fermentation patterns where at their nadir. Thus, the lack of any increase in VFA production with OAF does not dismiss its role in modulating rumen fermentations, as such higher production could have been balanced by a higher absorption. Whatever the driving mechanism could be, the improved feeding behavior and energy metabolism in early lactation could account for the effectiveness of OAF in favoring the recovery of normal body functions after stressing events (Nace et al., 2014), suggesting it to be effective in the prevention of metabolic disorders of TP, and could also account for an improvement of leukocytes functions with OAF. In fact, ameliorated rumen functions and decreased incidence of metabolic disorders could account for the effectiveness of OAF in reducing blood cortisol concentrations after calving reported by Hall et al. (2014), and this could explain the effect reported by Rowson et al. (2011) in augmenting the expression of L-selectin and improving diapedesis of PMN. In fact, cortisol level is dependent on the occurrence of stressing events, and its raise is known to shade the L-selectin from the surface of PMN, impairing their diapedesis (Burton et al., 1995). Furthermore, a positive effect on leukocytes functions could also arise from the reduced mobilization of body fats occurring with OAF. In fact, both NEFA and BHB are known to impair the viability of white blood cells (Stevens et al., 2011; Calder, 2013; Sordillo, 2016) and their reduction could explain the lower lactate production and the higher glucose concentration found in blood of TRT cows after the stimulation of leukocytes with LPS. In fact, production of lactate by neutrophils and monocytes is known to impair motility, killing capacity and effector functions of leukocytes, to suppress the inflammasome and the production of PICs (Sordillo, 2016; Zhang et al., 2016). Thus, a lower production of this metabolite in activated leukocytes could reflect their greater immune competence. On the other hand, the lower glucose consumption after the

stimulation with LPS could be driven from a more efficient metabolism of leukocytes while they cope with a biological stressor, reflecting an improved killing capacity of those cells. This is also consistent with the previously reported effectiveness of OAF in improving phagocytosis, ROM production and respiratory burst activity of PMN (Wang et al., 2009). Furthermore, the numerically higher lymphocytes number founded in blood of TRT animals during the entire experimental period is consistent with previous results (Wang et al., 2007), and also suggests an effect of the product on the migration of lymphocytes from lymphoid tissue to blood .

State these positive effects on lipomobilization and white blood cells functions, an improvement of liver function and a faster resolution of inflammatory phenomena, reflected from typical trends of plasma parameters (Castell et al., 1989), were expected with OAF at metabolic level. Nevertheless, the concentration of IL-1B and IL-6 were not affected by the treatment, even though the augmented expression of genes triggered from toll like receptors in PMNs has been widely reported as main effect of OAF (Nace et al., 2014). Furthermore, OAF did not affect the concentrations of α-globulins (haptoglobin, ceruloplasmin and SAA), named positive acute phase proteins (**APP**) as their hepatic synthesis increase during inflammation (Ceciliani et al., 2012). Less clear is the effect of OAF on albumin, PON and lipoproteins concentrations. These parameters are named negative APP, as they decrease during inflammation due to the shift of liver synthesis on positive APP (Bertoni et al., 2008). TRT animals had higher concentration of cholesterol during dry period, reflecting a greater hepatic synthesis of lipoproteins (Gruys et al., 2005) and a better liver shape prior to calving. The lower myeloperoxidase, ROMt and AOPP concentration reflects also a less marked leukocyte activation in the peripheral blood to occur in TRT animals during dry period. In fact, myeloperoxydase generates hypoclorous acid that is converted into ROM within the respiratory burst (Faith et al., 2008; Celi, 2011) while AOPP are markers of protein oxidation

triggered by this metabolite (Celi and Gabai, 2015). Furthermore, the lower LLA found in TRT animals during the same phase could reflect a less marked body effort to suppress the inflammasome restoring normal tissue functions (Sordillo, 2016; Zhang et al., 2016). These results suggest that greater cholesterol concentrations found in TRT animals before calving could depend on a lower activation of defense systems. Such an anti-oxinflammatory effect exerted by OAF before calving could arise from a better stabilization of PMN and monocytes, that need a stronger stimulus for mounting an inflammatory reaction in TRT animals. On the other hand, TRT animals had lower levels of PON and albumin in early lactation in comparison to CTR, indicating a greater impairment of liver function to occur with calving, and thus a reduction of positive effect of OAF in stabilize leukocytes ameliorating inflammatory status. Nevertheless, trends of cytokines and positive APP dismiss inflammation as the main cause of post-partal reduction of negative APP in TRT animals, and the higher zinc concentration they had during the whole experimental period suggest no acute manifestation to occur neither before calving. In fact this mineral is sequestered by the liver during inflammatory processes (Bertoni and Trevisi, 2013), and a reduction of its blood concentration should be evident whenever an inflammation occurs. Despite that, the above trends of positive APP clearly suggest an impairment of some liver activities to occur in TRT animals after calving, and this is further reflected from their lower post partal availability of antioxidant systems (SHp and FRAP), as antioxidant capacity directly depends on liver activity (Sordillo and Aitken, 2009; Celi, 2011). In fact, blood SHp mainly reflects albumin, lipoic acid, glutathione and cysteine concentration, while FRAP provides a measurement of antioxidant power provided by blood concentration of bilirubin, uric-acid, proteins and vitamins C and E (Benzie and Strain, 1996). A dysregulation of liver functions involved in controlling blood concentrations of such compounds could account for post partal trends of negative APP and antioxidant systems with OAF, despite the lack of any effect on the

concentration of bilirubin or enzymes related to amino acids metabolism in liver (AST-GOT, GGT, ALP) dismiss any impairment of liver function to occur (Rodriguez-Jimenez et al., 2018). Thus, we may hypothesize that the depletion of antioxidant systems observed with OAF could arise from a weak antioxidant activity of the product. In fact, antioxidant biosynthesis and release are regulated processes, as the administration of weak oxidants is known to up-regulate endogenous antioxidant mechanisms (Gesslbauer and Bochkov, 2017). Conversely, it could be hypothesized that weak antioxidant activity exerted by OAF could have reduced body requirements of endogenous antioxidant compounds, down-regulating their synthesis *de-novo*.

CONCLUSIONS

Administration of OAF at dry-off did not affect BW, BCS, milk yield, milk and rumen fluid composition, and neither affected neutrophils diapedesis. Nevertheless, it seemed to ameliorate the recovery of rumen functions after calving, improving the rumination time, the energy metabolism and reducing ketogenesis. Such an improvement could justify the greater efficiency of leukocytes to face biological stressors during the peripartum, as suggested from their lower lactate production and lower glucose consumption after an LPS challenge. Furthermore, an increased lymphocytes abundance at blood level also occurred with OAF. Despite these positive effects on immune cells, OAF seem to be ineffective in reducing the degree of inflammation over calving. A reduced abundance of albumin, PON and antioxidants also occurred with OAF after calving, suggesting some impairment of hepatic functions to occur. Nevertheless, the lack of any effect on main biomarkers related to liver function (bilirubin) and liver damage (GGT, AST-GOT, ALP) dismisses a real impairment of liver activities to occur with OAF. This could suggest OAF to act as a weak antioxidant reducing body requirements for endogenous antioxidants synthesis. Despite no effect of OAF has been detected on liver function and inflammation, positive effects in favoring the recovery of rumen functions, reducing lipomobilization after calving suggest OAF to be an effective strategy in preventing metabolic disorders of transition period. A deeper investigation on rumen fluid composition, collecting rumen samples immediately after calving and when rumen fermentations reach their peak, is required to elucidate its effect at rumen level, understanding its real mode of action in ameliorating energy metabolism and antioxidants biosynthesis in early lactation.

TABLES AND FIGURES

Table 1. Composition and characteristics of the experimental diets fed to cow during the 5 experimental phases. Between -55 and -48 days from calving (DFC) cows received only grass hay

Diet, % DM	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
DFC	-62; -55	-47; -7	-6; 0	0; 30	31; 42
Item					
Corn silage	28.5	18.6	23.6	24.0	23.3
Alfalfa hay	16.4	-	-	13.7	11.1
Grass hay	23.4	71.4	58.0	18.1	11.9
Concentrate (Dry period)	-	10.0	10.1	5	-
Concentrate (Lactation period)	31.7	-	8.3	44.2	53.7
Concentrate composition, % DM	Dry period			Lactation period	
Corn flour				40.0	
Barley flour				1.4	
Sorghum grain expanded				-	
Soybean meal		90.5		13.1	
Soybean dry rolled				-	
Sunflower meal				4.9	
Corn gluten feed				-	
Beet pulp				16.6	
Wheat bran				9.8	
Beet molasse slops				2.6	
Potato protein				2.2	
Hydrogenated palm oil				3.3	
Limestone				1.39	
Dicalcium phosphate				1.80	
Sodium bicarbonate				0.98	
Magnesium oxide		2.		0.64	
Sodium Chloride		1.		0.32	
Supplement[1]		5.		1.07	
Chemical composition					
$NE_{L,}$ Mcal*kg of DM^{-1}	1.59	1.45	1.53	1.60	1.63
Crude protein, % DM	14.9	13.6	14.5	16.2	17.2
Starch + sugar, % DM	23.7	16.8	19.3	26.0	18.3
Ether extract, % DM	3.80	1.80	2.40	4.48	5.08
NDF, % DM	39.4	49.3	45.5	35.7	32.6
MP[2], % CP	9.80	9.10	9.70	10.5	11.1
RUP[2], % DM	4.64	4.48	4.77	5.23	5.96

[1]Supplements were composited to provide 150000 UI of vitamin A, 10000 IU of vitamin D, 200 mg of vitamin E, 100 mg of vitamin K, 100 mg of vitamin H1 50 mg of vitamin B1, 0.5 mg of vitamin B12, 500 mg of vitamin PP, 4000 mg of coline, 350 mg of Mn, 800 mg of Zn, 40 mg of Cu, 20 mg of I, 1 mg of Co, 1 mg Se.
[2]Estimated using NRC 2001.

Table 2. Milk composition, rheological parameters and somatic cell count in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item, Unit	T[1]	Days from calving				SE[5]	P-Value		
		7	14	28	42		T[1]	t[2]	T*t[3]
Butterfat, mg 100 mL^{-1}	CTR	4.69	4.24	4.68	3.67	0.35	0.21	0.01	0.78
	TRT	4.33	4.14	3.82	3.36				
Fat output, kg	CTR	0.38	0.41	0.65	0.48	0.07	0.17	0.02	0.65
	TRT	0.27	0.37	0.48	0.44				
Total protein, mg 100 mL^{-1}	CTR	3.84	3.30	3.07	3.09	0.10	0.49	<0.01	0.66
	TRT	3.81	3.34	3.25	3.19				
Protein output, kg	CTR	1.21	1.18	1.33	1.36	0.06	0.61	<0.01	0.15
	TRT	1.15	1.18	1.38	1.50				
Fat/protein ratio, -	CTR	1.22	1.28	1.54	1.19	0.12	0.11	0.11	0.64
	TRT	1.14	1.24	1.19	1.05				
Lactose, mg 100 mL^{-1}	CTR	4.79	5.25	5.06	5.17	0.07	0.73	<0.01	0.03
	TRT	4.96 +	5.09	5.16	5.14				
Caseins, mg 100 mL^{-1}	CTR	2.84	2.50	2.28	2.33	0.08	0.42	<0.01	0.59
	TRT	2.85	2.51	2.45	2.40				
Titratable acidity, °SH 50 mL^{-1}	CTR	3.96	3.33	3.23	3.12	0.17	0.77	<0.01	0.93
	TRT	3.86	3.19	3.20	3.12				
Urea-N, mg dL^{-1}	CTR	31.2	34.7	26.8	31.2	3.11	0.64	0.08	0.66
	TRT	33.7	33.3	30.2	33.0				
Coagulation time (r), min	CTR	12.3	20.0	14.0	14.5	2.03	0.77	<0.01	0.68
	TRT	11.5	16.2	14.8	16.1				
Curd firmness (a30), mm	CTR	37.7	23.2	35.3	32.8	4.59	0.27	0.31	0.40
	TRT	43.4	33.6	35.6	34.7				
SCC[4], n mL^{-1}	CTR	293.4	-	37.7	-	91.4	0.47	0.18	0.31
	TRT	72.4	-	54.9	-				

[1]Treatment (CTR is control; TRT is treated).
[2]Time.
[3]Treatment x time interaction (* is $P < 0.05$; + is $P < 0.1$).
[4]Somatic cells count.
[5]Standard error.

Table 3. Ruminal pH, ammonia concentrations and volatile fatty acids molar proportion, determined at 28 days after calving in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item	Unit	T[1]		SE[3]	P-value
		CTR	TRT		
pH	-	6.78	6.54	0.10	0.14
Ammonia	mg L^{-1}	4.65	4.47	0.10	0.96
VFA total[2]	mmol L^{-1}	103.8	109.0	0.59	0.61
Acetic acid	mol 100 mol^{-1}	66.2	67.0	6.83	0.89
Propionic acid	mol 100 mol^{-1}	21.8	25.4	4.24	0.38
Butyric acid	mol 100 mol^{-1}	11.4	12.3	2.69	0.50
Isobutyric acid	mol 100 mol^{-1}	0.89	0.80	0.86	0.44
Valeric acid	mol 100 mol^{-1}	1.26	1.42	0.08	0.47
Isovaleric acid	mol 100 mol^{-1}	1.55	1.57	0.15	0.95
Caproic acid	mol 100 mol^{-1}	0.55	0.46	0.14	0.59
Enanthic acid	mol 100 mol^{-1}	0.04	0.03	0.11	0.36
L-lactic acid	mmol L^{-1}	35.3	33.7	0.01	0.88
D-lactic acid	mmol L^{-1}	34.8	33.7	6.81	0.91
Total lactic acid	mmol L^{-1}	70.1	67.4	6.78	0.89

[1]Treatment effect (CTR is control; TRT is treated).
[2]Total volatile fatty acids.
[3]Standard error.

Table 4. White blood cells profile in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item, Unit	T[1]	Days from calving						SE[2]	P-value		
		-62	-48	-21	-3	7	28		T[1]	t[3]	T*t[4]
WBC[5], K µL^{-1}	CTR	6.4	5.8	6.3	7.7	6.0	5.9	0.77	0.54	<0.01	0.59
	TRT	7.5	6.6	6.8	8.4	6.8	5.7	0.77			
Neu[6], K µL^{-1}	CTR	3.3	3.0	3.2	4.7	3.6	3.2	0.61	0.52	<0.01	0.69
	TRT	4.0	3.3	3.5	5.1	3.9	3.0	0.61			
Lym[7], K µL^{-1}	CTR	2.2	2.0	2.2	2.2	1.7	1.9	5.09	0.56	<0.01	0.04
	TRT	2.7	2.6	2.6	2.3	2.2	2.0	6.15			
HGB[8], g dL^{-1}	CTR	10.8	11.4	10.6	11.0	10.7	9.8	0.40	0.20	<0.01	0.42
	TRT	10.0	10.5	10.0	10.2	10.4	9.4	0.40			
MCV[9], fL	CTR	48.3	48.6	48.6	51.0	49.6	48.7	1.4	0.08	<0.01	0.78
	TRT	45.8	45.5	45.9	47.5	47.6	46.8	1.4			
MCH[10], pg	CTR	16.5	16.9	16.9	17.2	17.1	16.9	0.47	0.08	0.06	0.69
	TRT	15.7	15.7	16.0	16.5	16.4	16.4	0.47			
PLT[11], K µL^{-1}	CTR	323.8	273.6	280.0	305.8	313.8	420.4	38.2	0.07	0.02	0.84
	TRT	354.6	348.8	345.8	412.8	357.2	437.6	38.2			
MPV[12], fL	CTR	6.9	8.3	7.6	6.9	6.8	5.8	0.36	0.01	0.01	0.01
	TRT	6.1	5.7	6.3	6.5	5.9	5.5	0.45			
			**	*		+					

[1]Treatment effect (CTR is control and TRT is treated).
[2]Standard error.
[3]Time effect.
[4]Treatment x time interaction effect (+ is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[5]White blood cells.
[6]Neutrophils.
[7]Lymphocytes.
[8]Hemoglobin.
[9]Mean cell volume.
[10]Mean cell hemoglobin.
[11]Platelets.
[12]Mean platelet volume.

Table 5. Fold change values (expressed with respect to baseline) of interleukin-1, beta (IL-1) and interleukin-6 (IL-6) in a whole blood stimulation assay with a low or high dose of bacterial lipopolysaccharides in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item	D[2]	T[1]	Days from calving						SE[3]	Effect	P-Value
			-62	-45	-21	-3	7	28			
IL-1	L	CTR	3.5	9.9	10.0	20.2	18.7	6.9	11.3	T[1]	0.66
		TRT	5.2	9.9	13.1	10.2	9.8	9.7	11.3	t[4]	<0.01
	H	CTR	13.1	21.2	33.6	52.1	55.3	23.7	11.3	D[2]	<0.01
		TRT	20.0	40.9	39.9	51.8	59.6	32.3	11.3	T*t[5]	0.13
	Tot	CTR	8.3	15.5	21.8	36.1	37.0	15.3	9.3	T*t*D[6]	0.87
		TRT	12.6	25.4	26.5	31.0	34.7	21.0	9.3		
IL-6	L	CTR	1.34	1.61	1.27	1.92	1.57	1.91	0.39	T[1]	0.97
		TRT	1.69	1.65	1.56	1.33	1.78	1.60	0.39	t[4]	0.06
	H	CTR	1.90	2.19	1.99	2.10	2.40	3.07	0.39	D[2]	<0.01
		TRT	2.20	2.38	1.77	1.71	2.87	2.38	0.39	T*t[5]	0.10
	Tot	CTR	1.62	1.90	1.63	2.01	1.98	2.49	0.31	T*t*D[6]	0.95
		TRT	1.95	2.01	1.67	1.52	2.32	1.99	0.31		

[1]Treatment effect (CTR is control and TRT is treated).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Treatment x time interaction effect (+ is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[6]Treatment x time x dose interaction effect.

Table 6. Fold change values (expressed with respect to baseline) of glucose (Glu), L-lactic acid (LLA) and D-lactic acid (DLA) after a whole blood stimulation assay with a low or high dose of bacterial lipopolysaccharides in control dairy cows or cows receiving 65 $g*d^{-1}$ of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item	D[2]	T[1]	Days from calving						SE[3]	Effect	P-Value
			-62	-45	-21	-3	7	28			
Glu	L	CTR	0.96	0.96	0.94	0.96	0.93	0.97	0.04	T[1]	0.13
		TRT	0.98	0.98	0.99	0.91	1.08	0.97	0.04	t[4]	0.41
	H	CTR	0.95	0.94	0.89	0.91	0.88	0.93	0.04	D[2]	0.02
		TRT	0.92	0.93	0.96	0.85	1.00	0.91	0.04	T*t[5]	0.06
	Tot	CTR	0.95	0.95	0.91	0.93	0.91	0.95	0.03	T*t*D[6]	1.00
		TRT	0.95	0.95	0.98 +	0.88	1.04 **	0.94	0.03		
DLA	L	CTR	0.97	1.00	1.05	1.12	1.08	1.09	0.04	T[1]	0.62
		TRT	1.02	1.03	0.99	1.07	1.01	0.99	0.04	t[4]	0.01
	H	CTR	0.98	1.00	1.09	1.13	1.11	1.15	0.04	D[2]	0.04
		TRT	1.06	1.06	1.05	1.12	1.09	1.07	0.04	T*t[5]	0.08
	Tot	CTR	0.98	1.00	1.07	1.12	1.10	1.12	0.03	T*t*D[6]	0.99
		TRT	1.04	1.05	1.02	1.10	1.05	1.03 +	0.03		
LLA	L	CTR	1.02	1.02	1.02	1.04	1.06	1.06	0.02	T[1]	0.35
		TRT	1.04	1.02	1.03	1.04	1.02	1.00	0.02	t[4]	0.10
	H	CTR	1.03	1.03	1.08	1.07	1.10	1.14	0.02	D[2]	<0.01
		TRT	1.07	1.03	1.04	1.06	1.05	1.08	0.02	T*t[5]	0.10
	Tot	CTR	1.02	1.02	1.05	1.06	1.08	1.10	0.02	T*t*D[6]	0.84
		TRT	1.05	1.02	1.04	1.05	1.04	1.04 *	0.02		

[1]Treatment effect (CTR is control and TRT is treated).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Treatment x time interaction effect (+ is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[6]Treatment x time x dose interaction effect.

Table 7. Fold change values (expressed with respect to baseline) of nitrite (NO2), nitrate (NO3) and nitric oxides (NOx) after a whole blood stimulation assay with a low (L) or high dose (H) of bacterial lipopolysaccharides in control dairy cows or cows receiving 65 $g*d^{-1}$ of Omnigen-AF® as top-dress between -55 and 42 days from calving

Item	D[2]	T[1]	Days from calving						SE[3]	Effect	P-Value
			-62	-45	-21	-3	7	28			
NO2	L	CTR	1.02	0.99	1.03	1.11	0.96	1.13	0.07	T[1]	0.96
		TRT	0.97	0.97	1.23	1.23	1.03	0.97	0.07	t[4]	0.00
	H	CTR	1.04	1.00	1.03	1.18	0.99	1.06	0.07	D[2]	0.74
		TRT	1.05	1.01	1.01	1.14	1.02	0.95	0.07	T*t[5]	0.16
	Tot	CTR	1.03	1.00	1.03	1.15	0.97	1.10	0.05	T*t*D[6]	0.70
		TRT	1.01	0.99	1.12	1.18	1.03	0.96	0.05		
NO3	L	CTR	0.95	1.07	1.05	1.06	1.04	1.01	0.12	T[1]	0.96
		TRT	0.99	1.01	0.98	1.02	1.02	0.96	0.12	t[4]	0.99
	H	CTR	1.20	1.05	1.20	1.05	1.04	1.05	0.12	D[2]	0.03
		TRT	1.14	1.40	1.13	1.02	1.11	1.23	0.12	T*t[5]	0.94
	Tot	CTR	1.07	1.06	1.12	1.05	1.04	1.03	0.08	T*t*D[6]	0.78
		TRT	1.06	1.20	1.05	1.02	1.07	1.09	0.08		
NOx	L	CTR	0.98	1.05	1.05	1.07	1.02	1.05	0.07	T[1]	0.73
		TRT	0.98	1.01	1.03	1.05	1.03	0.97	0.07	t[4]	0.97
	H	CTR	1.14	1.08	1.15	1.08	1.02	1.05	0.07	D[2]	0.03
		TRT	1.11	1.17	1.07	1.04	1.09	1.14	0.07	T*t[5]	0.94
	Tot	CTR	1.06	1.06	1.10	1.08	1.02	1.05	0.05	T*t*D[6]	0.79
		TRT	1.05	1.09	1.05	1.04	1.06	1.06	0.05		

[1]Treatment effect (CTR is control and TRT is treated).
[2]Dose (L is low, and H is high).
[3]Standard error.
[4]Time.
[5]Treatment x time interaction effect (+ is $P < 0.1$; * is $P < 0.05$; ** is $P < 0.01$).
[6]Treatment x time x dose interaction effect.

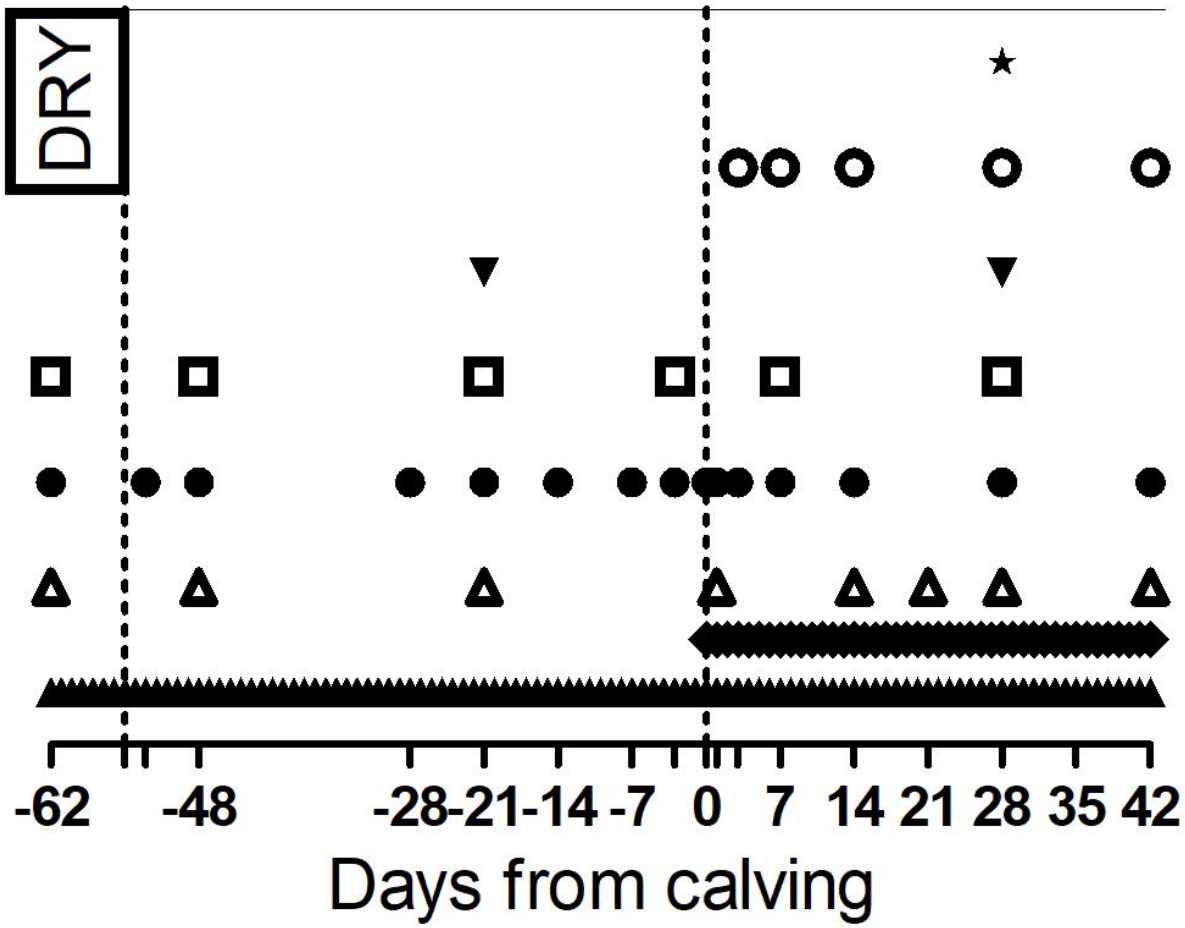


Figure 4. Scheduled time points, expressed as day from calving (DFC), for dry-off (DRY), treatment administration (-), rumen sample collection (★), milk sample collection (○), blood sample collection for the interferon gamma release assay (▼), carrageenan skin test performance and blood sample collection for white blood cells profile and whole blood stimulation assay (□), blood sample collection for metabolic profile (●), milk yield measurement (◆), body weight and body condition score determination (△), dry matter intake and rumination time determination (▲). Empty ticks indicate -55; -53; -3; 1 and 3 DFC respectively.

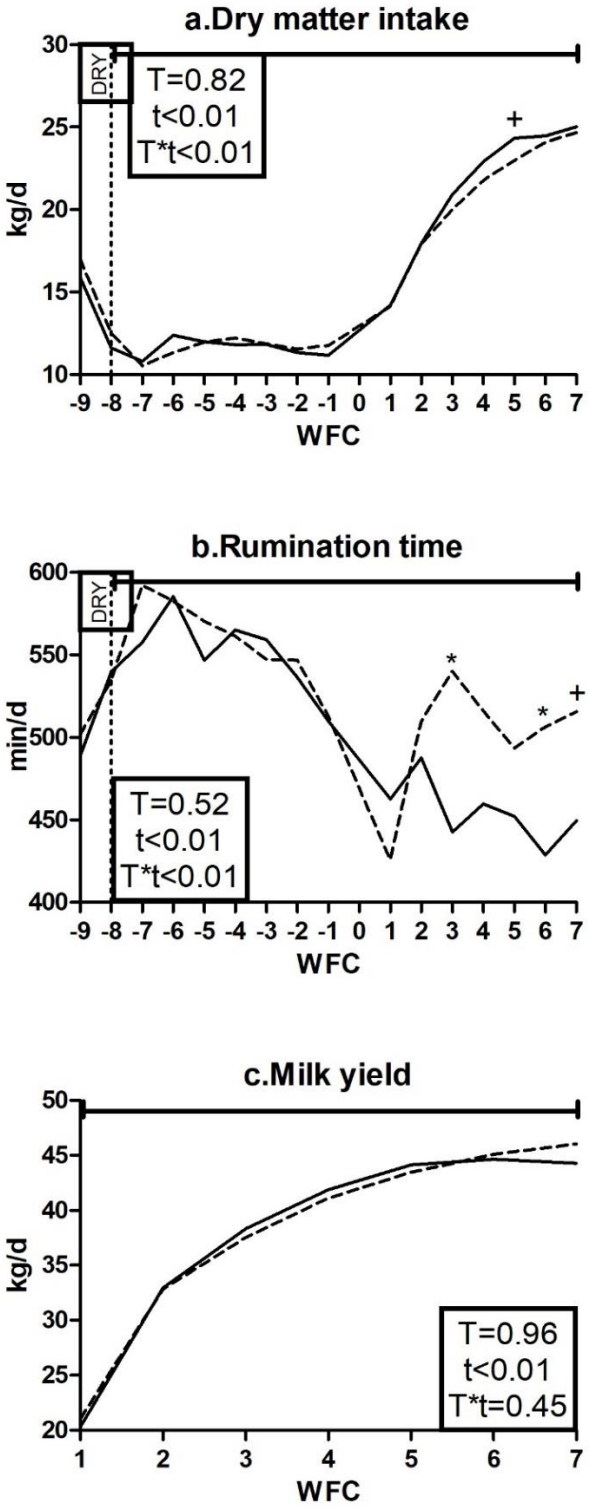


Figure 5. Average week values of dry matter intake (a), rumination time (b) and milk yield (c) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect (* is $P < 0.05$; † is $P < 0.1$); WFC is weeks from calving; DRY is dry-off day (-55 days from expected calving).

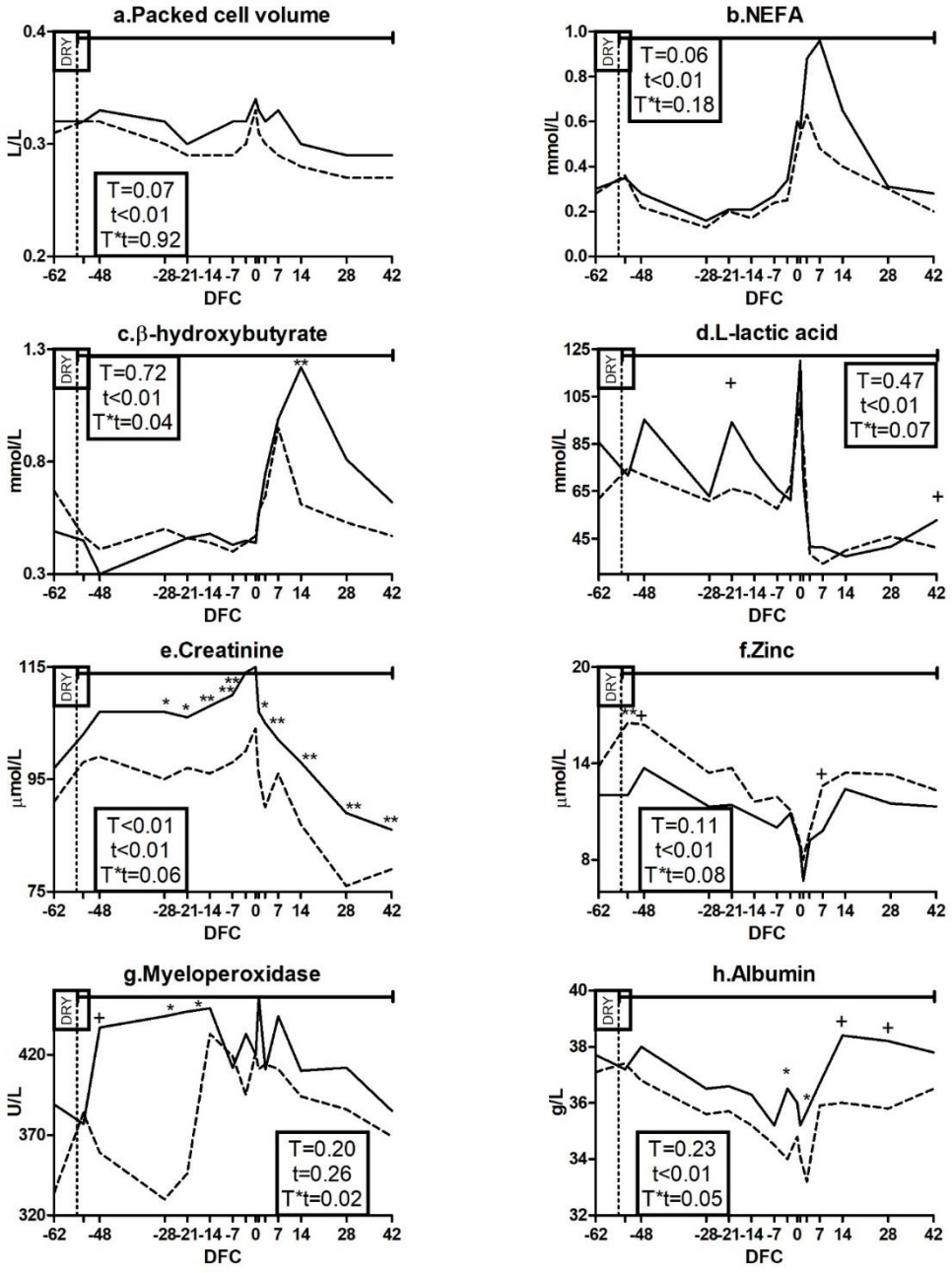


Figure 6.Time course of packed cell volume (a) and plasma concentrations of not esterified fatty acids (NEFA; b), β-hydroxybutyrate (c), L-Lactic acid (d), creatinine (e), zinc (f), myeloperoxidase (g) and albumin (h) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); DFC is days from calving; DRY is dry-off day (-55 days from expected calving).

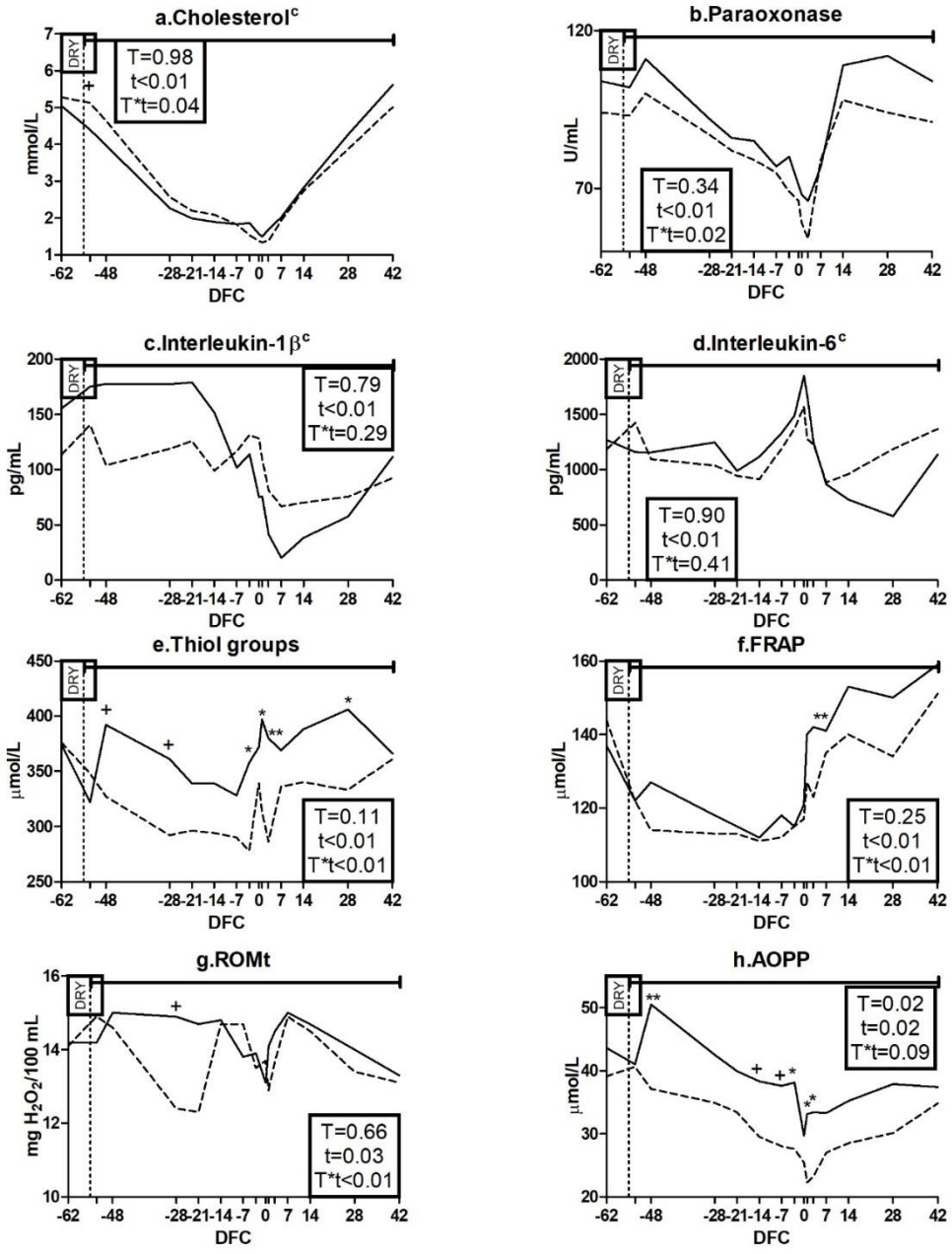


Figure 4.Time course of plasma concentrations of cholesterol (a), paraoxonase (b), interleukin 1-β (c), interleukin-6 (d), thiol groups (e), ferric reducing antioxidant power (FRAP; f), total reactive oxygen metabolites (ROMt; g) and advanced oxidation of proteins product (AOPP; h) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect (** is $P < 0.01$; * is $P < 0.05$; † is $P < 0.1$); DFC is days from calving; DRY is dry-off day (-55 days from expected calving); ^{c}indicate that parameter has been covariate on -62 value.

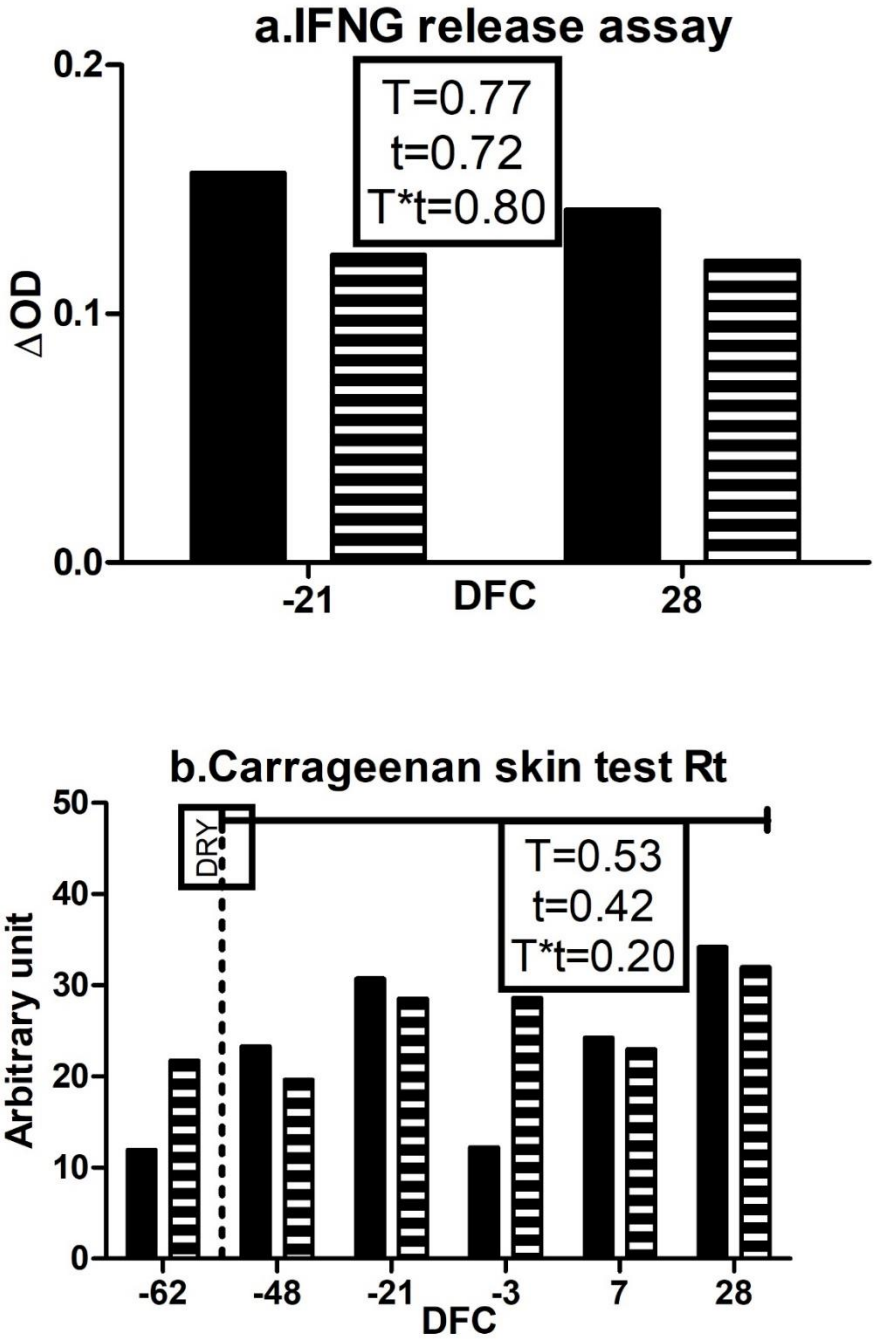


Figure 5. Optical density difference (ΔOD) between avian PPD-stimulated and control wells in the interferon gamma (IFNG) release assay (a) and total peripheral response (Rt) after the carrageenan skin test (b) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect; DFC is days from calving; DRY is dry-off day (-55 days from expected calving).

SUPPLEMENTARY FILE

Supplementary file 1. Trends of body weight (BW) and body condition score (BCS) in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 days from calving

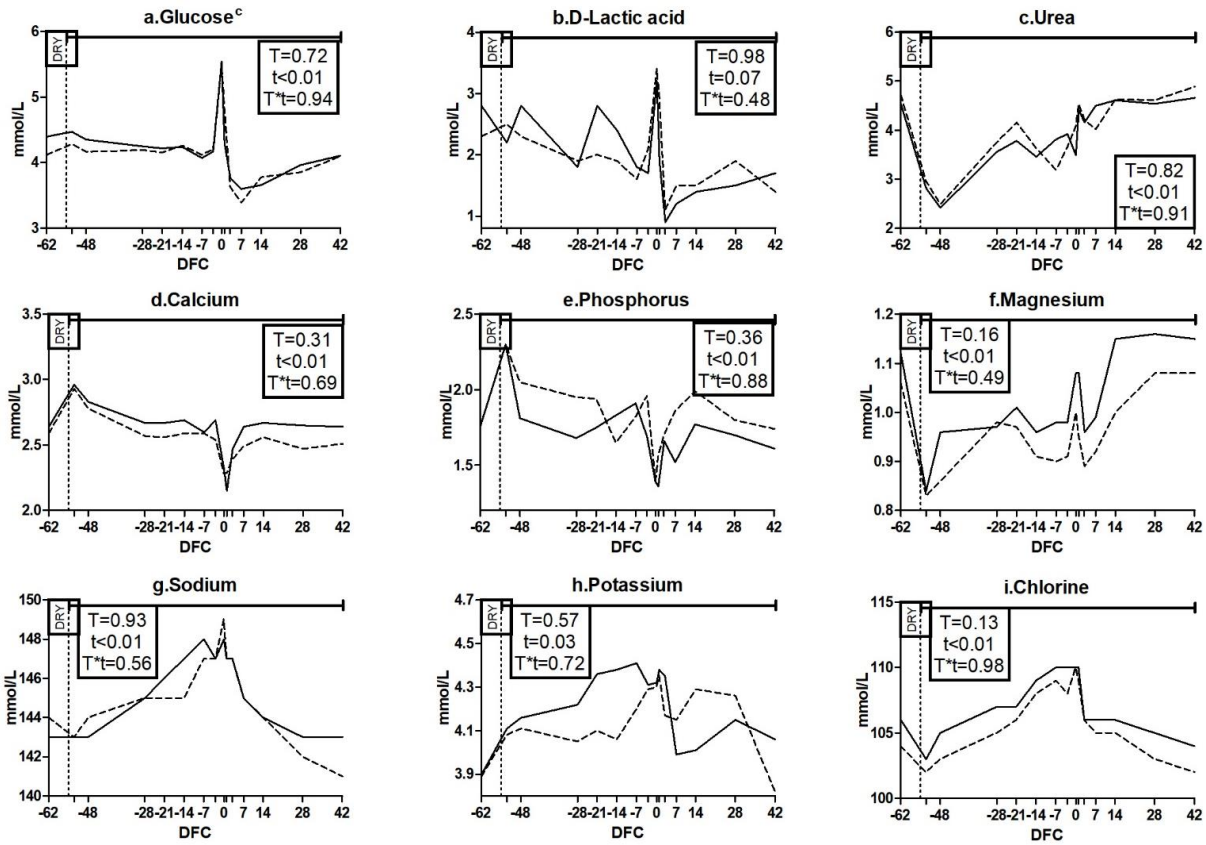
Item, Unit	T[1]	Days from calving								P-Value		
		-65	-45	-21	1	14	21	28	45	T[1]	t[3]	T*t[4]
BW, Kg	CTR	644.6	649.8	683.6	630.6	587.6	582.2	582.9	585.9	0.96	<0.01	0.99
	TRT	638.2	643.2	679.0	631.4	593.5	585.4	583.8	578.5			
BCS, -	CTR	2.55	2.54	2.56	2.51	2.28	2.20	2.19	2.15	0.54	<0.01	0.88
	TRT	2.41	2.43	2.45	2.44	2.26	2.13	2.13	2.04			
ΔBCS[2], -	CTR				0.32					0.94	-	-
	TRT				0.31							

[1]Treatment effect (CTR is control and TRT is treated).
[2]Difference between BCS value at calving and 28 DFC.
[3]Time.
[4]Treatment x time interaction effect.

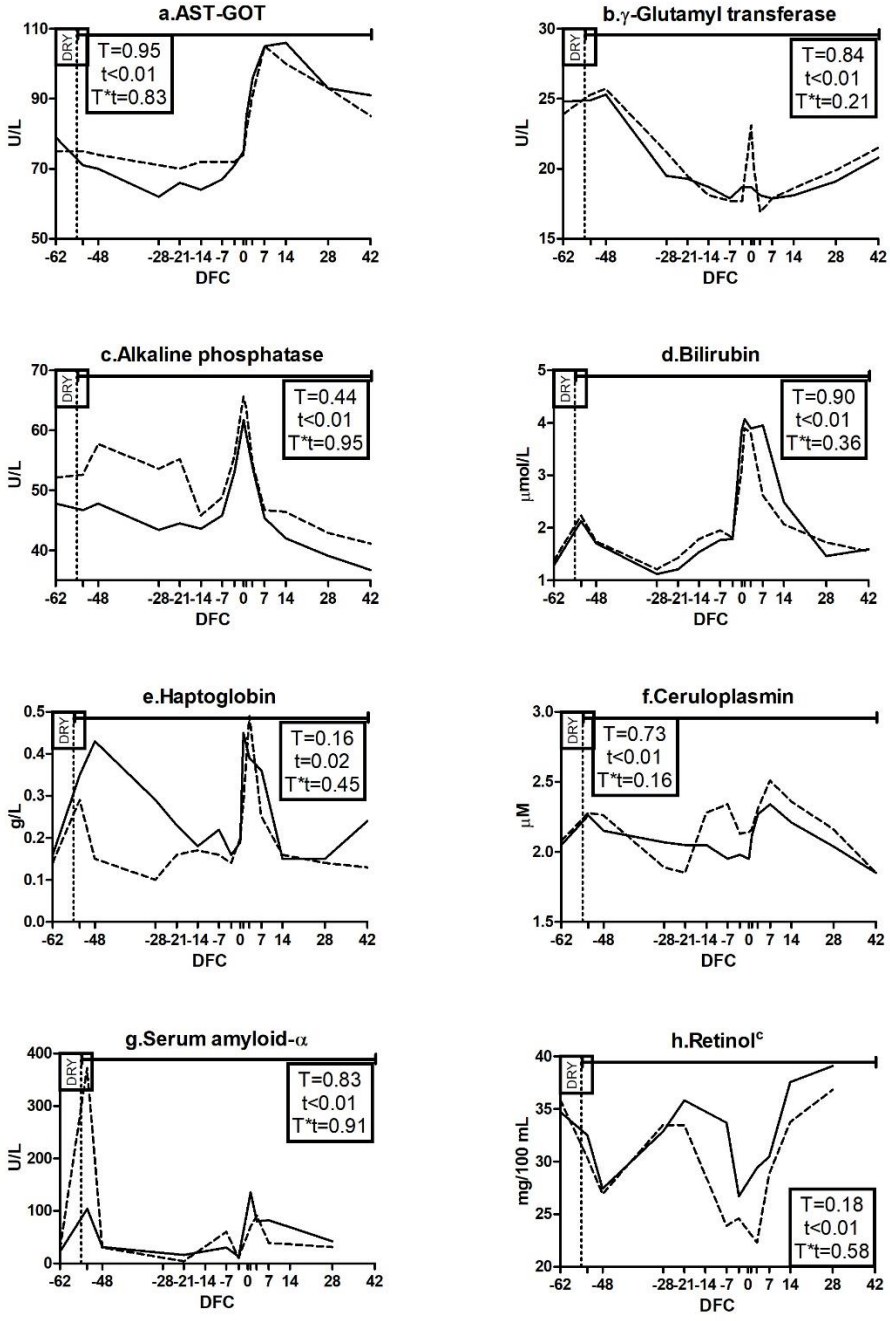
Supplementary file 2. Incidence of diseases observed between -62 and 42 days from calving (DFC) in control dairy cows or cows receiving 65 g*d^{-1} of Omnigen-AF® as top-dress between -55 and 42 DFC

T[1]	Healthy[2]	Problems occurrence, n								
		RP[3]	ME/ED[4]	MF[5]	DA[6]	MA[7]	PN[8]	DH[9]	KET[10]	Total
CTR (5 cows)	0	0	0	0	0	0	0	2	3	5
TRT (5 cows)	4	0	0	0	0	0	0	0	1	1
P	<0.01	-	-	-	-	-	-	0.11	0.2	<0.01

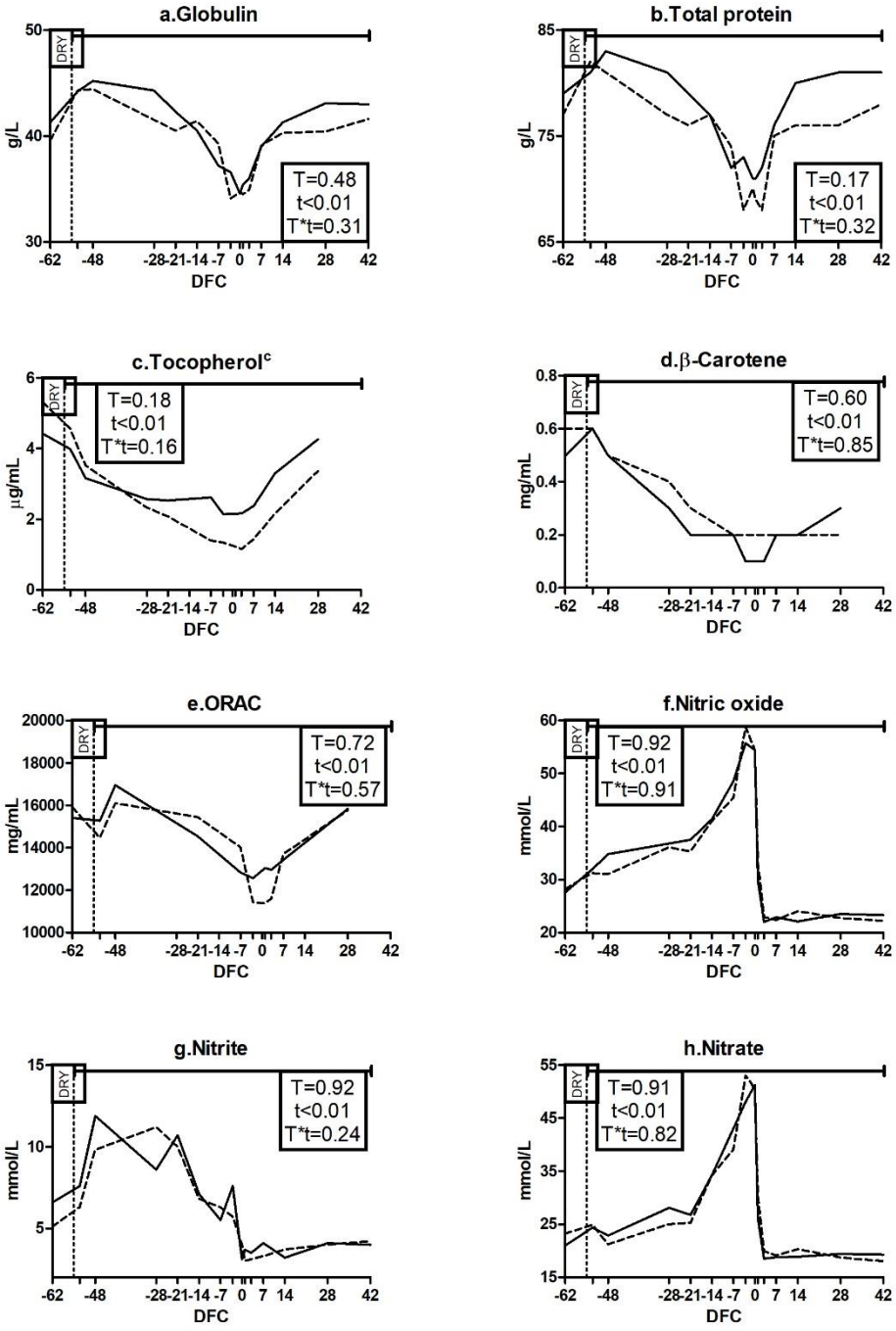
[1]Treatment (CTR is control and TRT is treated).
[2]Cows that did not have any clinical disease between -62 and 42 DFC.
[3]Retained placenta.
[4]Endometritis or metritis.
[5]Milk fever.
[6]Displacement of abomasum.
[7]Mastitis.
[8]Pneumonia.
[9]Diarrhea.
[10]Ketosis (blood BHB higher than 1.4 mmol*L^{-1}).



Supplementary file 3.Time course of plasma concentrations of glucose (a), D-lactic acid (b), urea (c), calcium (d), phosphorus (e), magnesium (f), sodium (g), potassium (h) and chlorine (i) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect; DFC is days from calving; DRY is dry-off day (-55 days from expected calving); [c]indicates that parameter has been covariate on -62 values.



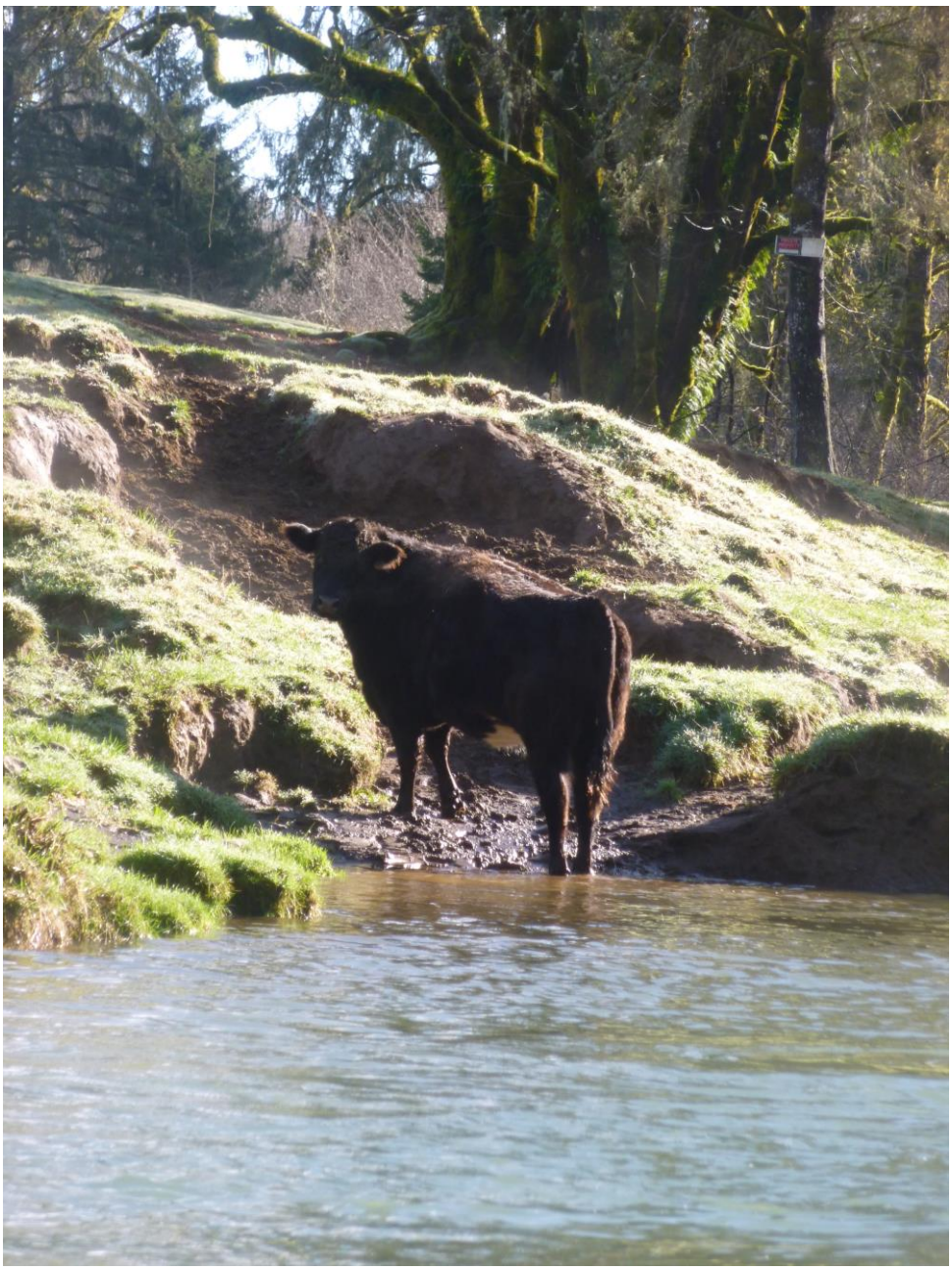
Supplementary file 4.Time course of plasma concentrations of aspartate aminotransferase – glutamate oxaloacetate transaminase (AST-GOT; a), γ-glutamyl transferase (b), alkaline phosphatase (c), bilirubin (d), haptoglobin (e), ceruloplasmin (f), serum amyloid-α (g) and retinol (h) in control dairy cows (CTR; solid line) or cows receiving 65 g*d^{-1} of Omnigen-AF as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect; DFC is days from calving; DRY is dry-off day (-55 days from expected calving); [c]indicates that parameter has been covariate on -62 values.



Supplementary file 5.Time course of plasma concentrations of globulin (a); total protein (b), tocopherol (c), β-carotene (d), oxygen reactive antioxidant capacity (ORAC; e), nitric oxide (f), nitrite (g) and nitrate (h) in control dairy cows (CTR; solid line) or *cows receiving* 65 g*d^{-1} of Omnigen-AF as top-dress (TRT; dotted line) between -55 and 42 days from calving. Upper solid line indicates timing of treatment administration; T is treatment effect; t is time effect; T*t is the treatment x time interaction effect; DFC is days from calving; DRY is dry-off day (-55 days from expected calving); [c]indicates that parameter has been covariate on -62 values.

CHAPTER V - FINAL REMARKS

General considerations on altered immune functions during peripartum of dairy cows



INTRODUCTION

In the last decades, immune dysfunctions have been reported in dairy cows during the phase named transition period (**TP**), which includes the three weeks prior to calving and the first three weeks of lactation (Kehrli et al., 1989; Drackley, 1999). Such alterations consist in two distinct phenomena: 1) a reduced competence of the innate immune system, triggering a hypo-responsive state in leukocytes, and 2) the occurrence of systemic inflammations, that activates leukocytes triggering the acute phase response. Despite opposite effects they have on leukocyte functions, those two phenomena suddenly appears together during TP of dairy cows (Bertoni and Trevisi, 2013). The risk ratio of both metabolic and infectious diseases in early lactation is strictly related to the degree of immune dysfunction. In turn, the occurrence of a disease could further impair leukocytes functions, increasing the likelihood of other diseases (Ingvartsen, 2006). This vicious circle increases drugs costs and could impair fertility of the animals, frequently resulting in their culling. A wide literature background has been developed trying to elucidate the causes of immune dysfunction, but the driving cause remain unclear. This work was aimed to investigate the changes that occurs on dairy cow's immunity during peripartum and their effect on metabolism, productive performances, and health status.

THE ROLE OF DRY-OFF ON IMMUNE DYSFUNCTIONS

Our data suggests that roots of immune dysfunctions could sink way before TP, and that dry-off could have a fundamental role in their development. In fact, dry-off has been revealed as a challenging phase in high-yielding cow's career, related to deep changes in feeding behavior, metabolism and immune parameters. A systemic inflammation occurred at dry-off in all the animals, impairing liver function and triggering oxidative stress conditions during the early dry period. Such an inflammation could probably arise from leukocytes contribution in the involution phase of mammary gland, as indicated from the decreased neutrophils and monocytes amounts in blood after dry-off, which reflects their migration in peripheral tissues, probably also in mammary gland (Putman et al., 2018). Animals with an average milk yield higher than 15 L d^{-1} in the last week prior to dry-off had the worst condition, and this could probably be related to the deeper metabolic changes they faced at dry-off time consequently to milking interruption, to the udder pain due to milk accumulation and to the greater amount of parenchymatic tissue to be reabsorbed. Metabolic stress at dry-off has already been related to the production of cortisol, that is known to impair immune competence when released in massive quantity (Bertulat et al., 2013). Sudden changes in feeds composition and rumen functions, as those occurring during the dry period, could affect the integrity of rumen epithelia, allowing the migration of bacterial components to internal tissues, as Minuti et al. (2014) had well demonstrated in the transition from dry to lactation phase.

IMMUNE FUNCTIONS AND KETOSIS

Our results on cows affected from sub-clinical ketosis (blood beta-hydroxybutyrate -**BHB**- higher that 1.2 mmol/L) in early lactation, revealed higher prepartal plasma concentrations of pro inflammatory cytokines (**PICs** – Interleukin-1, beta and Interleukin-6) and myeloperoxidase, an enzyme that is related to the respiratory burst activity of neutrophils (Faith et al., 2008), and higher concentrations of reactive oxygen metabolites. Furthermore, they had lower level of plasma minerals and higher creatinine, suggesting an impaired kidney functionality, and higher concentration of gamma-glutamyl transferase (**GGT**), suggesting a liver damage to occur. Leukocytes from the same cows had greater production of interferon gamma (**IFNG**) after stimulation with *Mycobacterium avium* in the same period, suggesting ketosis to be preceded from a metabolic preeclamptic-like status. Greater IFNG production may account for insulin resistance and increased plasma concentration of NEFA and BHB observed in KET cows. The anorexic power exerted by NEFA and BHB and the high circulating glucose levels reduced DMI around calving and, together with the energy requirement related to activation of immune system in dry period, worsen the negative energy balance (**NEB**) in early lactation. Such a condition induces severe milk yield losses and boosts the mobilization of lipid sources. The impaired liver function and the activation of the immune system during the dry period consequential to preeclampsia accrues the physiologic acute phase response after calving, further impairing oxidizing capacity in liver. Anti-inflammatory regulatory mechanisms, both at liver and leukocytes level (Biswas and Lopez-Collazo, 2009; Akbar et al., 2015), could account for the reduced production of PICs and increased production of anti-inflammatory mediators (such as lactate) in leukocytes of KET cows after a stimulation with lipopolysaccharides (**LPS**). Lower PICs detected before ketosis onset suggested the development of an endotoxin tolerance status during dry period. It consists in a transient unresponsive state against further challenges with endotoxins, leading

to the upregulation of anti-inflammatory genes in leukocytes (Biswas and Lopez-Collazo, 2009). During ketosis, leukocytes function can be further impaired by the combination of higher lipomobilization (Sordillo, 2016) and of a greater expression of anti-inflammatory genes at liver level to cope with inflammation and prevent fatty liver condition (Akbar et al., 2015).

OMNIGEN-AF AS A STRATEGY TO COPE WITH IMMUNE DYSFUNCTIONS

The administration of immune-stimulant products could be an effective strategy to minimize negative effects of immune dysfunctions in TP. Omnigen-AF (**OAF**) has been widely investigated in this respect (Nickerson et al.; Wang et al., 2004; Ortiz-Marty et al., 2013). A positive effect on leukocyte function and a consequent reduction of the incidence of infectious diseases in TP has been widely reported with OAF (Wang et al., 2007; Nace et al., 2014). Although a direct effect of OAF on neutrophils gene expression has been reported, our data suggests an indirect effect of the additive on immune function. In our experiment, the administration of 65 g/d of OAF from dry-off (55 days prior to calving) to 45 days after parturition did not affect neutrophils diapedesis at the carrageenan skin test and neither had any effect on the amelioration of liver metabolism and inflammation of dairy cows over calving. Nevertheless, it seemed to ameliorate the recovery of rumen functions after calving, improving the rumination time, the energy metabolism and reducing ketogenesis. Such an improvement could justify the greater efficiency of leukocytes to face biological stressors during the peripartum, as suggested from their lower lactate production and lower glucose consumption after an LPS challenge. Furthermore, an increased lymphocytes abundance at blood level also occurred with OAF. Despite these positive effects on immune cells, OAF seem to be ineffective in reducing the degree of inflammation over calving. A reduced abundance of negative acute phase proteins and antioxidants also occurred with OAF after calving, suggesting some impairment of hepatic functions to occur. Nevertheless, the lack of any effect on main biomarkers related to liver function (bilirubin) and liver damage (GGT, aspartate-amino transferase, alkaline phosphatase) dismisses a real impairment of liver activities to occur with OAF. This could suggest OAF to act as a weak antioxidant reducing body requirements for endogenous antioxidants synthesis.

GENERAL CONCLUSION

In conclusion, our data allow us to state that: 1) dry-off could affect metabolism and trigger an acute phase response, especially when milk yield was higher than 15 L/d in the week prior to milking interruption 2) an activation of immune system during dry period could trigger an endotoxin tolerance status, that could contribute in decreasing immune cells functions around calving 3) altered immune functions before calving, and IFNG production in particular, could boost the development of sub clinical ketosis in early lactation 4) administration of OAF could be effective in reducing rumen alteration after stressing events, as dry-off and calving, and could act as a weak antioxidant.

Nevertheless, our data does not allow us to 1) clearly identify the role of acute phase response occurred at dry-off in triggering endotoxin tolerance prior to ketosis onset 2) fully elucidate the driving mechanism of the preeclamptic-like status occurred in our cows prior to ketosis, 3) fully understand the real mode of action of OAF on rumen fermentations.

A wider study considering the whole peripartal period should be performed to clearly elucidate the effect of milk yield and other stressing events occurring at dry-off on the onset of diseases in early lactation. Furthermore, a wider experimental group should be enrolled to clearly identify the role of preeclamptic-like status in the onset of such diseases (immune dysregulation and ketosis in particular). Finally, an experimental design involving multiple rumen samples during the administration of OAF should be adopted to test its effects on rumen fermentation and metabolism. The adoption of specific tools aimed to elucidate the interactions of the product with microbiota and immune cells at the mucosal level, and their relationship with the acute phase response at metabolic level, should be essential to elucidate the exact mode of action of the molecule on immune cells during the whole peripartum period.

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APPENDIX

Appendix 1. Abbreviations used throughout this thesis and their definitions

ABBREVIATION	DEFINITION	ABBREVIATION	DEFINITION
ΔBCS	Body condition score difference	MD*t*D	Milk yield at dry-off x time x dose interaction effect
ΔOD	Optical density difference	MDA5	Melanoma Differentiation-Associated protein 5
a30	Curd firmness at 30 min	MHC	Major histocompatibility complex
AIC	Anti-inflammatory cytokines	MPO	Myeloperoxidase
ALP	Alkaline phosphatase	mRNA	Messengers-RNA
AOPP	Advanced oxidation protein products	NADPH	Nicotinamide adenine dinucleotide-phosphate
APP	Acute phase proteins	NEB	Negative energy balance
AST-GOT	Aspartate amino transferase-glutamate oxaloacetate transaminase	NEFA	Non-esterified fatty acids
BCS	Body condition score	NEL	Net energy for lactation
BHB	Beta-hydroxybutyrate	NFC	Not fiber carbohydrates
BIRC1	Inhibitor of apoptosis domain-containing protein 1	NF-κB	Nuclear factor-κB
cAMP-BP	Cyclic adenosine monophosphate response element binding protein	NLR	Nucleotide binding oligomerization domain-like receptors
CD	Cluster of differentiation	NO2	Nitrite
CIITA	Class II major histocompatibility complex transactivator	NO3	Nitrate
CLA	Conjugated linoleic acid	NOx	Nitric oxides
CLR	C-type lectin receptors	OAF	Omnigen-AF
COX	Cyclooxygenases	ORAC	Oxygen reactive antioxidant capacity
CST	Carrageenan skin test	PAMPs	Pathogen associated molecular patterns
D	Dose effect	PCV	Packed cell volume
DAMPs	Damage associated molecular patterns	PEB	Positive energy balance
DCs	Dendritic cells	peNDF	Physical effective-neutral detergent fiber

DFC	Days from calving	PG	Prostaglandin
DFD	Days from dry-off	PI	Physiological imbalance
DHA	Docosahexaenoic acid	PIC	Pro-inflammatory cytokines
DLA	D-lactic acid	PMN	Polymorphonuclear cells
DM	Dry matter	PON	Paraoxonase
DMI	Dry matter intake	PPARA	Peroxisome proliferation activated factor, alpha
EPA	Eicosapentaenoic acid	PPARD	Peroxisome proliferation activated factor, delta
FGF21	Fibroblast growth factor 21	PPARG	Peroxisome proliferation activated factor, gamma
GGT	Gamma-glutamyl transferase	PPD	Purified protein derivative
GH	Growth hormone	PRR	Pattern of recognition receptor
H	High dose	PUFA	Poly-unsaturated fatty acids
HLA	Human leukocytes antigen	r	Rennet clotting time
HM	High milk yield at dry-off	RIG	Retinoic acid-inducible gene
Hs	Health status effect	RLR	RIG-like receptors
Hs*t	Health status x time interaction effect	ROM	Reactive oxygen metabolites
Hs*t*D	Health status x time x dose interaction effect	RP	Retained placenta
IFNA	Interferon, alpha	SAA	Serum amyloid, alpha
IFNG	Interferon, gamma	SCC	Somatic cells count
IGF-1	Insulin-like growth factor-1	SELL	Selectin, L
Igs	Immunoglobulins	SFA	Saturated fatty acids
IL-10	Interleukin-10	SHp	Thiol groups
IL-12	Interleukin-12	t	Time effect
IL-17	Interleukin-17	T	Treatment effect
IL-1B	Interleukin-1, beta	T*t	Treatment x time interaction effect
IL-2	Interleukin-2	T*t*D	Treatment x time x dose interaction effect
IL-4	Interleukin-4	Tc	T-cytotoxic cells
IL-8	Interleukin-8	Th	T-helper cells
L	Low dose	TLR	Toll-like receptor
LDA	Left displacement of abomasum	TNFA	Tumor necrosis factor, alpha
LLA	L-lactic acid	TP	Transition period

LM	Low milk yield at dry-off	Treg	T-regulatory cells
LOX	Lipoxygenases	TX	Thromboxane
LPS	Lipopolysaccharides	UFA	Unsaturated fatty acids
LT	Leukotrienes	VFA	Volatile fatty acids
LX	Lipoxins	VLDL	Very low-density lipoproteins
MD	Milk yield at dry-off effect	WBA	Whole blood stimulation assay
MD*t	Milk yield at dry-off x time interaction effect	WBC	White blood cells

Appendix 2. Publications edited by the thesis author

DATE	**JOURNAL/CONFERENCE**, publication
January 3rd, 2019	**Gene X** - Busato S., Mezzetti M., Logan P., Aguilera N. and Bionaz M. 2018. "What's the norm in normalization? A frightening note on the use of RT-qPCR in the livestock science". Gene X. 1:100003. doi:10.1016/j.gene.2018.100003
June 24th, 2018 – June 27th, 2018	**Annual Meeting of the American Dairy Science Association Knoxville, Tennessee, USA.** - Mezzetti M., Jaaf S., Busato S., Premi M., Trevisi E., Bobe G., and Bionaz M. 2018. "Calves born from cows fed with alfalfa enriched with selenium have higher Se in blood and higher phagocytosis". 2018 ADSA Annual Meeting Abstract#154 - Jaaf S., Mezzetti M., Krueger A., Batty B., Belveal J., Premi M., Foster J., Trevisi E., Bobe G., Estill C. and Bionaz M. 2018. "Effect of selenium-enriched hay on Se concentration in blood and milk, immune function, and performance in dairy cows during the transition period". 2018 ADSA Annual Meeting Abstract#268 - Busato S., Aguilera N., Mezzetti M., and Bionaz M. 2018. "What's the norm in normalization? A note on the use of RTqPCR in livestock-related studies". 2018 ADSA Annual Meeting Abstract#429
June 22nd, 2018 – June 24th, 2018	**Annual meeting of the Italian Association of Veterinary Sciences (SISVet). Torino, Italy** - Minuti A., Amadori M., Mezzetti M., Lovotti G., Piccioli-Cappelli F. and Trevisi E. 2018. "The interferon gamma response to Mycobacterium avium in vitro can be correlated with a higher risk of clinical ketosis in dairy cows". 2018 SISVet Annual Meeting Abstract
June 28th, 2017 – July 1st, 2017	**Annual meeting of the Italian Association of Veterinary Sciences (SISVet). Naples, Italy** - Trevisi E., Minuti A., Mezzetti M., Soares J.F.F., Riva F., Bani P. and Amadori M. 2017. "Monensin affects the innate immune response in the rumen". 2017 SISVet Annual Meeting Abstract
June 13th, 2017	**Annual meeting of the Association for Science and Animal Productions (ASPA). Perugia, Italy** - Trevisi E., Minuti A., Mezzetti M., Ferrari A., and Piccioli-Cappelli F. 2017. "Supplements of Aloe arborescens improve health and inflammo-metabolic status of transition dairy cows". 2017 ASPA Annual Meeting Abstract - Piccioli-Cappelli F., Maiocchi M., Minuti A., Mezzetti M., and Trevisi E. 2017. "Effect of rumen-protected methionine and choline supplementation during dry

	period on dairy goats metabolic and inflammatory profile". 2017 ASPA Annual Meeting Abstract
	- Trevisi E., Piccioli-Cappelli F., Mezzetti M., and Minuti A. 2017. "Relationship between Liver Functionality Index and fertility in dairy cows". 2017 ASPA Annual Meeting Abstract
April, 2017	**Bianco e Nero**
	- Trevisi E., Mezzetti M. 2017. "Ridurre i rischi nel periparto migliorando l'immunità". Bianco e Nero, 68(2):78-82
2015	**Italian Journal of Animal Science 14(s1)**
	- Trevisi E., Piccioli-Cappelli F., Mezzetti M., Lovotti G. and Bani P. 2015. "Effect of the ruminal slow-release of monensin during the transition period of dairy cows on health status, energy metabolism and inflammatory conditions". Italian Journal of Animal Science 14(s1), 73–74