UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXIV

S.S.D: AGR/19

TRANSITION PERIOD OF DAIRY COWS AND INFLAMMATION: A NOVEL INDEX TO ASSESS THE INDIVIDUAL RESPONSE, PRE-CALVING TREATMENTS AIMING TO MITIGATE IT AND CONSEQUENCES ON PRODUCTIVE AND REPRODUCTIVE PERFORMANCES

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Academic Year 2010/2011

RINGRAZIAMENTI

Dopo 10 anni di Università Cattolica a Piacenza, forse vicino a perdere lo status di studente, desidero ringraziare tutti coloro che mi hanno aiutato nel superare anche questi tre anni di dottorato. Ad iniziare dal sostegno immateriale e materiale della mia famiglia.

Grazie poi ai prof. Bertoni e Trevisi, che mi hanno aiutato e insegnato molto in questi anni, spero di ricambiarli in futuro.

Un doveroso ringraziamento va anche a tutti gli altri membri dell'IZ, in particolare alla quotidiana compagnia della "stanza dei computer"...

Infine un saluto a tutti i compagni di avventura che, sulla famosa stessa barca in questi giorni giungono all'approdo finale.

Infine (ma *dulcis in fundo*) ringrazio, in modo particolare, chi ogni giorno ci dà la forza di affrontare la vita sempre in maniera positiva. Dante chiudeva la sua Commedia con «*L'amor che move il sole e l'altre stelle*», e Benedetto XVI nella sua enciclica *Deus Caritas Est* ci ricorda che questo Amore è Dio stesso. Arrigo Boito, nel Simon Boccanegra musicato da Giuseppe Verdi, fa urlare alla massima potenza al protagonista, esasperato dalle lotte interne genovesi, pace e amore! E allora nella prossima pagina, visto che nel mondo d'oggi ce n'è molto bisogno, urliamo anche noi con lui…

«E vo gridando: pace!

E vo gridando: amor!»

Simon Boccanegra
Atto I, scena XII
Musica: Giuseppe Verdi
Libretto: Arrigo Boito
1881

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PERIPARTUM OF DAIRY COWS

During the last decades genetic selection allowed a huge increase in milk production of dairy cows in a short period of time, especially for the widespread and most productive Holstein breed. It is reported that such a genetic improvement came only partially with a consistent and quick adaptation of all the other factors involved in dairy cows rearing. The "new" high-production dairy cow has to face new issues, unknown to the "old" animals. Feeding and management have to be carefully taken into account to provide a good response to requirements raised by genetic improvements. Research provided in the course of time new techniques to cover the increased requirements, and new structures were framed to better house and take care of animals. The adaptation of the cow to the higher requirements may be rather simple in some life stages characterized by a not so high production and by a constant metabolism (e.g. established lactation). However, around calving the cow is facing a challenge due to sudden and strong modifications in her metabolism and life in general. This is the so-called transition period of dairy cow (or *peripartum*), defined by Grummer (1995) as the period of time from 3 weeks before calving through the first 3 weeks after calving. Most of the troubles which may occur in the cow's life usually concentrates in this short period (Trevisi, 2010). However, this is not only a temporary problem, but it may also negatively affect the whole following lactation causing an increased risk of diseases, a lower milk yield and resulting in an increase of costs. For these reasons it is important to focus the efforts on this physiological stage to find suitable solution to reduce the count of occurring problems and their severity. Feeding management has major importance in this challenge, since an unbalanced diet may worsen ongoing problems or rise new. Furthermore, it is clear that an incorrect feeding management during peripartum may negatively affect the negative energy balance, resulting in a worsening of the reproductive performances (Bertoni *et al.*, 2009; Butler *et al.*, 1981; Wathes *et al.*, 2007) and consequently adding costs for farmers.

It is not an easy task to find the causes of the problems occurring in the peripartum period since it is well known that many of them have a multi-factor etiology. One of these may be an insufficient response to stress. In this phase there are several possible stress sources for the cow, such as the calving itself, group changing, not proper availability of feed or water, not suitable housing. The prevention of any stress is an essential prerequisite to the animal welfare in general, but in particular in this phase (Calamari, 2003). It is important to point up that the consequences to stress factors not always are clinical illnesses, but many times they take subclinical forms implying subtle consequences that anyhow may cause losses in cow's health and/or milk yield. An important stress is due to the physiological changings occurring in the organism during dry period. After dry off, cow enters in a phase characterized by no requirements for milk production but with an increasing importance of the foetus. In late pregnancy its size can be great enough to influence cow's metabolism, in particular for energy requirements. Calving itself implies a number of endocrine variations, often coming with other changes in cow's life (e.g. group and diet change, different housing). Later, with the onset of lactation, requirements (especially energy and protein) dramatically increase, forcing the cow to draw energy from body reserves (Drackley, 1999; Overton, 2001). The mechanisms that control the physiological status switch from homeostasis to homeoresis (Bauman, 2000), implying a number of important endocrine variations (mainly involving GH and insulin) that make more nutrients available in the mammary gland than in other tissues.

MAIN METABOLIC AND RELATED EVENTS OCCURRING IN TRANSITION PERIOD OF DAIRY COWS

1. **Inadequate feed intake** at the end of pregnancy and onset of lactation. During the dry period energy and protein requirements lower, as there are no needs by the udder for milk production. In this condition, as in late lactation, the cow is able to gather some energy reserves in adipose tissues. However, also the daily intake is lower than in lactation, causing a small reduction in the size of rumen,

and particularly in papillae number and size (Goff and Horst, 1997). Approaching calving the DMI undergoes a further reduction caused by several possible factors mainly linked to physiological changes far to be clarified. This allows the possibility that a negative energy balance may occur already before calving. For instance, the reduction of feed intake at the end of pregnancy may be explained by the presence of clinical or subclinical diseases (Trevisi *et al.*, 2002). Immediately after calving requirements dramatically increase, and the cow attempts to match it with reserves mobilization and by increasing the feed intake. However the small size of rumen doesn't allow the intake of large amounts of feed and the reduced number/size of papillae cannot quickly soak up the increased amount of volatile fatty acids (Goff and Horst, 1997).

Furthermore, glucose undergoes a major requirement immediately after calving mainly due to the production of lactose in the mammary gland. The tremendous gap between glucose requirement and glucose availability in early lactating cows was estimated by Drackley *et al.* (2001) as 500 g/d. The organism tries to fill the gap by increasing the gluconeogenesis, using as substrate mainly amino acids and glycerol from body reserves. This is a different condition in respect to the cow afar from calving with a correct feed intake, when the main energy contributor is the propionate from rumen and glycerol is limited to a marginal amount.

Further factors affecting the lowering of DMI in the peripartum are clinical or subclinical health problems.. The mechanism that exerts this effect is partly due to the release of cytokines characterized by anorectic effect (e.g. Tumor Necrosis Factor-α, Interleukin-1, Interleukin-6) which occurs during the inflammatory response. There is just a few bibliography dealing with cytokines in dairy cows: a recent paper from (Trevisi et al., 2011) describes the IL-6 response to metabolic stress in periparturient dairy cows. However, the action of some cytokines in lowering appetite and consequently the feed intake is a well-known mechanism (Grimble, 1990; Johnson, 1998; Kelley *et al.*, 2003). The consequence of a not adequate feed intake is a marked mobilization of body reserves and so likely the impossibility to achieve the maximum productive potential.

2. **Mobilization of body fat reserves** to support lactation. This phenomenon is due to the marked energy deficit in postpartum and it is promoted by the growth hormone (GH) plasma level increase and insulin decrease. The mobilized non-esterified fatty acids (NEFA) may be used by peripheral tissues, in particular they are processed in liver where they can be esterified in triacylglycerols and excreted in blood within very low density lipoprotein (VLDL). NEFA are usually oxidized in mitochondria to get energy, but Drackley (2001) argues that an alternative pathway, in peroxisomes, is activated when NEFA are present in large amount. When the apolipoproteins production is impaired in the liver, the triglycerides (TG) excretion is reduced, thus they are gathered in the hepatocytes, causing liver steatosis (Drackley, 1999). This pathology has important implications on the liver functionality and on the overall health status. In order to meet the increased mobilization requirement after calving, a proper body condition score is desirable. Indeed, several authors report that an excessively fatty cow may be more easily affected by problems at calving time or later (Bernabucci *et al.*, 2005; Pryce *et al.*, 2001; Reid *et al.*, 1986).
3. **Oxidative stress.** According to Sies (1997), an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage, is termed "oxidative stress". A number of studies shows as a variety of reactive oxygen species (ROS) are produced by normal metabolic processes and by certain leukocyte populations during defense against disease. However, less is known about how oxidative stress can affect health status and well-being, particularly during times of high metabolic activity. In these conditions the considerable increase in oxygen requirements results in a raise of ROS production. In the periparturient dairy cow an imbalance between increased production of ROS and the availability of antioxidant defenses needed to reduce ROS accumulation may expose cows to increased oxidative stress. Early studies suggested a role of oxidative stress in the etiologies of dairy cattle disorders since supplementation with certain antioxidants could ameliorate the severity of a variety of metabolic and infectious diseases (Miller *et al.*, 1993). There are now several recent studies

to support the concept that oxidative stress is an important factor in the enhancement of the immune and inflammatory responses through an increased susceptibility of dairy cattle to a variety of health disorders, particularly during the transition period (Allison and Laven, 2000; Bernabucci *et al.*, 2005; Castillo *et al.*, 2005; Sordillo, 2005; Wilde, 2006).

4. **Immunosuppression status**. The immune response is the organism's reaction to the presence and multiplication of pathogens or to antigenic substances. Two kinds of response are observed: innate and acquired. The first one is composed by a number of non-specific defense mechanisms, such as physic-chemical walls (e.g. skin), phagocytic cells (e.g. macrophages, neutrophils), plasma proteins (e.g. complement and mediators of inflammation), soluble factors (substances exerting actions on other cells, e.g. some cytokines). Innate response is characterized by a quick action (between 0 and 96 hours from stimulus), high efficiency without giving any immunologic memory. The acquired response takes a specific action against different stimuli. Pathogens are recognized and specific cells (e.g. T lymphocytes) are recruited in order to fight them. First response time is delayed with respect to the innate response than the innate system, but once a pathogen is recognized, the immune memory is acquired and can also be everlasting. Several works report as immune system is partly impaired during the peripartum of dairy cow. Goff *et al.* (1997) argue that changes in plasma hormones levels (mainly progesterone) may alter the function of leukocytes. Such an effect take an action just the day before and after calving, when progesterone is markedly reduced. Conversely, Kehrli and collaborators (Kehrli *et al.*, 1989; Kehrli *et al.*, 1989; Kehrli *et al.*, 1999) stated that the decrease in immune function would begin already before calving and lasting through 2-3 weeks after it, and showed the reduction in the activity of neutrophils and macrophage. This loss of function may derive by the gene expression reduction of about 30 genes (Burton *et al.*, 2001) partly due to the increase in steroidal hormones and partly to stress phenomena. Stabel *et al.* (2003) describe the possibility of a connection with other factors (e.g. feeding, negative energy

balance) and point up that the reduced immunity negatively affects the cow's capability to resist to new infections often occurring in this period.

5. **Inflammation**. It is generally assumed that the decreased efficiency of the immune system in late pregnancy is one of the major causes that make cows more susceptible to infections and perhaps metabolic diseases in this stage (Drackley *et al.*, 2001; Goff, 2006). However, an inflammatory status – a specific defense mechanism associated to the innate immune system - often occurs in periparturient cows even without any clinical symptom (Bertoni *et al.*, 2008; Cappa *et al.*, 1989; Petersen *et al.*, 2004; Sordillo *et al.*, 2009). Several factors may contribute to trigger the inflammatory response around calving in a clinical or subclinical form: infectious or metabolic diseases, parasites, trauma, endotoxins from the gut. The common factor of these causes is likely represented by the pro-inflammatory cytokines release (Grimble, 1990), whose main effects concern liver synthesis, nutrient partitioning, anorexia, and reproductive activity through the triggering of the acute phase response (Moshage, 1997). The amount and the release time of cytokines are the main factors affecting the severity of the triggered effects. One of the major of them is the stimulation of the acute phase response (Elsasser *et al.*, 2000; Fleck, 1989), which in liver is characterized by the increased production of some proteins (positive acute phase proteins, +APP) and the impairment in the production of some other proteins (negative acute phase proteins, -APP). The determination in plasma of +APP (e. g. haptoglobin, ceruloplasmin) may be very helpful to detect the presence of an inflammatory status, also in case of a subclinical form. The assessment of –APP (e. g. albumins, retinol binding protein, apolipoproteins) can give some information on the response of the organism to the ongoing inflammation.

   Pro-inflammatory cytokines exert an important effect in the nutrients channeling, e.g. promotion of heat production (fever) and worsening the anorexic status of cows at calving (Johnson *et al.*, 2001; Kelley *et al.*, 2003). The combination of all these effects comes out in a worsening of the (likely already negative) energy balance (Pryce *et al.*, 2001; Trevisi *et al.*, 2009) and in the

increased risk of liver lipidosis (Bertoni *et al.*, 2006; Katoh, 2002), which may also be causes of reduced reproductive efficiency (Bertoni *et al.*, 2008; Butler, 2000).

All these metabolic events may have a role in worsening existing problems in the animal's health. A situation of clinical or subclinical illness is characterized by the release of pro-inflammatory cytokines (IL-1, IL-6, TNFα; Feghali *et al.*, 1997), protein mediators causing anorexia and consequently a lower availability of nutrients for the mammary gland, together with higher requirements due to the need of tissues to counteract the illness. In this condition of "metabolic stress" the energy provided by reserves and intake often is not sufficient to cover all the metabolic expenses, resulting in a lower milk production and in a weaker ability to counteract illnesses. A so critical framework describes as it is as crucial as difficult to avoid any problem during the peripartum, which may also negatively affect the whole lactation and even the cow's career.

DISEASES AND HEALTH DISORDERS IN PERIPARTURIENT DAIRY COWS

The occurrence of health problems (mainly caused by inadequate feeding, infections and trauma) increases in the peripartum period of dairy cows, favored by the sudden and major endocrine and metabolic changings taking place in the cow's organism in this phase. This condition needs special cares, but a late intervention is not the only reason which can worsen the situation. At this moment, an intervention is almost impossible for those cows which do not show any evident problem, but may suffer a subclinical disease status.

Liver is a major organ in processing energy for the body. Fat reserves are mobilized as NEFA whenever energy requirements are increased. Plasma NEFA are mainly uptaken by liver (Reynolds *et al.*, 2003), where they are oxidized or esterified in triglycerides and consequently excreted in the blood flow within lipoproteins. An insufficient lipoprotein release on the first day of lactation cause the possible onset of severe (triglycerides > 20 % of liver dry matter) or mild lipidosis (triglycerides between 10 and 20 %) in 50-60% of cows (Grummer, 1993). However, the liver is

also an important organ in protein production, either in normal conditions and after inflammatory events that trigger the acute phase response. If the presence of inflammation is added to the normal conditions, it is known that the liver undergoes an extra effort in the attempt to satisfy all the needs resulting in an increase in the release of some proteins involved in the acute phase response (positive acute phase proteins, +APP) and in the decreased release of some proteins synthesized in normal conditions (negative acute phase proteins, -APP; Gruys *et al.*, 2005).

In dairy cow, liver functionality is not usually impaired during the dry period and, accordingly, the usual protein systems are not altered. On the contrary, after calving, the risk of ketosis and/or liver steatosis increases. Indeed, Herdt (1988) reports as a steatosis condition may arise in a few hours already immediately before calving; this is certainly caused by the liver functionality impairment and it is often connected to a lower DMI and to an important plasma NEFA increase after lipomobilization (Grummer, 1993). Furthermore, the partial inability of the target tissues to completely oxidize NEFA contributes to prevent the drop of their plasma level. Most of NEFA have to be esterified in triglycerides and excreted in the blood within lipoproteins, but it has been reported the reduced ability of periparturient cows to produce apolipoproteins (Bertoni *et al.*, 1983; 1984; Calamari *et al.*, 1989) and consequently to excrete fats from liver, resulting in an increased risk of steatosis. Drackley *et al.* (1999; 2001) stated as a major importance in this process is due to the liver impairment to oxidize NEFA in peroxisomes. A decrease in the production of apolipoproteins is usual in bovines, but Reid *et al.* (1986) interestingly argue that such a decrease comes with an higher frequency of diseases and a plasma increase of +APP (Bertoni *et al.*, 2006; Bradford *et al.*, 2009). Thus, it is probable the connection between diseases, cytokines release, acute phase response and deviation of protein synthesis in liver.

Among the tremendous changes in the cow metabolism at calving, the onset of lactation causes a massive and quick increase in Ca requirement (Horst *et al.*, 1997), occurring when DMI is depressed. If the mobilization from bones is not sufficient as well, the cow may be affected by milk fever, an impairment of muscular motility

due to lack of Ca necessary for the normal muscular motility. The onset of such a problem may be harmful for the animal if not quickly solved, even if it arises in a subclinical form.

Furthermore, in early lactation, the cow is usually subjected to a sudden change in diet composition: the need to cover requirements for milk production makes the diet richer in more fermentable starch. Moreover, the rumen decreased, during the dry period, part of its capability to absorb volatile fatty acids (VFA). The increased production of VFA and their decreased absorption cause their accumulation in rumen with a consequent mild lowering of pH (ruminal acidosis). After this, rumen microbial profile changes and the new predominant microorganisms cause an increase in the production of lactic acid, causing a further pH drop. The critical pH threshold for acute acidosis is usually assumed to be <5.0, while for sub-acute acidosis is <5.5 (Nocek, 1997). As a consequence of the altered bacterial flora, endotoxins can be released and in some conditions moved into the blood. Their accumulation in some target tissues may cause further problems and/or clinical diseases, i.e. in the hoof, where the effects triggered by the accumulation of endotoxins may determine areas of tissue necrosis, and consequently lamenesses (Nocek, 1997). However, in most of cases the acidosis is subclinical, but it may in any case determine some important consequences on the cow's health, such as lack of appetite.

Ruminal acidosis may be a contributory cause in the development of a severe post-calving pathology, the displacement of abomasum (DA). Other risk factors that may contribute to manifest this pathology are ketosis, retained placenta, metritis and hypocalcaemia. DA shows when the abomasum, outstretched for the high content in gas and/or liquid, changes its place first from its normal position on the right ventral part of the abdomen to the left or right side (LDA, RDA). Consequences are the total stop of the passage rate of feed and of digestion, no milk production, lack of appetite, general suffering of the animal. Abomasum displacements cause economic loss in dairy herds through treatment costs (surgery), premature culling, lost production, and death. Current treatment costs range from

$100 to $200 per case, and 10% of the cows that are diagnosed with displaced abomasum are culled or die before the next test day. Treated cows that remain in the herd produce 350 kg less milk the following month than cows without a displaced abomasum (Shaver, 1997).

Moreover, major health problems can derive after calving from the reproductive system, which is not always able to go back to a normal condition without facing any issue. Immediately after calving, cow may suffer an abnormal retention of foetal membranes beyond 24h after calving (retained placenta, RP), whose causes are multi-factorial and still partly unknown. Literature reports as possible factors the alterations in plasma levels of hormones or lipid mediators and the oxidative stress (Kankofer, 2002). An important role seems to be played by prostaglandins (Leidl *et al.*, 1980), a family of lipid mediators involved in several biochemical pathways, included uterus motility and ovarian function. RP may be a contributory cause of other diseases of reproductive system, such as metritis and endometritis. These kind of inflammation of the uterus lining is in most of cases due to a bacterial infection and may be cause of important losses in milk yield and may also negatively affect the fertility of the cow (LeBlanc, 2008). The overall costs of post-calving reproductive diseases were reviewed by Bellows *et al.* (2002), who calculated an average cost of $ 52.6 per cow per year.

EFFECTS OF TREATMENTS TO AMELIORATE THE RESPONSE TO INFLAMMATION

The inflammatory status occurring in peripartum period is cause of several negative consequences. Many attempts have been done to reduce them, in particular through treatments with cyclooxygenase antagonists. In an experiment (Bertoni *et al.*, 2004), the administration of acetylsalicylic acid during the first 5 days of lactation lowered the main plasma inflammatory indices (haptoglobin and ceruloplasmin) and maintained the synthesis of usual liver proteins (albumin, lipoprotein and retinol-binding protein), suggesting a better liver functionality. In addition, milk yield and reproductive performance were significantly improved. These results underline the

marked negative effects due to inflammation occurred around calving (likely due to the release of pro-inflammatory cytokines around calving) even in clinically healthy cows, and confirm that the inhibition of some cytokine effects (i.e. eicosanoid production) could be an efficient approach to improve the health status of dairy cattle at calving time. Good results were obtained using a more selective anti cyclooxygenase-2 (meloxicam; Trevisi *et al.*, 2003) concerning body condition score (BCS) and fertility, but some adverse effects were highlighted on milk yield and in the diseases incidence (e. g. metritis).

Another attempt was carried out administering the interferon-α (IFN-α) Trevisi *et al.* (2009) to periparturient cows (10 IU/kg of BW daily during the last two weeks of pregnancy or 0.5 IU/kg of BW daily until 5 DIM), but the inflammatory status worsened. This result is in contrast with previous works on monogastric (Amadori, 2007; Begni *et al.*, 2005), thus authors argued as this effect might be due to the prolonged contact of the administered cytokine with the oral lymphoid tissue during chewing that determined the unwanted increase of the cytokines effects and thus a shift of the physiological effect to pro-inflammatory condition. Moreover, during this experiment, an increase in pro-inflammatory cytokines was found before calving not only in treated cows, but also in controls, providing a further evidence that a systemic inflammatory status exists in transition cows.

Alternative way to reduce the release of pro-inflammatory cytokines in the peripartum is the administration of phyto-extracts with anti-inflammatory effects. Tedesco *et al.* (2004) administered silymarin to periparturient dairy cows, a natural acknowledged hepato-protector (10 g as water suspension from -10 to 15 days from calving). They noticed beneficially effects of the treatment on lactation performances and body condition without pointing out any adverse effect. Trevisi *et al.* (2008) obtained promising results with an extract of *Echinacea angustifolia* administered *per os* from 30 days before calving through 14 days after it. In particular, positive effects were shown on the response to inflammation (lower reduction of -APP) and on the immune system (lower levels of neutrophils after

calving and lower neutrophil/lymphocytes ratio), with improvements in energy balance, milk yield and composition.

Another possibility of intervention is represented by the administration of essential long chain fatty acids protected from the rumen bio-hydrogenation in the diet of periparturient dairy cows. Conjugated linoleic acid (CLA) was shown to have beneficial effects on inflammatory status and energy balance (Trevisi *et al.*, 2009), on milk yield (Bernal-Santos *et al.*, 2003), but it may exert a depressive effect on the production of milk fat (Baumgard *et al.*, 2001). Moreover, the administration of ω3 fatty acids in the diet showed positive effects in several experiments on periparturient dairy cows, e.g. Moussavi *et al.* (2007) displayed improvements on milk production and on feed intake, while Petit *et al.* (2006) had a good effect on fertility.

INFLAMMATION, AN OVERVIEW

GENERAL MECHANISMS OF INFLAMMATION

Despite it is very hard to find a brief and complete definition of inflammation (Scott *et al.*, 2004), it may be defined as the reaction of a living tissue (or an organ) due to physical, chemical or biological nature harmful stimuli (stressors), including all the changes in the distal vascular bed (microcirculation) of blood and connective tissue, aiming to eliminate the cause and to repair damaged tissue. Distinctive signs of inflammation are well-known since very long time, and were described for the first time by Aulus Cornelius Celsus (I century AD) as *rubor*, *tumor*, *calor*, *dolor*. About a century later Galen of Pergamon, a Greek physician, surgeon and philosopher, added to these definitions a fifth one, *functio laesa* (disturbance of function; Rather, 1971). All these terms refer to clear and perceptible conditions, which nowadays we can refer precisely to physiological events. *Rubor*, latin term for reddening, is due to active and passive tissular hyperaemia, a condition of highly filled vessel due to a greater blood influx and to haemoconcentration (*inspissatio sanguinis*), causing a reduced blood influx up to stasis. *Tumor* (swelling) rises later and derives part from hyperaemia and part from the exudate accumulation. The last sign, *dolor* (pain), is caused by the action of swelling on nerve endings. All these events trigger the *functio laesa*, term that describes compromised function of the tissue or organ hit by inflammation.

The whole mechanism involves a number of biochemical mediators and causes cellular alterations, whose extent is usually related to the initial cause. After this cause, pro-inflammatory cytokines are released, and the vascular system and inflammatory cells are activated. These responses in turn are associated with the production of more cytokines and other inflammatory mediators which diffuse to the extracellular fluid compartment and circulate in the blood. The cytokines activate receptors on different target cells leading to a systemic reaction resulting in the activation of the hypothalamic-pituitary-adrenal axis, reduction of growth hormone secretion and a number of physical changes clinically characterized by

fever, anorexia, negative nitrogen balance and catabolism of muscle cells (Gruys *et al.*, 2005). Nevertheless, an activation of the inflammatory response may be altered and excessive, causing a negative effect on the organism.

The acute phase response (APR) is a prominent systemic reaction of the organism to local or systemic disturbances in its homeostasis caused by infection, tissue injury, trauma or surgery, neoplastic growth or immunological disorders (Gordon *et al.*, 1985; Gruys *et al.*, 1998). The acute phase is characterized by the rapid influx of blood granulocytes, typically neutrophils, followed swiftly by monocytes that mature into inflammatory macrophages that subsequently proliferate and thereby affect the functions of resident tissue macrophages (Ricciotti *et al.*, 2011). In blood, a number of variations in proteins (acute phase proteins) can be observed, mainly due to the altered hepatic metabolism (Cousins, 1985; Dinarello, 1983; Gruys *et al.*, 1994). During this response, liver is engaged in the production of the positive acute phase proteins (+APP), whose plasma level increases at the expense of the proteins usually synthesized, which decrease (negative acute phase proteins; -APP).

EICOSANOIDS FROM Ω6 FATTY ACIDS AS MEDIATORS OF INFLAMMATION

Several causes may trigger a set of reactions at local level producing small molecules like cytokines and eicosanoids. Eicosanoids are 20-carbon fatty acids mostly derived from arachidonic acid (20:4 ω-6), considered the primary eicosanoid precursor in mammalian cells (Funk, 2001). Following different pathways various classes can be originated the most important are prostaglandins (PGs), leukotrienes (LTs), lipoxins (LXs) and thromboxanes (TXs). Every eicosanoid exerts its specific action: for example PGI_2 (prostacyclin) is a potent inhibitor of platelet aggregation, PGE_2 induces pain, heat and fever, TXA_2 stimulates platelet aggregation and $PGF_{2\alpha}$ is known for its positive effect on oxytocin release.

These molecules play a fundamental role in the duration and intensity of inflammatory response (Kinsella *et al.*, 1990; Tilley, 2001) and can be divided

according to their activity, in more or less pro-inflammatory eicosanoids. Prostaglandin class mostly derive from arachidonic acid (20:4 ω-6; ARA) that, through two different pathways involving 5-lypooxygenase (LOX) and cyclooxigenase-2 (COX-2) enzymes, generates several mediators (e.g. hydroperoxy-eicosatetraenoic acid and prostaglandin from 2 and 4 series; Calder, 2006) that exert the maximum pro-inflammatory activities. In competition with ARA cascade, two other cascades independent and parallel, originate from eicosapentaenoic acid (EPA; 20:5 ω3) and dihomo-γ-linoleic acid (DGLA; 20:3 ω-6). EPA (more important) and DGLA (less important) cascades result mainly in the production of eicosanoids characterized by a weaker pro-inflammatory activity (series 1, 3, 5 PG, TX, LT). Moreover, docosahexaenoic acid (DHA; 22:6 ω3), an important EPA-derived fatty acid, can compete for COX-2 and LOX enzymes, forming mediators (protectins and resolvins D series) taking part in the resolution phase of inflammation (Serhan *et al.*, 2006). Ultimately, an increase in ω3/ARA ratio means higher incorporation of ω3 fatty acids in phospholipids at the expense of ARA, resulting in a decreased production of more pro-inflammatory eicosanoids.

According to this mechanism, an adequate supplementation of long chain ω3 fatty acids before inflammatory events, may be useful to optimize the inflammatory response and to reduce its adverse effects by changing PG production ratio, as previously suggested (Calder, 2002; Mori *et al.*, 2004). Polyunsaturated fatty acids, and in particular long chain ω3 may be potential tools able to modulate the response to inflammatory phenomena (Calder, 2005).

INFLAMMATION RESOLUTION

The mechanisms of inflammation resolution remained unclear for decades, but recently a number of studies gave some light on this important phase. A number of mediators is involved in this process: their origin, action, and pathways have been recently clarified (Ariel *et al.*, 2007).

The resolution phase begins already a few hours after the beginning of inflammatory response, when granulocytes promote the switch of arachidonic acid–

derived prostaglandins and leukotrienes to lipoxins, initiating the termination sequence of their recruitment. When the recruitment of neutrophils is stopped, the programmed death of these cells (apoptosis) takes place. At the same time ω3 polyunsaturated fatty acids give origin to a number of mediators named resolvins and protectins, which critically shorten the period of neutrophil infiltration by initiating apoptosis. Apoptotic neutrophils are then cleared by macrophages and anti-inflammatory and reparative cytokines are released (e. g. transforming growth factor- β1, TGF- β1). The last step of resolution phase (and of the overall inflammatory response) is the departure of macrophages through the lymphatics (Serhan *et al.*, 2005).

Action of non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) is a class of drugs exerting their action through the inhibition of prostaglandins production. The first studies on these compounds were carried out in the XIX century by Maclagan (1876), who reported the beneficial effects of salicylic acid in the treatment of rheumatisms. In the following years the company of Frederick Bayer was actively engaged in finding a derivative characterized by an efficacy as good as the salicylic acid. Some time later, the molecule of acetylsalicylic acid was shown effective and was given the name "Aspirin" by the Bayer's chief pharmacologist Heinrich Dreser (1899). About a century later Vane (1971) for the first time described the connection between aspirin and cyclooxygenase (COX) activity and consequently to the prostaglandin metabolism. A recent review of the same author (Vane, 2003) summarizes all the studies carried out in the following years on different species and experimental designs which confirmed his theories.

A homogeneous, enzymatically active cyclooxygenase or prostaglandin endoperoxide synthase (PGHS) was isolated in 1976 (Hemler *et al.*, 1976) and it is found in greatest amounts in the endoplasmic reticulum of prostanoid-forming cells (Smith, 1986). It exhibits COX activity that both cyclizes arachidonic acid and adds the 15-hydroperoxy group to form prostaglandin G_2 (PGG_2). The hydroperoxy

group of PGG_2 is reduced to the hydroxy group of PGH_2 by a peroxidase. Both COX and hydroperoxidase activities are contained in the same dimeric protein molecule. Aspirin selectively acetylates the hydroxyl group of one serine residue (Ser 530) located 70 amino acids from the C terminus of the enzyme (Roth *et al.*, 1975). This acetylation leads to irreversible COX inhibition; thus, a new enzyme must be synthesized before more prostanoids are produced. When the purified enzyme is acetylated, only the COX, not the hydroperoxidase, activity is inhibited. The stoichiometry of this reaction is 1:1, with one acetyl group transferred per enzyme monomer of this dimeric protein. At low concentrations, aspirin acetylates PGHS rapidly (within minutes) and selectively. At high concentrations, over longer time periods, aspirin will also non-specifically acetylate a variety of proteins and nucleic acids (Smith, 1989). Acetylation of the enzyme by aspirin places a bulky substituent on the Ser 530 oxygen that inhibits binding of arachidonic acid (DeWitt *et al.*, 1990).

Synergic action of aspirin and ω3 fatty acids on eicosanoids production

Considering the results showed above, the efficacy of the simultaneous administration of aspirin together with ω3 fatty acids may be evaluated on the eicosanoids metabolism (Engström *et al.*, 2001). Serhan *et al.* (2002) reported as in several experiments on different species the administration of EPA and DHA in combination with aspirin gave origin to the 17R-hydroxy-DHA, that is transformed by neutrophils into two sets of novel di- and tri-hydroxy products (resolvins, Rv) which exert an inhibitory effect on some cytokines. Later, Arita *et al.* (2005) gave some further explanations on resolvins and their functions. Resolvin E1 (RvE1) is biosynthesized in vivo from EPA via trans-cellular biosynthetic routes during cell–cell interactions, and thus it is formed in vivo during multicellular responses such as inflammation and microbial infections. RvE1 protects tissues from leukocyte-mediated injury and counter-regulates pro-inflammatory gene expression. These newly identified Rv may underlie the beneficial actions of ω3 PUFAs especially in chronic disorders where unresolved inflammation is a key mechanism of pathogenesis.

AIM OF THE THESIS

The transition period of dairy cows is still the most important phase of their productive cycle, when most of pathologies, and probably the most severe, appear. The inflammatory response is a mechanism that occurs after several kind of stimuli (e.g. infections, trauma) with the purpose to remove the injurious stimuli and to initiate the healing process. Nevertheless, it causes also an extra effort for the whole body that subtracts energy and nutrients from other primary functions (e.g. milk production, reproduction). In the periparturient dairy cow, it could be very harmful, in particular if too strong and prolonged.

In order to avoid negative consequences, first of all it is important to limit the occurrence of inflammatory processes adopting correct procedures (e.g. vaccinations, hoof trimming, to avoid digestive disturbs and other metabolic diseases, besides any kind of stress factors). However, whenever they take place, the key is to make the body less responsive to inflammation and in any case to promptly treat with specific drugs. Thus, the main issues of this thesis are:

a) to identify a combination of markers able to assess both the inflammatory status around calving and the cow's response to it. According to previous attempts, carried out in the past with a similar purpose, our aim has been to set up a new index characterized by an easy application in field conditions;
b) to verify the possibility to reduce the consequences of inflammation by using some of the tools already proposed in literature:
    - in the first experiment algae-derived ω3 fatty acids were administered daily from three weeks before through three weeks after calving;
    - in the second one fish-derived ω3 fatty acids were administered the last 3 weeks of pregnancy alone or in combination with lysine acetylsalicylate (last 7 pregnancy days) to take advantage of their well-known effects, as well as their supposed synergic actions against inflammation.

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CHAPTER 1

A novel index to quickly assess the severity and the consequences of the inflammatory status in the periparturient dairy cow

INTRODUCTION

Inflammation is a process mediated by the release of pro-inflammatory cytokines after an external stimulus (e.g. infections, trauma) that may occur at any time (Feghali and Wright, 1997). In some physiological stages inflammation is a common occurrence. In particular, it is demonstrated that dairy cows suffer an inflammatory condition during their transition period (Cappa *et al.*, 1989), that exerts several negative effects on metabolism, performances and fertility (Trevisi *et al.*, 2011). The level of inflammation may be very different among cows, and only a part of them show clinical signs together with the inflammatory status. Thus, several cows appear clinically healthy but may suffer a subclinical inflammatory status (Bertoni *et al.*, 2008) demonstrated by the important raise of the positive acute phase proteins (Gruys *et al.*, 2005; Murata, 2004; Petersen *et al.*, 2004).

The mechanism of inflammation triggers the release of a number of mediators, such as eicosanoids and cytokines. In particular, cytokines exert many effects, some of which may cause detrimental consequences on some crucial aspects of the periparturient dairy cow life (e.g. anorexia, fever). Among the effects exerted by cytokines, it is important the action that triggers the production of +APP, causing the so-called acute phase response (APR). APR is not only characterized by the raise of the plasma levels of +APP, but also by the reduced production of some other proteins usually synthesized by the liver in normal conditions (-APP). The plasma changes of +APP are useful indices to describe the severity of the inflammatory status, while –APP describe the extent and the consequences of the response to inflammation. However, it is a common believe that the consideration of single plasma parameters may be insufficient to evaluate the whole complexity of the inflammatory event and its consequences. Thus, some attempts have been done in the past to set up indices considering different parameters: Bonnefoy *et al.* (1998) proposed an index (Prognostic Inflammatory and Nutritional Index, PINI) useful to score the overall health of humans considering two blood markers of inflammatory (C-reactive protein and alpha(1)-acid glycoprotein) and of nutritional (albumin and

transthyretin) states. Their aim was to forecast hospital mortality and outcome of patients hospitalized in an acute geriatric unit. Toussaint *et al.* (1995) suggest the use of an Acute Phase Index (API) to better describe the inflammatory status and consequently to give further elements to assess the quality of slaughtered animals. Gruys *et al.* (2005) proposed another index composed either by +APP and –APP, the Nutritional and Acute Phase Indicator, NAPI = (value of a rapid positive APP × value of a slow positive APP)/(value of rapid negative APP × value of a slow negative APP).

Trevisi *et al.* (2001) and Bertoni *et al.* (2006) proposed two new indices (Liver Activity Index, LAI and Liver Functionality Index, LFI) that contributes to give an idea of the extent of the inflammatory response in dairy cows during the first month of lactation. The main limitation of these indices is the delay of assessment: a result is available only one month after calving, when several negative effects are already spent. Thus, the aim of this research was to identify a new index able to evaluate both the severity of the inflammatory status occurring after calving in the periparturient dairy cow and its consequences, selecting the most proper parameters with the statistical support of the principal component analysis. The secondary aim is a simplification and a shortening of the time of diagnosis (at 7 DIM, 3 weeks earlier than LFI and LAI), in order to make it easily applicable in a field situation.

MATERIALS AND METHODS

This study complied with Italian and European rules on animal experimentation and ethics.

BARN CHARACTERISTICS, ANIMALS AND TREATMENTS

This work involved 56 cows reared in the experimental barn of the Università Cattolica (CERZOO) located in the Northern Italy (Piacenza) over 3 years. Cows were reared in loose stall with cubicles and milked twice a day (12 hours gap). Sufficient space was available as number of cubicles and manger places to avoid any

competition for feed and/or rest. Cows were checked for the last month of pregnancy through the first of lactation as described in the next paragraph, and underwent a similar management and suffered similar stress factors due to sample collection. Dry and lactating cows were fed once a day two different TMR, which only underwent slight changes in the years due to different lots of the same feed (Table 1).

CLINICAL CHECKS

During the experiment, animals health conditions were checked every day by general inspections and monitored by a computerized Afimilk system (S.A.E. Afikim, Kibbutz Afikim, Israel), based on automatic recording of activity and milk production through a leg transponder. Cows underwent a thorough gynecological examination at about 10 and 30 DIM, or when required by a suspect of pathology. Each cow was submitted to the following assessments: 1) Body condition scoring, using a 5-point scale (ADAS, 1986), starting about 35 d before the expected calving date and, then, every 14 days to 35 DIM. 2) Milk yield and its conductivity, measured and recorded by the Afimilk computer-controlled automated system at every milking. 3) Blood samples, collected approximately at -28, -21 (pre-treatment), -14, -10, -7, -3 (before calving), 1 (day after calving), 3, 7, 10, 14, 21, 28, 35 (post-treatment) days from calving. Every sample was collected in the morning before feeding, from a jugular vein in two vacuum tubes (Vacuette, Greiner Bio-One GmbH, Kremsmunster, Austria) containing lithium-heparin as anticoagulant. Tubes were cooled immediately after collection in an ice-water bath until their arrival in laboratory. After a small aliquot of blood was taken to determine packed cell volume (centrifugation at 12000 RPM for 11 minutes), tubes were centrifuged at 3520 × g for 16 minutes at 4°C; plasma samples were divided in 5 aliquots, stored at -20°C (4) or -80°C (1). On these samples were determined: I) Inflammatory response indexes: positive acute phase proteins (+APP; haptoglobin, ceruloplasmin) and negative acute phase proteins (-APP; albumin, cholesterol as lipoprotein index). II) Liver indexes: total bilirubin , aspartate amino-tranferase (GOT), γ-glutamil

transferase (GGT), alkaline phosphatase (ALP), paraoxonase (PON). III) Energy metabolism indexes: glucose, NEFA, BHB. IV) Protein metabolism indexes: urea, creatinine. V) Oxidative stress response: total reactive oxygen metabolites (ROM). VI) Minerals (Ca, P, Mg, Na, K, Cl, Zn). VII) Vitamins (assayed only on 41 cows): retinol (index of its carrier protein), tocopherol, β-carotene. VIII) Other parameters (total proteins, globulins).

Glucose, total protein, albumin, total cholesterol, total bilirubin, creatinine, urea, Ca, P, Mg, GOT, GGT and ALP were detected at 37°C by a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA) using commercial kits purchased by Instrumentation Laboratory (IL Test), at the conditions described in Appendix 2. Globulins were calculated as the difference between total protein and albumin. Electrolytes (Na^+, K^+, and Cl^-) were detected by the potentiometer method (Ion Selective Electrode connected to ILAB 600). Zn and NEFA were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). Haptoglobin, BHB, and ceruloplasmin were analyzed using methods described by Bertoni *et al.* (1998), adapted to ILAB 600 conditions. Plasma retinol, tocopherol and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2.3 μm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL); a UV detector set at 325 nm (for retinol) or 290 nm (for tocopherol); and 80:20 methanol:tetrahydrofurane as the mobile phase. ROM were measured using a method patented by Diacron International S.r.l. (Grosseto, Italy) and expressed as mg of hydrogen peroxide per 100 mL of plasma. Plasma PON activity was assessed by adapting the method of Ferré *et al.* (2002) to the ILAB 600, as described by Bionaz *et al.* (2007).

DATA HANDLING AND STATISTICAL ANALYSIS

All data in this paper are presented in the form mean ± standard deviation.

The days relative to calving are reported as classes, thus their variability is ± 3 days.

Principal component analysis (PCA)

To perform the choice of the parameters involved in the index and the best day of sampling, the statistical analysis of the principal component (PRINCOMP procedure, SAS Inst. Inc., Cary, NC) was carried out considering as variables the plasma concentrations of the main parameters involved in the inflammation after calving (at 3, 7, 10 DIM). In the Table 3 are reported the eigenvalues (which quantify the variability explained by every single component as a percentage of the total variability explained by all the components involved in the model) of the two principal components. The most important parameters found on principal component 1 (negatively correlated) were: haptoglobin at 7 DIM (-0.27) and ROM at 7 DIM (-0.26). On the same principal component, but positively correlated are found PON at 10 DIM (0.33), PON at 7 DIM (0.31), cholesterol at 10 DIM (0.30) and cholesterol at 7 DIM (0.26).

Index construction

Every cow was assigned a score for each parameter basing on extreme values given in Table 2. According to these extreme values, the linear equations for each parameter were calculated, in order to assign a score to the values of the parameters:

Haptoglobin score = -8,33 × (haptoglobin value) + 12,5

ROM score = -1,43 × (ROM value) + 25,7

Cholesterol score = 10 × (cholesterol value) – 20

PON score = 0,17 × (PON value) – 10

The sum of the scores of the four parameters gives the final index named Post-calving Inflammatory Response Index (PIRI):

PIRI = haptoglobin score + ROM score + cholesterol score + PON score

The PIRI index assigns higher values to cows characterized by a less severe inflammation and by a lower consequences to inflammation.

Analysis of variance

The cows were ranked according their score and divided in tertiles. A statistical analysis was carried out to verify significant differences among tertiles for every checked parameter: data were submitted to repeated measures variance analysis using a mixed model (MIXED procedure, SAS Inst. Inc., Cary, NC; Littell *et al.*, 1998). Before analysis the normality of distribution was verified for each parameter through skewness and kurtosis calculation according to the Shapiro test (SAS Inst. Inc.). When necessary, data were normalized through logarithmic (ALP, haptoglobin, BHB), root-square (bilirubin, NEFA) or inverse (GOT) transformations.

The layout of our statistical model can be summarized as follows:

$Y_{iklm} = \mu + G_i + T_k + GT_{ik} + B_{l(im)} + e_{iklmn}$

where Y_{ijklm} = m^{th} observation of the l^{th} cow B_l within the i^{th} tertile G_i, at the k^{th} day relative to calving T_k; μ = total average; G_i = effect of the i^{th} tertile; T_k = effect of the k^{th} day relative to calving (the number of levels being defined as a function of pregnancy phase and actual variable); GT_{ik} = effect of the interaction between the i^{th} tertile and the k^{th} day relative to calving; $B_{l(im)}$ = effect of the l^{th} cow within the i^{th} tertile; e_{iklmn} = random effect or error.

Calculation of other indices

The Liver Functionality Index (Bertoni *et al.*, 2008) was calculated for the same set of cows of PIRI index, while the Liver Activity Index (Bertoni *et al.*, 2006) was calculated on only 41 cows out of 56. A simple correlation with PIRI index was calculated for these two indices.

RESULTS

Post-calving Inflammatory Response Index. The whole population of cows presented an averaged PIRI score of 22.74±7.96. The correlation between PIRI index and LFI

index (Table 4) was r = 0.60 ($P<0.001$), while between PIRI and LAI was r = 0.55 ($P<0.001$).

To investigate the possible usefulness of this index, cows were ranked according to its value and divided in tertiles: LO-PIRI, IN-PIRI and HI-PIRI. The average PIRI scores (Table 4) were: 13.76±4.29 in LO-PIRI, 23.33±1.49 in IN-PIRI and 31.17±3.59 in HI-PIRI. In the next sections are described the main differences found between LO-PIRI and HI-PIRI tertiles. IN-PIRI tertile will be mentioned only in case its trend differed from the median level.

Health status and performances. As shown in Table 5, more cows in LO-PIRI (n = 7) suffered post-calving health troubles than in the other tertiles (n = 5 in IN-PIRI and n = 2 in LO-PIRI). Furthermore, the total problems suffered by LO-PIRI cows was 10, while HI-PIRI cows suffered only 3 post-calving health issues. The LO-PIRI cows showed a more marked drop of BCS (Figure 2) from 14 days to calving to 35 DIM (-0.57±0.17 points *vs* -0.43±0.15 points in HI-PIRI; $P<0.01$). The total milk yield in the first 28 days of lactation was higher in HI-PIRI (1050±136 kg) than in LO-PIRI (966±172 kg). The daily production (Figure 1) was significantly higher in HI-PIRI cows than in LO-PIRI cows between 7 and 14 DIM, with the widest difference recorded at 8 DIM (37.7±4.9 kg/d in HI-PIRI *vs* 33.2±6.1 kg/d in LO-PIRI; $P<0.05$).

Inflammatory status parameters. Some of these parameters (haptoglobin, ROM, cholesterol, PON; Figures 11, 15, 4, 14) are included in the PIRI index, and significant differences between extreme tertiles are expected at least immediately after calving as a consequence of the PIRI calculation method. In the first 10 days of lactation all the four parameters showed significant differences between LO-PIRI and HI-PIRI, and some of these parameters were different also later. Moreover, cholesterol, PON and ROM showed statistically significant differences already before calving. In detail, the plasma concentrations of haptoglobin (Figure 11) before calving kept average values <0.3 g/L, while immediately after calving the levels increased in all the tertiles reaching a peak at 3 DIM. LO-PIRI had a stronger increase, with higher levels from the day after calving to 10 DIM (0.16±0.13 g/L in

HI-PIRI *vs* 0.69±0.38 g/L in LO-PIRI at 7 DIM; $P<0.001$). ROM (Figure 15) showed a general tendency to increase after calving, keeping higher levels during lactation in respect to pregnancy. HI-PIRI showed during all the considered period lower plasma concentrations in respect to LO-PIRI. In particular, the maximum difference was found at 3 DIM (11.25±2.29 mg H_2O_2/100 mL in HI-PIRI *vs* 15.22±2.55 mg H_2O_2/100 mL in LO-PIRI; $P<0.001$). Cholesterol (Figure 4) showed in all cows the usual decreasing trend in late pregnancy, followed by a recovery during the first month of lactation. HI-PIRI cows showed higher levels already at the beginning of the experimental period (3.11±0.69 mmol/L in HI-PIRI *vs* 2.62±0.59 mmol/L in LO-PIRI; $P<0.05$ at 28 days before calving), and kept the difference until the end. However, the difference was wider starting from 7 DIM (2.56±0.44 mmol/L in HI-PIRI *vs* 1.88±0.38 mmol/L in LO-PIRI; $P<0.001$). Paraoxonase (Figure 14) showed a general trend similar to cholesterol. The levels were higher in HI-PIRI than in LO-PIRI for the whole experiment, and reached the maximum gap after calving (120.36±25.48 U/mL in HI-PIRI *vs* 69.08±18.09 U/mL in LO-PIRI at 10 DIM; $P<0.001$).

Among the parameters related to inflammation not included in the PIRI index, particularly interesting are the trends of ceruloplasmin (Figure 5), which showed a similar trend between LO-PIRI and HI-PIRI, with an increase immediately after calving followed by a slight reduction. HI-PIRI cows kept lower values for all the checked period, and at 7 DIM the values were 3.11±0.39 μmol/L in HI-PIRI and 3.55±0.68 μmol/L in LO-PIRI ($P<0.05$). Albumin (Figure 7) did not show any difference between groups in late pregnancy. After calving, its plasma concentration dropped in LO-PIRI cows much more than in HI-PIRI, and showed also a delayed recovery. The greatest difference was found at 10 DIM (37.16±1.40 g/L in HI-PIRI *vs* 34.72±3.32 g/L in LO-PIRI; $P<0.001$). The values of retinol (Figure 16) decreased in the last month of pregnancy, reaching the minimum immediately after calving and then recovering the pre-calving levels. HI-PIRI kept higher levels than LO-PIRI for the whole checked period. The difference was maximum at 7 DIM (43.71±11.87 μmol/L in HI-PIRI *vs* 25.87±5.21 μmol/L in LO-PIRI; $P<0.001$).

Bilirubin (Figure 9) started an increasing trend some weeks before calving in all the cows, and peaked in LO-PIRI at 1 DIM (9.14±6.14 mmol/L), while in HI-PIRI the peak was achieved 3 days after calving (4.63±2.03 mmol/L). The difference between HI-PIRI and LO-PIRI was significant between 14 days before calving to 10 days after it and maximum gap was observed at 1 DIM (4.63±2.03 mmol/L in HI-PIRI *vs* 9.14±6.14 mmol/L in LO-PIRI; $P<0.001$).

Energy metabolism parameters. Glucose (Figure 3) showed a tendency to be higher in HI-PIRI at 7 DIM (3.69±0.26 mmol/L in HI-PIRI vs 3.41±0.83 mmol/L in LO-PIRI; $P<0.10$). NEFA levels (Figure 12) increased in all cows around calving, peaking at 3 DIM. LO-PIRI cows showed a stronger increase around calving and the level of NEFA turned out significantly higher from 3 days before calving to 10 DIM. The maximum difference between tertiles was observed the day after calving (0.47±0.21 mmol/L in HI-PIRI *vs* 0.99±0.47 mmol/L in LO-PIRI; $P<0.001$). BHB (Figure 13) showed a similar trend around calving in all the tertiles, starting to increase 3 days before calving and peaked at 3 DIM. In HI-PIRI, BHB was significantly lower: 0.75±0.15 mmol/L in HI-PIRI vs 1.28±0.89 mmol/L in LO-PIRI ($P<0.01$).

Other plasma parameters. Globulin (Figure 8) showed during the first month of lactation higher levels in LO-PIRI, with the maximum difference reached at 14 DIM (38.78±4.85 g/L in HI-PIRI *vs* 43.53±5.36 g/L in LO-PIRI; $P<0.01$). ALP (Figure 10) displayed significantly higher levels in HI-PIRI already 28 days before calving (58.31±13.41 U/L in HI-PIRI vs 42.12±20.69 U/L in LO-PIRI; $P<0.01$) and kept the significance until calving. In the first month of lactation it tended to be higher in HI-PIRI until 28 DIM, while at 35 DIM values were similar. Urea, Ca, P, Mg, Na, K, Cl, Zn, GOT, GGT, creatinine did not show any statistical differences among tertiles.

DISCUSSION

The new index was set up in order to create a field-applicable tool mainly to identify the cows characterized by subclinical pathologies before these result in severe metabolic damages to the cow.

Effectiveness of PIRI in the detection of inflammation severity. The application of PIRI index to the set of 56 cows showed that LO-PIRI suffered more health problems after calving than HI-PIRI cows (Table 5) and the values of haptoglobin (Figure 11), ceruloplasmin (Figure 5), ROM (Figure 15) and bilirubin (Figure 9) showed that the inflammatory status in LO-PIRI was more marked than in HI-PIRI. Retinol (Figure 16), cholesterol (Figure 4) and PON (Figure 14) showed that the LO-PIRI cows suffered more severe consequences of inflammation. Interestingly in LO-PIRI cows the pre-calving plasma concentrations of cholesterol and PON were already significantly lower and ROM significantly higher than in HI-PIRI cows. This may imply a different starting situation of the cows, with a predisposition of LO-PIRI cows to be affected by health issues. The partition of cows according to PIRI index showed as the cows which suffered milder inflammation had an higher milk yield, as previously shown by Bertoni *et al.* (2006). Interestingly, the same cows were also characterized by a lower lipomobilization suggested by the lower BCS drop (Figure 2) and confirmed by the significantly higher post-calving plasma concentrations of NEFA and BHB (Figures 12 and 13). The higher levels of glycaemia (Figure 3) in the first week after calving confirmed a better picture of energy metabolism in HI-PIRI cows.

Comparison with other complex indices: LAI and LFI. Considered the effectiveness of the index to split the cows according to the severity of their inflammatory status, was evaluated its affinity to other indices which had similar purposes. Although the highly significance of the correlation, the good but not very high values of r ($r = 0.60$ with LFI and $r = 0.55$ with LAI) suggest that PIRI describes something partly different from what LAI and LFI describe. Indeed, PIRI includes some parameters characterized by different significances, e.g. haptoglobin as an indicator of

inflammatory status and ROM, involved in the oxidative stress. Furthermore, the plasma concentration of these parameters is considered at different times (only 7 DIM in PIRI vs trend in the first month of lactation in LFI and LAI). Thus, the PIRI index aims to assess also the severity of the inflammatory status and not only its consequences (as it was in LFI and LAI). However, an implication of PIRI may be the identification as "problem animals" the subjects that are later able to recover by themselves, anyway an eventual treatment given to these animals may speed the recovery up.

Field applicability of the PIRI index. In comparison to LFI and LAI, the PIRI index is able to promptly identify cows with inflammatory problems at the beginning of lactation, while LFI and LAI give a result only after the end of the first month of lactation. In particular, PIRI is useful to identify animals with subclinical health problems that might later worsen and show clinical pathologies after 7-10 DIM. Such these cows could in this way be treated since the second week of lactation in order to prevent the occurrence of more severe pathologies.

CONCLUSION

The results reported show that the Post-calving Inflammatory Response Index is a useful tool to identify cows affected by inflammatory problems in the first days after calving. In particular its structure takes into account either the inflammatory status (+APP) and its consequences (-APP), allowing a better assessment of the whole inflammatory event in respect to the previous indices LFI and LAI which only considered -APP. The fairly good correlation with LFI and LAI, considered the differences in the composition of the indices, suggests that also PIRI could be a good index to describe inflammation in post-calving of dairy cows.

Furthermore, an important improvement is the possibility to have a response already at 7-10 days after calving, allowing to take early the needed decisions on eventual proper treatments or drug administration. However, LFI and LAI may give later a better description of the inflammatory status suffered by the cow during the first month of lactation.

The extent of the population considered in this work to set up the index was good, but all cows were from the same barn. To give PIRI more strength and to have a further and stronger validation, it is necessary the involvement of more cows from different farms.

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Table 1 - Nutritional values of the diets fed as TMR for close-up and lactating cows. Small fluctuation of these values are expected due to different lots

COMPONENTS [% of DM]	Dry cows	Lactating cows
Grass hay	49.63	7.37
Alfalfa hay	-	11.64
Wheat straw	16.5	-
Corn silage	25.6	32.9
Corn meal (67%) and corn flakes (34%)	-	24.85
Cottonseed	-	8.68
Soybean meal	7.4	-
Mineral and vitamin supplementation[§]	0.9	-
Commercial concentrate[#]	-	14.56
CHEMICAL COMPOSITION		
NEL [Mcal/kg of DM]	1.29	1.58
CP [% of DM]	12.44	16.11
NDF [% of DM]	56.57	33.88
Starch + sugar [% of DM]	12.58	26.83
Ether extract [% of DM]	1.91	4.79

[§] 42.9% Ca_2PO_4; 28.6% urea; 14.3% MgO; 7.1% NaCl; 7.1% mineral and vitamin supplement (1500000 IU/kg vitamin A; 150000 IU/kg vitamin D; 7000 IU/kg vitamin E; 10 mg/kg Co; 70 mg/kg I; 1100 mg/kg Mn; 500 mg/kg Cu; 23 mg/kg Se; 4000 mg/kg Zn)

[#] 58800 IU/kg vitamin A; 5200 IU/kg vitamin D; 102 IU/kg vitamin E; 0.4 mg/kg Co; 2.4 mg/kg I; 150 mg/kg Mn; 35 mg/kg Cu; 0.5 mg/kg Se; 150 mg/kg Zn

Table 2 - Values for each parameter included in PIRI index assigned maximum (10) and minimum (0) scores

Parameter	Max score = 10	Min score = 0
Haptoglobin	0.3	1.5
ROM	11	18
Cholesterol	3	2
PON	120	60

Table 3 - Eigenvalues from PCA analysis sorted by PRIN1 values

PARAMETER	DIM	PRIN1	PRIN2
PON	7	0.3357	0.0315
PON	10	0.3274	-0.0016
Retinol	7	0.3235	-0.0513
Retinol	3	0.2902	-0.0777
Albumin	10	0.2864	0.1569
PON	3	0.2620	0.0620
Albumin	7	0.2477	0.1744
Cholesterol	7	0.1817	-0.1690
Cholesterol	10	0.1809	-0.1924
Zn	3	0.1653	0.0542
Zn	7	0.1575	0.1661
Ceruloplasmin	7	0.1035	0.3690
Ceruloplasmin	10	0.0880	0.3444
Cholesterol	3	0.0757	-0.2281
Zn	10	0.0709	0.1397
Ceruloplasmin	3	0.0673	0.3021
Albumin	3	0.0578	0.1079
ROM	10	0.0200	0.3426
ROM	7	-0.0307	0.3653
ROM	3	-0.0762	0.2803
Bilirubin	10	-0.0885	0.1633
Bilirubin	7	-0.1581	0.1269
Haptoglobin	3	-0.1780	0.0400
Haptoglobin	10	-0.1925	0.0415
Bilirubin	3	-0.2284	0.1489
Haptoglobin	7	-0.2520	0.0922

Table 4 - Mean values of PIRI index in every tertile and mean values of LFI and LAI for the same tertiles of PIRI. Correlations among PIRI, LFI and LAI

		PIRI	LFI	LAI
LO-PIRI	MEAN	13.76	-2.22	-0.38
	SD	4.29	3.18	0.35
IN-PIRI	MEAN	23.33	-0.89	-0.06
	SD	1.49	2.58	0.64
HI-PIRI	MEAN	31.17	1.26	0.36
	SD	3.59	1.61	0.28

CORRELATIONS

	r	P<
PIRI-LFI	0.60	0.0001
PIRI-LAI	0.55	0.0001

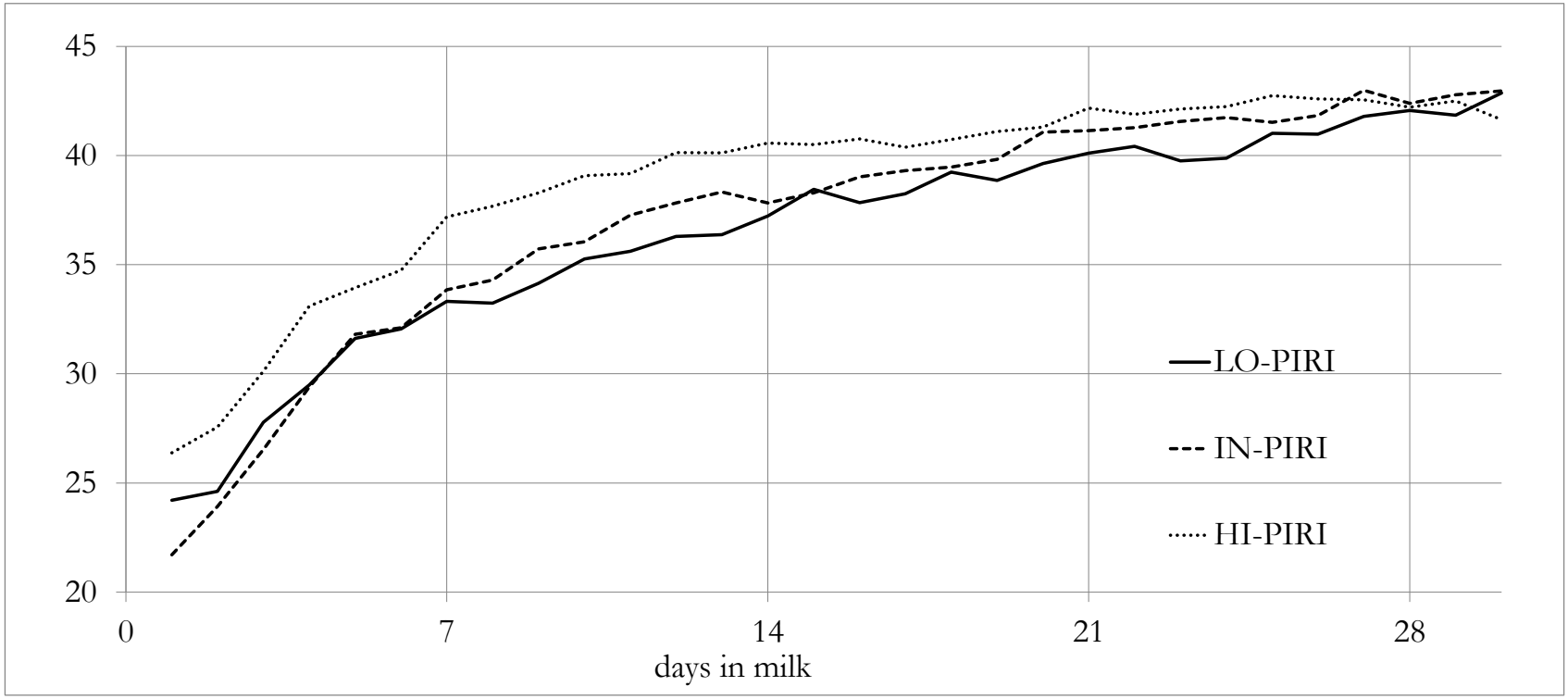
Table 5 - Count of clinical health problems found in each tertile of cows and count of animals affected by problems; comparison with LFI index

	MILK FEVER	RP	METRITIS	MASTITIS	KETOSIS
HI-PIRI	1	1	1	-	-
IN-PIRI	1	1	3	-	-
LO-PIRI	1	2	3	2	2

PIRI	N. OF COWS
HI-PIRI	2
IN-PIRI	5
LO-PIRI	7

LFI	N. OF COWS
HI-LFI	5
IN-LFI	2
LO-LFI	7

Figure 1 - Milk yield [kg/d] of dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
HI-PIRI vs LO-PIRI							*	*	*	*	+	+	+	+																
HI-PIRI vs IN-PIRI																														
IN-PIRI vs LO-PIRI																														

Figure 2 - Trend of body condition score (BCS) of dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.

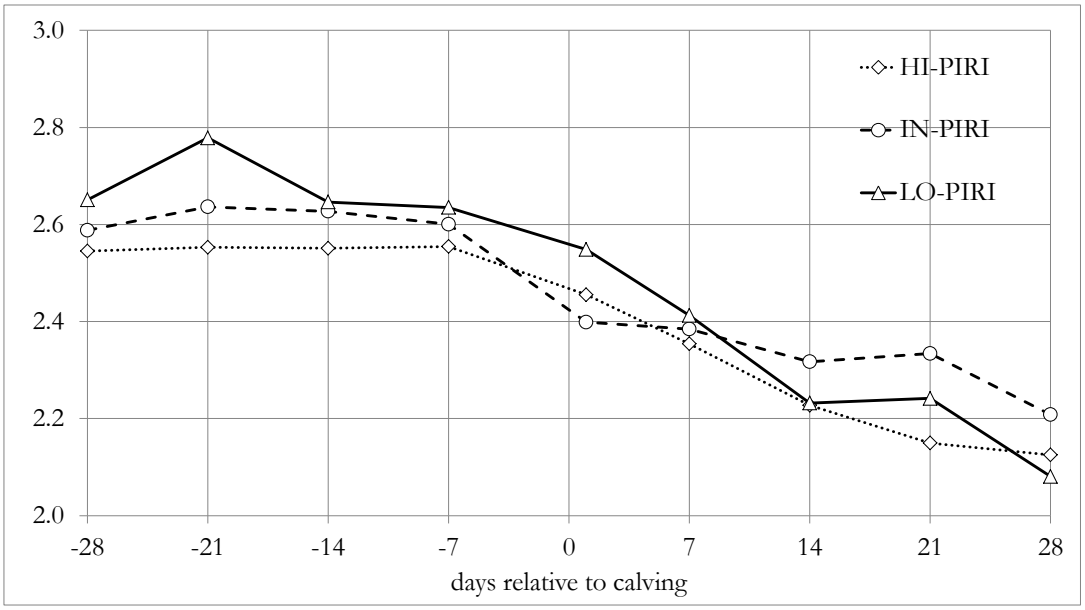
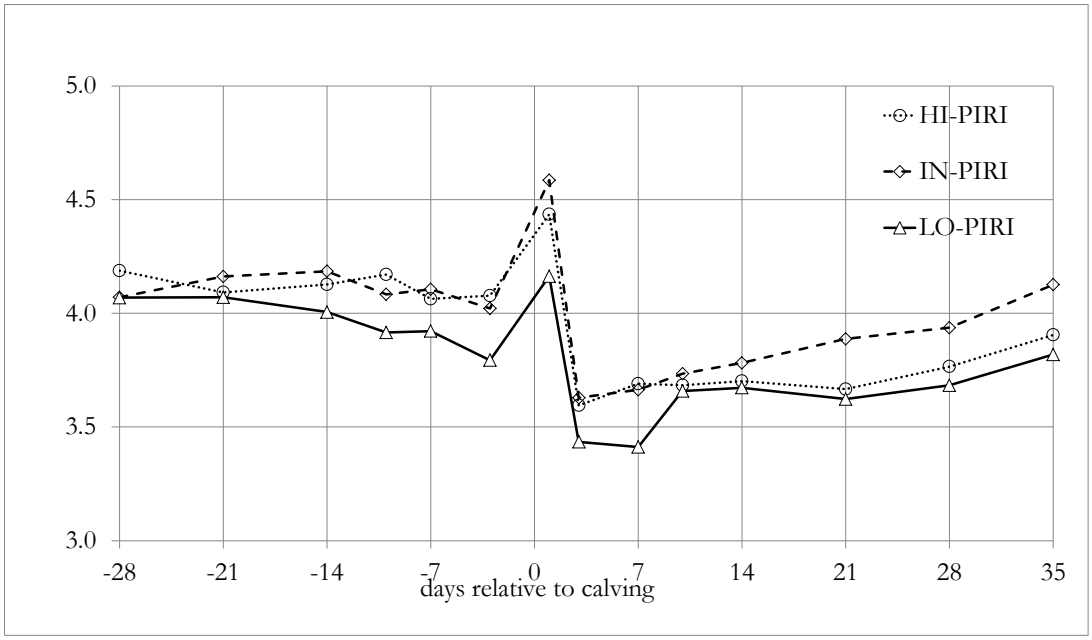


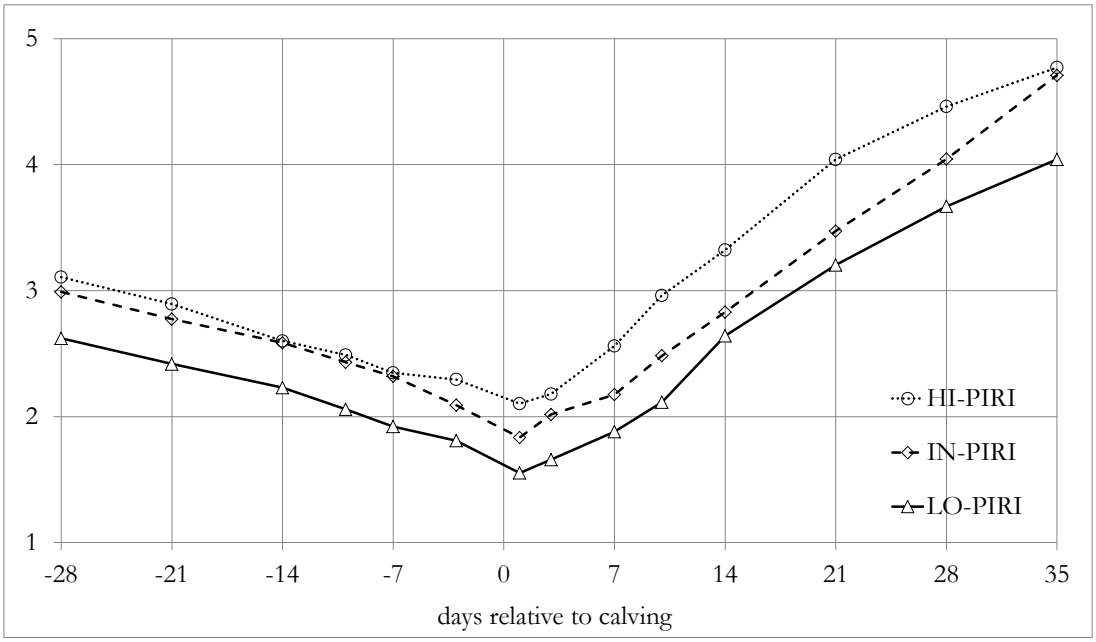
Figure 3 - Pattern of changes of glucose [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						+			+					
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI							**		+			+	+	+

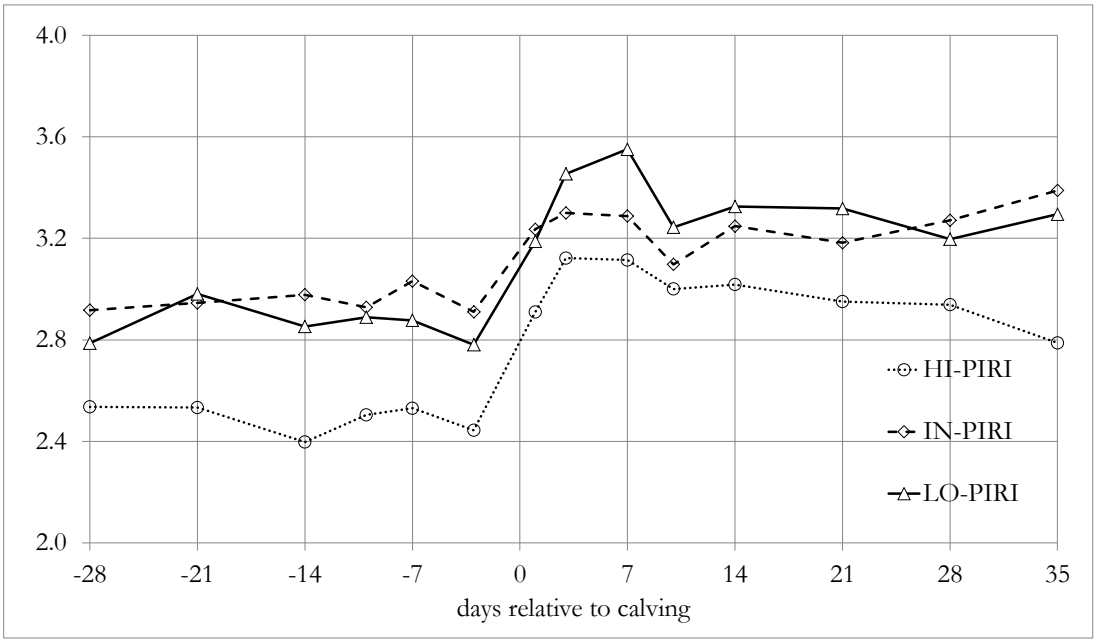
Figure 4 - Pattern of changes of cholesterol [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	*	*	+	*	*	*	*	**	***	***	***	***	***	***
HI-PIRI vs IN-PIRI									+	*	*	**	*	
IN-PIRI vs LO-PIRI	+	+	+	+	+			+		+			+	*

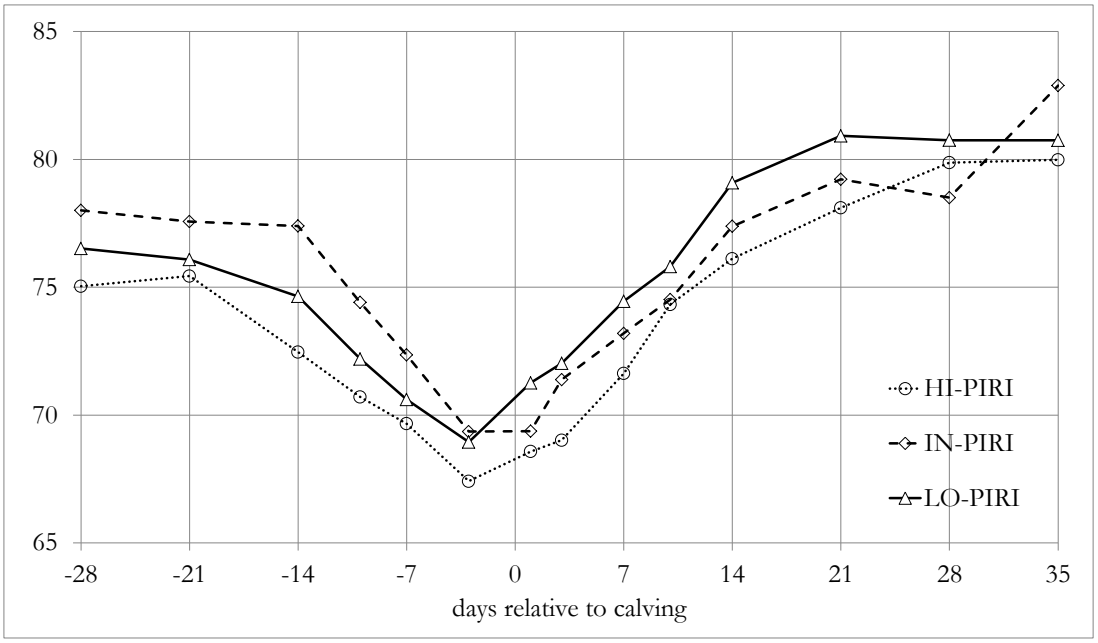
Figure 5 - Pattern of changes of ceruloplasmin [μmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI		*	*						*			+		**
HI-PIRI vs IN-PIRI	+	*	**	+	*	*	+							**
IN-PIRI vs LO-PIRI														

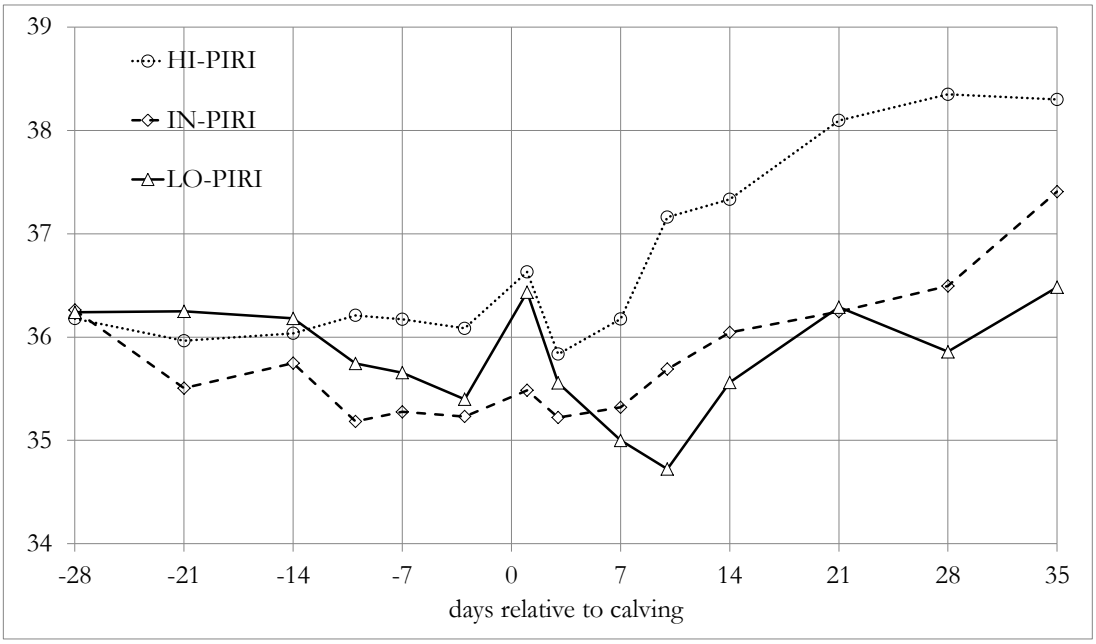
Figure 6 - Pattern of changes of total protein [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI							+	+			+			
HI-PIRI vs IN-PIRI			**	*										+
IN-PIRI vs LO-PIRI														

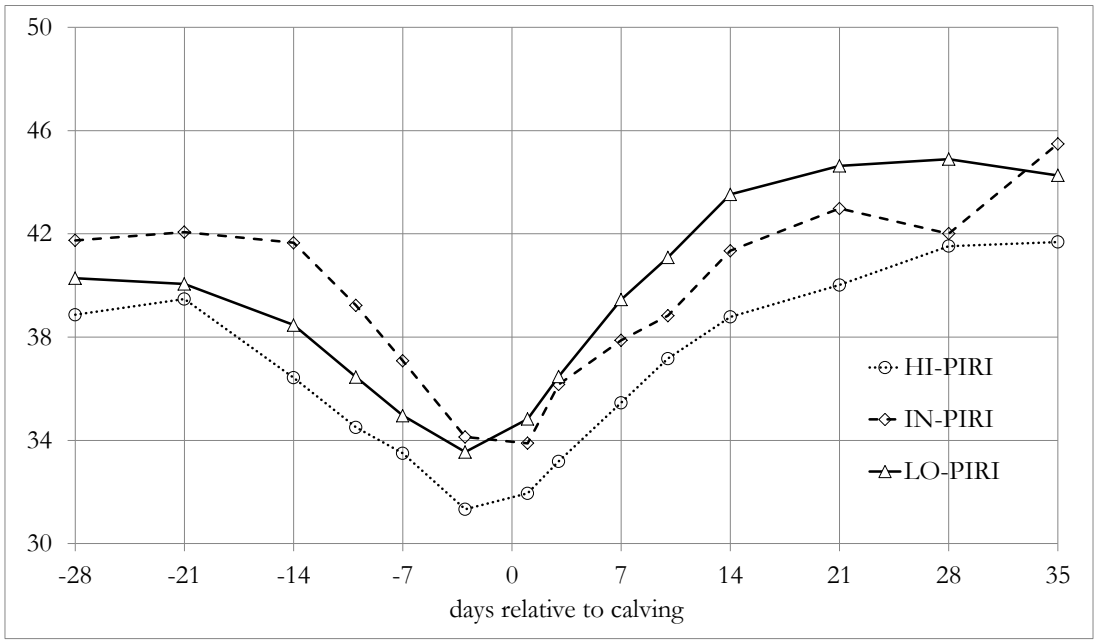
Figure 7 - Pattern of changes of albumin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI									+	***	**	**	***	*
HI-PIRI vs IN-PIRI							+			*	+	**	**	
IN-PIRI vs LO-PIRI							+							

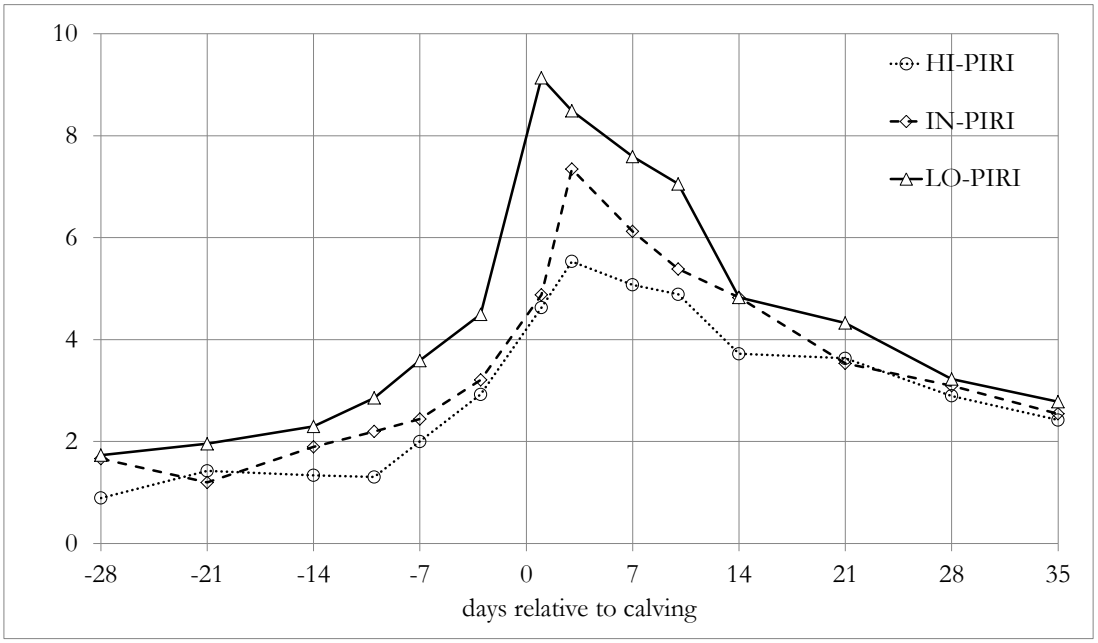
Figure 8 - Pattern of changes of globulin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI							+	+	*	*	**	*	+	
HI-PIRI vs IN-PIRI			**	**	*									*
IN-PIRI vs LO-PIRI			+											

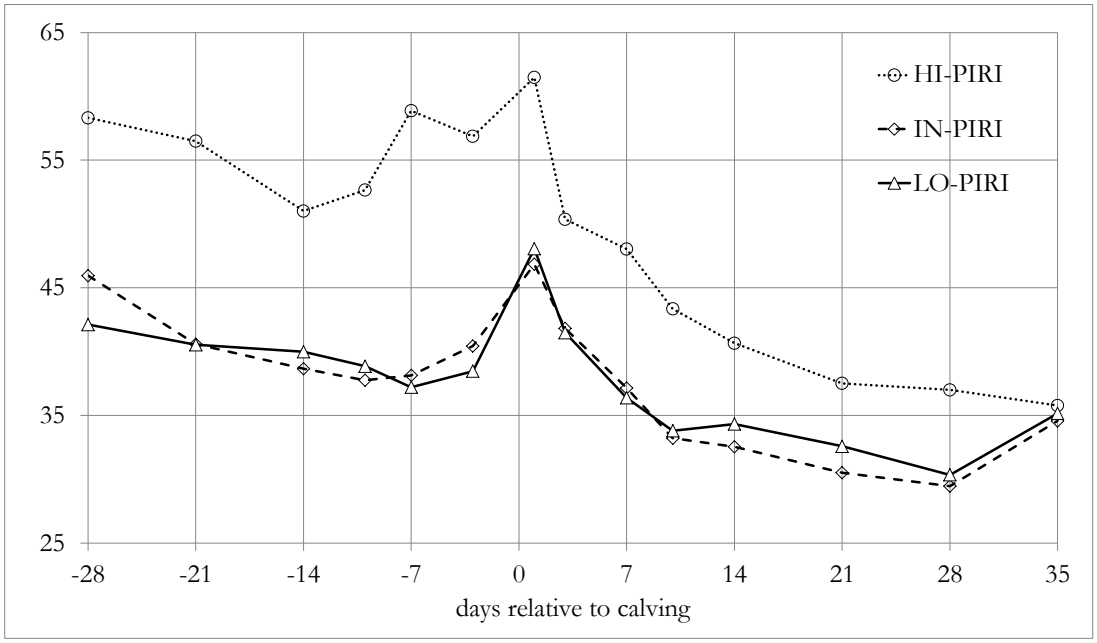
Figure 9 - Pattern of changes of bilirubin [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI			+	*	*	+	***	**	**	**				
HI-PIRI vs IN-PIRI	+			*				+						
IN-PIRI vs LO-PIRI							***		+	+				

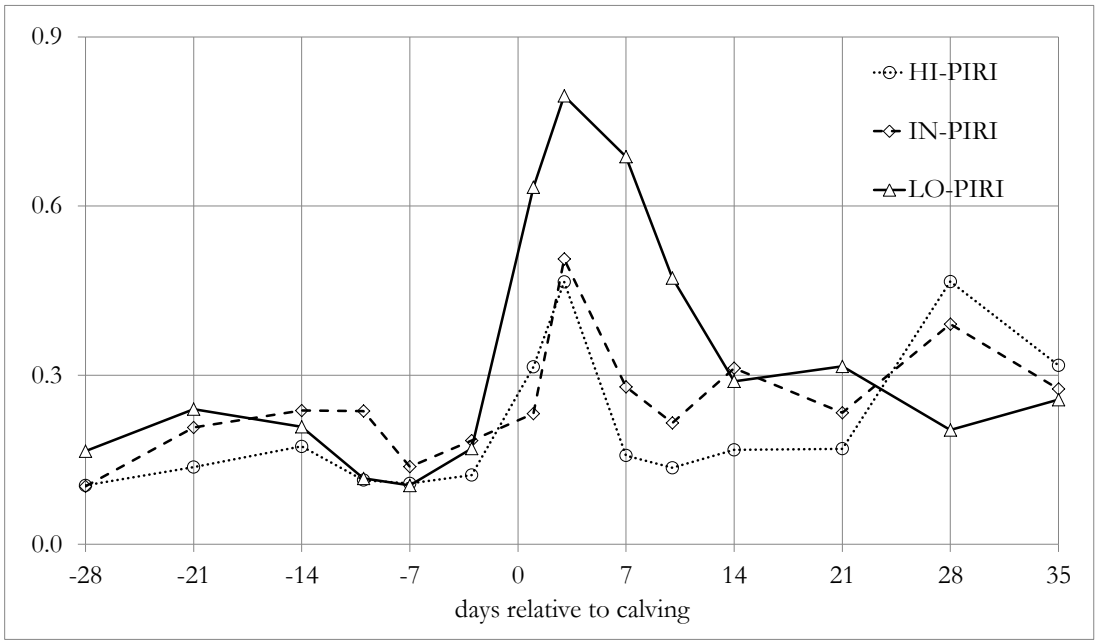
Figure 10 - Pattern of changes of alkaline phosphatase [U/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	**	**	*	*	***	**	+		*	+	+		+	
HI-PIRI vs IN-PIRI	*	**	**	**	***	**	*		+	*				
IN-PIRI vs LO-PIRI														

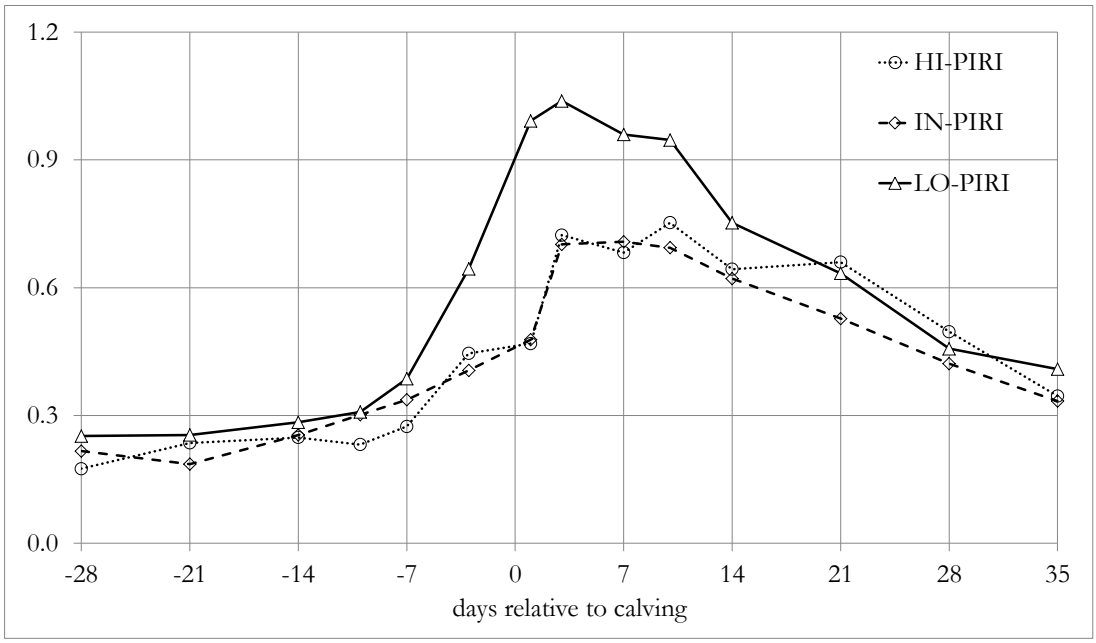
Figure 11 - Pattern of changes of haptoglobin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI							*	**	***	**		+		
HI-PIRI vs IN-PIRI											*			
IN-PIRI vs LO-PIRI							**	*	***	+			+	

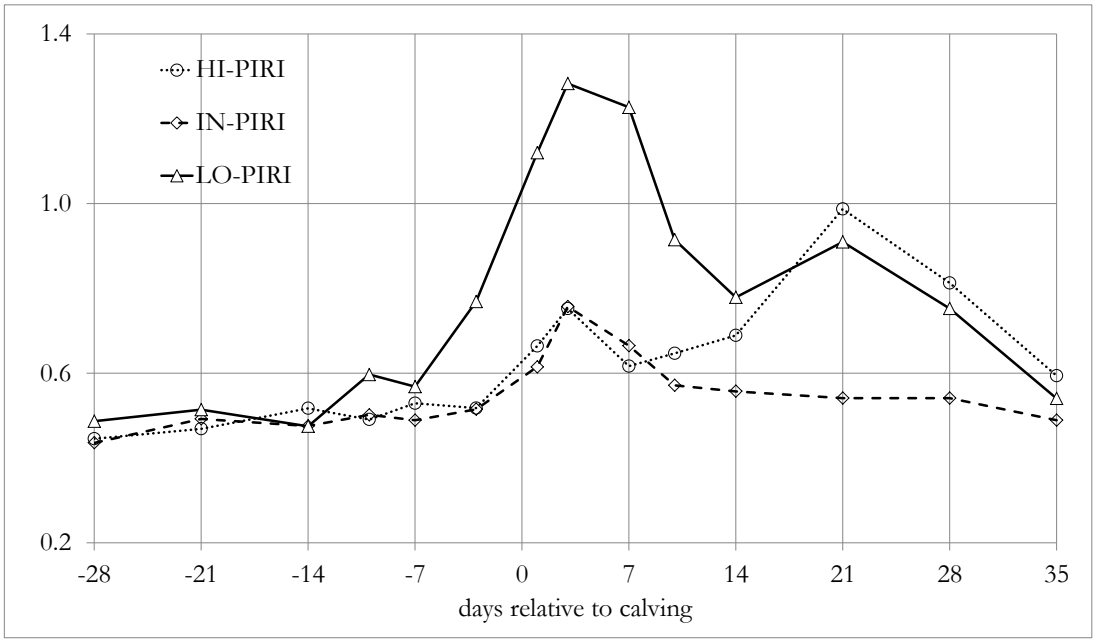
Figure 12 - Pattern of changes of NEFA [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						+	***	**	**	+				
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI						*	***	***	**	*				

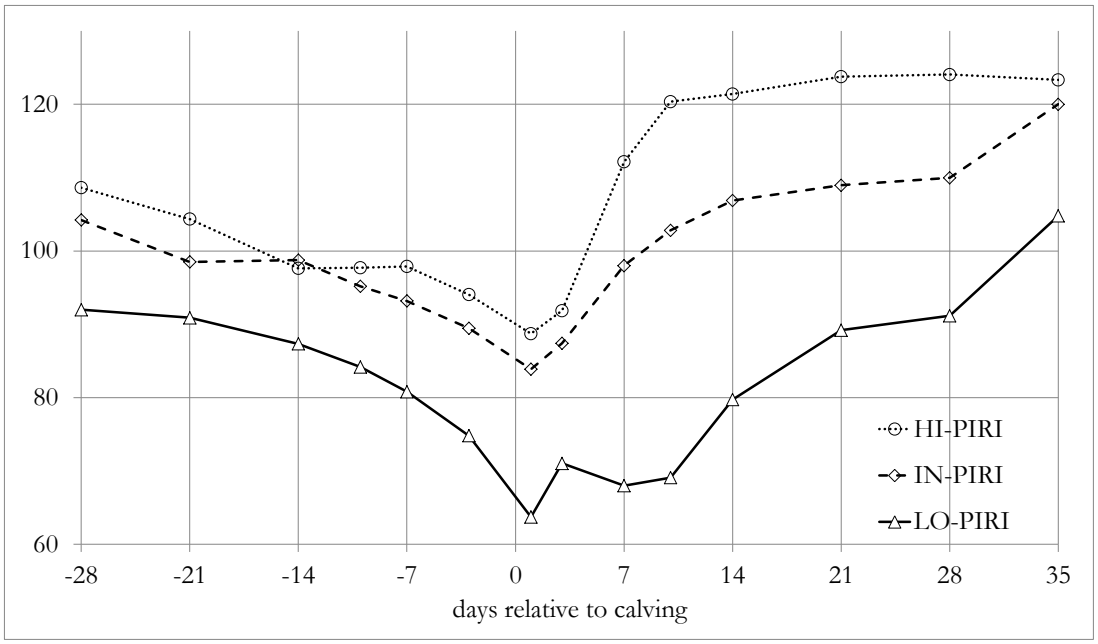
Figure 13 - Pattern of changes of β-hydroxybutyric acid [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						*	***	**	***					
HI-PIRI vs IN-PIRI												*		
IN-PIRI vs LO-PIRI						*	***	***	***	+		*	+	

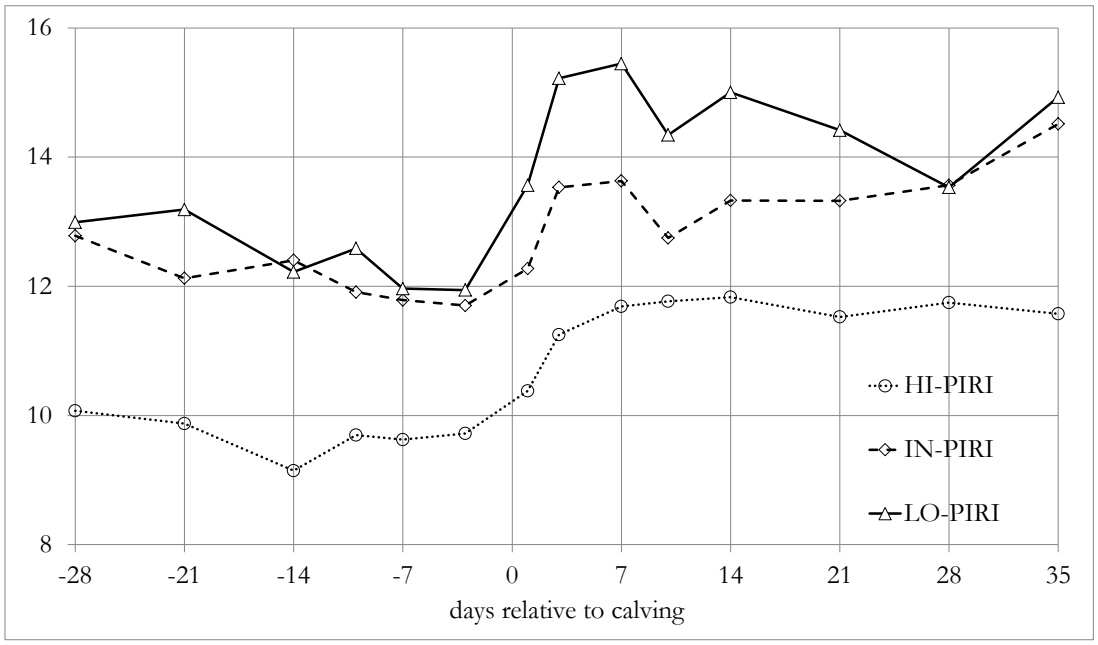
Figure 14 - Pattern of changes of paraoxonase [U/mL] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	*	+		+	*	**	**	**	***	***	***	***	***	**
HI-PIRI vs IN-PIRI									*	*	*	*	*	
IN-PIRI vs LO-PIRI	+				+	*	**	*	***	***	***	**	**	

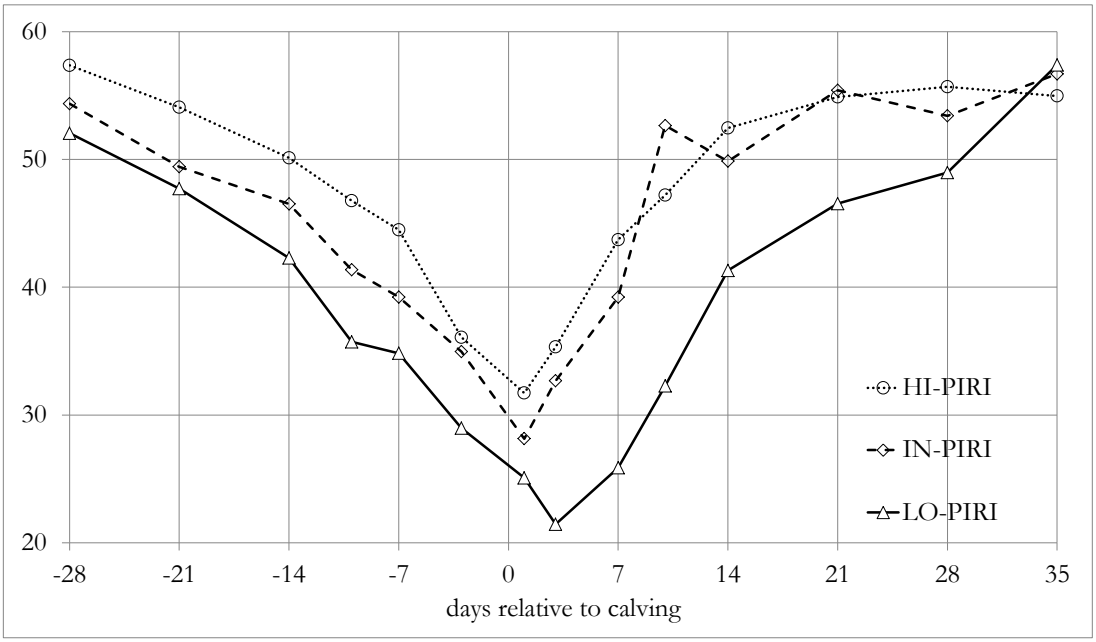
Figure 15 - Pattern of changes of total Reactive Oxygen Metabolites [mg H_2O_2/100 mL] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	**	***	***	**	*	*	***	***	***	**	***	**	+	**
HI-PIRI vs IN-PIRI	**	**	***	*	*	*	*	*	*			+	*	**
IN-PIRI vs LO-PIRI								+	*	+	+			

Figure 16 - Pattern of changes of retinol [μmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI								**	***	***	*			
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI								*	**	**	+	+		

CHAPTER 2

Attenuation of inflammatory response phenomena in periparturient dairy cows by the administration of an ω3 rumen protected supplement containing vitamin E

This chapter has been published as a paper by the *Italian Journal of Animal Science*, vol. 10:e61, n.4 (2011); pagg. 277-286. DOI: 10.4081/ijas.2011.e61 http://www.aspajournal.it/index.php/ijas/article/view/ijas.2011.e61

Italian Journal of Animal Science 2011; volume 10:e61

PAPER

Attenuation of inflammatory response phenomena in periparturient dairy cows by the administration of an ω3 rumen protected supplement containing vitamin E

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Abstract

The aim of this research was to study the consequences of ω3 fatty acids (FA) administration around calving on inflammatory response and on productive performances. In this period dairy cows undergo a metabolic challenge, coming with an inflammatory-like status triggering the release of pro-inflammatory mediators (e.g. eicosanoids, cytokines). rumen-protected ω3 FA in transition period seems to attenuate the effects of subclinical inflammations and to improve the energy balance.

Introduction

The endocrine-immune-metabolic system of periparturient dairy cows is strongly challenged and cows are often unable to react properly. For this reason, cows in their first two months of lactation undergo the highest frequency of metabolic (e.g. milk fever, ketosis, liver lipidosis) and infectious diseases (Goff *et al*., 1997; LeBlanc *et al*., 2006). The latter often occurs after calving and seems mainly related to the inability of cows to readily adapt their biologic systems to the new situation, causing an increased susceptibility to environmental stimuli (e.g. stress, pathogens, feeding changes; Drackley *et al*. 2005). In any case, these troubles can induce the release of pro-inflammatory cytokines (e.g. IL-1; IL-6, TNF-α), which are responsible of reduced appetite, fever, increased energy expenditures and fat mobilization (Elsasser *et al*., 1995). In addition, the ability of cows to increase their feed intake at the beginning of lactation is much lower than milk yield rise, resulting in a

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Key words: Dairy cow, ω3 diet supplement, Inflammation, Plasma ω3 fatty acids.

Acknowledgments: the authors acknowledge Co.S.A.P.A.M. scrl (Peschiera Borromeo, MI, Italy) for product purchase and Mr. Tiziano Geroldi for support provided during the experiment carrying out.

Received for publication: 13 July 2011.
Revision received: 11 October 2011.
Accepted for publication: 15 October 2011.

Italian Journal of Animal Science 2011; 10:e61
doi:10.4081/ijas.2011.e61

et al., 2008). On the contrary, the oral administration of IFN-α (a suggested anti-inflammatory cytokine) in the last three weeks of preg-

ABSTRACT

The aim of this research was to study the consequences of ω3 fatty acids (FA) administration around calving on inflammatory response and on productive performances. In this period dairy cows undergo a metabolic challenge, coming with an inflammatory-like status triggering the release of pro-inflammatory mediators (e.g. eicosanoids, cytokines). Eicosanoids synthesis may be modulated by altering the ratio of their precursors (ω3 and ω6 FA). Ten cows received 22 g/d of rumen-protected ω3 FA from -21 to +21 days from calving (OPT), while 10 (CTR) received no supplement. Cows were frequently monitored for health status, body condition score (BCS), blood (metabolic, inflammatory and FA profiles), milk yield. OPT (*vs* CTR) showed a similar milk production, a numerically smaller BCS drop, lower postpartum levels of non-esterified fatty acids ($P<0.05$), β-hydroxybutyric acid ($P<0.1$) and creatinine ($P<0.05$), suggesting a milder post-calving reserves mobilization. All cows underwent an inflammatory condition around calving, but OPT showed a milder response, as suggested by lower levels of bilirubin ($P<0.05$), and by the higher level of Liver Functionality Index ($P<0.10$). Plasma concentration of ω3 FA (eicosapentaenoic and docosahexaenoic acids) increased in OPT during treatment ($P<0.01$ *vs* CTR). Since ω3 FA are the main replacers of arachidonic acid in membrane phospholipids, their increased levels in plasma of OPT cows may have cut the formation of arachidonic-derivatives (pro-inflammatory mediators), countering the beginning of the inflammation. Hence, the administration of rumen-protected ω3 FA in transition period seems to attenuate the effects of subclinical inflammations and to improve the energy balance.

INTRODUCTION

The endocrine-immune-metabolic system of periparturient dairy cows is strongly challenged and cows are often unable to react properly. For this reason, cows in their first two months of lactation undergo the highest frequency of metabolic (e.g.

milk fever, ketosis, liver lipidosis) and infectious diseases (Goff *et al.*, 1997; LeBlanc *et al.*, 2006). The latter often occurs after calving and seems mainly related to the inability of cows to readily adapt their biologic systems to the new situation, causing an increased susceptibility to environmental stimuli (e.g. stress, pathogens, feeding changes; Drackley *et al.* 2005). In any case, these troubles can induce the release of pro-inflammatory cytokines (e.g. IL-1; IL-6, TNF-□), which are responsible of reduced appetite, fever, increased energy expenditures and fat mobilization (Elsasser *et al.*, 1995). In addition, the ability of cows to increase their feed intake at the beginning of lactation is much lower than milk yield rise, resulting in a marked negative energy balance that cause a noticeable lipomobilization (Drackley, 1999; Drackley *et al.*, 2001). These events can trigger new and more serious pathologies as well as many endocrine-metabolic modifications. Indeed, pro-inflammatory cytokines could reiterate the inflammatory phenomena, if not promptly stopped (Dinarello, 2000), and the organism's reaction of response to such a negative situation may be harmful itself if too strong and long.

Periparturient cows, even without clinical signs, experience an inflammatory-like condition around calving, shown by the marked rise of positive acute phase proteins at calving or immediately after, as well as the reduction of negative acute phase proteins (Bionaz *et al.*, 2007; Bertoni, 2008). Moreover, we recently demonstrated that cows with higher pro-inflammatory cytokines before calving (Trevisi *et al.*, 2009; Trevisi *et al.*, 2011), had also lower levels of feed intake, milk yield and some negative acute phase proteins (–APP). Some attempts to modulate inflammatory response in this critical period have been done. Non-steroidal anti-inflammatory drugs (NSAIDs) administration in post-partum period improved both metabolic status and performance of dairy cows (Trevisi *et al.*, 2005; Trevisi *et al.*, 2008). On the contrary, the oral administration of IFN-α (a suggested anti-inflammatory cytokine) in the last three weeks of pregnancy (Trevisi *et al.*, 2009) failed to display any metabolic or performance improvement. The issue still remains relevant in the management of dairy cows, therefore additional tools able to modulate inflammatory response, avoiding negative aspects and keeping positive ones, are

needed. In this respect, potential candidates might be the conjugated linoleic acid (Butz *et al.* 2007; Ringseis *et al.* 2008), phytoextracts (Choi *et al.*, 2003; Trevisi *et al.*, 2008), and polyunsaturated fatty acids (Calder, 2006a; Ballou *et al.*, 2009), in particular the long chain ω3.

These fatty acids are involved in the modulation of the inflammatory process; ω3 can mitigate the inflammatory response, by decreasing the formation of pro-inflammatory prostaglandins (series 2 and 4), as well as minimizing the formation of the Nuclear Factor kB (NFkB; Rimbach *et al.*, 2002; Thurnham, 2004). This nuclear factor enhances the gene expression leading to the formation of pro-inflammatory cytokines and cyclooxygenases (COX; i.e. enzymes producing eicosanoids, mediators of inflammation). Antioxidants (e.g. flavonoids), another class of nutraceutical substances able to relieve the inflammatory process, also use a mechanism that inhibits COX-2 activity (O'Leary *et al.*, 2004). However, not always ω3 fatty acids (FA) have been shown to be effective in this regard. Farran *et al.* (2008) administered flax seed to steers and observed an increase of ω3 FA plasma level but, after LPS challenge, no significant effects on haptoglobin and fibrinogen were evenly observed. Ballou *et al.* (2009) administered unprotected fish oil to periparturient dairy cows and observed an increase of ω3 FA concentration in liver phospholipids, but unfortunately no parameters related to inflammatory status were assessed in this research.

Furthermore, the occurrence of an inflammatory stimulus triggers a set of reactions at local level producing several mediators, like cytokines (proteins) and eicosanoids (prostaglandins=PG, leukotrienes=LT, lipoxins=LP and thromboxanes=TX, lipids). These molecules play a fundamental role in the regulation of the duration and intensity of inflammatory response (Kinsella *et al.*, 1990; Tilley, 2001) and can be divided according to their activity, in anti- and pro-inflammatory eicosanoids. Prostaglandins mostly derive from arachidonic acid (20:4 ω6; ARA) that, through two different pathways involving 5-lypooxygenase (LOX) and COX-2 enzymes, generates several mediators (e.g. hydroperoxy-eicosatetraenoic acid, prostaglandin from 2 and 4 series; Calder, 2006) that exert pro-inflammatory

activities. In competition with ARA cascade, two other cascades originated from eicosapentaenoic acid (EPA; 20:5 ω3) and dihomo-γ-linoleic acid (DGLA; 20:3 ω6), involved in the production of eicosanoids with anti-inflammatory (or less inflammatory) activity (series 1, 3, 5 PG, TX, LT). Moreover, docosahexaenoic acid (DHA; 22:6 ω3), an important EPA-derived fatty acid, can compete for COX-2 and LOX enzymes, forming mediators (protectins and resolvins of D series) taking part in the resolution phase of inflammation. Therefore, a reduction in ω6/ω3 ratio means higher incorporation of ω3 FA in phospholipids at the expense of ω6, resulting in a decrease of pro-inflammatory eicosanoids production. According to this mechanism, an adequate supplementation of long chain ω3 FA before inflammatory events, seems useful to optimize the inflammatory response and to reduce its adverse effects by changing PG production ratio, as previously suggested (Calder, 2002; Mori *et al.*, 2004). In this context, it appears noteworthy to investigate the effects of the prolonged administration of long chain ω3 FA before and during the inflammatory challenge. Thus, the aim of the research was to examine the role of these ω3 FA administered from 3 weeks before to 3 weeks after calving on the common inflammatory response occurring during the peripartum of dairy cows and on their physiological adaptation and performances.

MATERIALS AND METHODS

This study complied with Italian and European rules on animal experimentation and ethics.

BARN CHARACTERISTICS, ANIMALS AND TREATMENT

The trial took place in the Università Cattolica experimental barn (CERZOO) located in the Northern Italy (Piacenza) during autumn-winter season and involved 20 multiparous Friesian dairy cows reared in loose stall with cubicles and milked twice a day (12 h gap). Dry and lactating cows were fed with two different TMR diets described in Table 1. Cows housed in the same pen and with low somatic cell

count at dry off were attributed to two homogeneous groups, according to body condition, calving period, production potential, parity body weight. The first one (n=9, OPT) received an algae-derived oil administration of 112.5 g/cow/d, corresponding to 250 g/cow/d of commercial product (Optimate, Agritech, Tipperary, Ireland; imported and distributed by Cosapam Soc. Coop. S.r.l. Peschiera Borromeo, Italy). The supplement was individually offered to each cow, without any possibility of competition and it was completely ingested in a few minutes. It was fed once a day immediately before the TMR distribution, mixed with about 0.5 kg of fresh cows TMR. The administration of algae-derived oil started around the 21st day before the expected calving date and lasted until 21 days in milk (DIM). CTR cows (n=11) were also daily captured as OPT group and before calving received 0.75 kg of fresh cows TMR.

The oil was rumen-protected by the NET (Nutrient Enrobing Technology; Agritech, Tipperary, Ireland) technology. The whole detailed FA composition of the product is described in Table 2. The daily administration contained per cow about 18.5 g of ω3 (34.5% of EPA, 34.1% of DHA, 31.4% others) and 5.5 g of ω6 FA, with an ω6/ω3 ratio of 0.3. Noteworthy is the presence of 4000 IU/kg of vitamin E in the supplement, which corresponds to an administration of 1000 U/cow/d.

CLINICAL CHECKS

During the experiment, the animals health conditions were checked every day by general inspections and monitored by a computerized Afimilk system (S.A.E. Afikim, Kibbutz Afikim, Israel), based on automatic recording of activity and milk production through a leg transponder. In addition, rectal temperature was measured the day after calving and twice a week from 14 day before to 14 days after calving. Cows were also submitted to a thorough gynecological examination at about 10 and 30 DIM. The dry matter intake of dry and lactating cows pens was fortnightly estimated. Moreover, each cow was submitted to the following assessments: I) body condition scoring, using a 5-point scale (ADAS, 1986), starting about 35 days before the expected calving date and, then, every 14 days to 42 DIM; II) milk yield and its

conductivity, measured and recorded by the Afimilk computer-controlled automated system at every milking; III) milk samples at 7, 14, 28, 42 DIM, from the morning milking, in order to assess fat, protein and lactose content (MilkoScan FT 120, Foss Electric, Hillerød, Denmark), and somatic cell count (SCC; Fossomatic 180, Foss Electric); iv) blood samples, collected approximately at -28, -21 (pre-treatment), -14, -10, -7, -3 (before calving), 1 (day after calving), 3, 7, 10, 14, 21 (end of treatment), 28, 35, 42 (post-treatment) days from calving.

Every sample was collected in the morning before feeding, from a jugular vein and in two vacuum tubes (Vacuette, Greiner Bio-One GmbH, Kremsmunster, Austria), one containing lithium-heparin as anticoagulant, and the other silicon (no anticoagulant). Lithium-heparin tubes were cooled immediately after collection in an ice-water bath until their arrival in laboratory. After a small aliquot of blood was taken to determine packed cell volume (centrifugation at 12000 RPM for 11 min), tubes were centrifuged at 3520 × g for 16 min at 4°C; plasma samples were divided in 5 aliquots, stored at -20°C (n=4) or -80°C (n=1).

In accordance with Bionaz *et al.* (2007) on these samples were determined: I) inflammatory response indexes: positive acute phase proteins (+APP; haptoglobin, ceruloplasmin) and negative acute phase proteins (-APP; albumin, cholesterol as lipoprotein index); II) liver indexes: total bilirubin, aspartate amino-tranferase (GOT), γ-glutamil transferase (GGT), alkaline phosphatase (ALP), paraoxonase (PON); III) energy metabolism indexes: glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB); IV) protein metabolism indexes: urea, creatinine (Finco, 1997); V) oxidative stress response: total reactive oxygen metabolites (ROM); VI) minerals (Ca, P, Mg, Na, K, Cl, Zn); VII) vitamins: retinol (index of its carrier protein; Blomhoff *et al.*, 1987; Erikstrup *et al.*, 2009), tocopherol, β-carotene; VIII) other parameters (total proteins, globulins).

Glucose, total protein, albumin, total cholesterol, total bilirubin, creatinine, urea, Ca, P, Mg, GOT, GGT and ALP were detected at 37°C by a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) using commercial kits purchased by Instrumentation Laboratory (IL Test), as previously described by

Bionaz *et al.* (2007). Globulins were calculated as the difference between total protein and albumin. Electrolytes (Na+,K+, and Cl−) were detected by the potentiometer method (Ion Selective Electrode connected to ILAB 600). Zn and NEFA were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). Haptoglobin, BHB, and ceruloplasmin were analyzed using methods described by Bertoni *et al.* (1998), adapted to ILAB 600 conditions. Plasma retinol, α-tocopherol and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2.3 μm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL, USA); a UV detector set at 325 nm (for retinol) or 290 nm (for tocopherol); and 80:20 methanol:tetrahydrofurane as the mobile phase. ROM were measured using a method patented by Diacron International S.r.l. (Grosseto, Italy) and expressed as mg of hydrogen peroxide per 100 mL of plasma. Plasma PON activity was assessed by adapting the method of Ferré *et al.* (2002) to the ILAB 600, as described by Bionaz *et al.* (2007). Plasma FA were detected in plasma at -21, -3, 21 and 42 days from calving and expressed as % of total FA measured in accordance with the method described by Visioli *et al.* (2003): for first lipids were extracted (Folch *et al.*, 1957) and quantified by microgravimetry. Later, FA methyl esters were prepared from total lipids extracts, and their plasma concentrations were determined by gas chromatography made quantitative by the use of 19:0 and 21:0 as internal standards.

DATA HANDLING AND STATISTICAL ANALYSIS

All data in this paper are presented in the form: mean ± standard deviation.

Eight cows (5 in CTR and 3 in OPT) received antibiotic and/or anti-inflammatory treatments, thus they have been excluded from the statistical analysis.

Data were submitted to repeated measures variance analysis using a mixed model (MIXED procedure, SAS Inst. Inc., Cary, NC; Littell *et al.*, 1998). Before analysis the normality of distribution was verified for each parameter through skewness and kurtosis calculation according to the Shapiro test (SAS Inst. Inc.). When necessary, data were normalized through logarithmic (cholesterol, haptoglobin, BHB, DHA),

quadratic (glucose, milk production, % ω3 HUFA/total HUFA) or root-square (bilirubin, DHA/ARA ratio) transformations. For body condition score (BCS) analysis, the initial value was used as covariate.

The layout of our statistical model can be summarized as follows:

$Y_{iklm} = \mu + G_i + T_k + GT_{ik} + B_{l(im)} + e_{iklmn}$

where Y_{ijklm} = m^{th} observation of the l^{th} cow B_l within the i^{th} treatment G_i, at the k^{th} time to calving T_k; μ = total average; G_i = effect of the i^{th} treatment (2 treatments: OPT and CTR); T_k = effect of the k^{th} time to calving (the number of levels being defined as a function of pregnancy phase and actual variable; levels were thus respectively 15, 50, and 8 for blood parameters, milk production, and BCS data); GT_{ik} = effect of the interaction between the i^{th} treatment and the k^{th} time to calving; $B_{l(im)}$ = fixed effect of the l^{th} cow within the i^{th} treatment; e_{iklmn} = random effect or error.

The analysis was carried out using 3 covariance structures: Autoregressive, Compound symmetry, and Spatial Power. These were ranked according to their AIC (Akaike's Information Criterion; Akaike, 1974), choosing as better the lowest one (Littell *et al.*, 1998). For each treatment, least squares means were computed, and preplanned pairwise comparisons (PDIFF option, SAS Inst. Inc.) were carried out when the F-test of one of the main factors (time, treatment, treatment×time) was significant at $P<0.10$. Statistical significance was designated as $P<0.05$, tendencies were declared at $P<0.10$.

In order to assess the different response of each cow to inflammatory status during the first month of lactation, two complex indexes (Liver Functionality Index, LFI and Liver Activity Index, LAI) were calculated, basing on some blood parameters related to inflammation. LFI (Bertoni *et al.*, 2006) considers blood changes of albumin, lipoproteins (measured as total cholesterol) and total bilirubin (its clearance enzymes are synthesized by the liver) from the 3rd to the 28th DIM. LFI calculation is carried out in 2 steps; the first one considers the values of the three parameters observed at 3 DIM (V3) and changes between 3 and 28 (V28) DIM. For albumin and cholesterol these two components equally concurred (50%)

to the partial LFI result (Alb-I and Chol-I =0.5×V3+0.5× (V28-V3)), while for bilirubin the level at 3 DIM represents 67% and the reduction between 3 and 28 DIM the remaining 33% of the partial LFI index (Bili-I=0.67×V3+0.33× (V28-V3)). In the second step, these partial indexes were standardized according to average values observed in healthy cows, and LFI was calculated according to the following formula:

LFI = [(albumin index – 17.71)/1.08 + (cholesterol index – 2.57)/0.43 – (bilirubin index – 6.08)/2.17].

The LAI index (Bertoni *et al.*, 2008) includes the average blood level at 7, 14 and 28 DIM of some proteins synthesized by the liver: albumin, lipoproteins (measured as total cholesterol), and Retinol Binding Protein (RBP, measured as retinol). Data of these 3 blood parameters were transformed into units of standard deviation obtained for each cow as follows: the mean value of the herd population of each plasma parameter (albumin, total cholesterol, and RBP) was subtracted from each cow value at 7, 14, and 28 DIM and divided by the corresponding standard deviation. The final LAI value of each cow is the result of the arithmetical mean of all the partial values. Thus, low LFI or LAI values are associated with a large inflammatory response and vice versa.

Finally, Pearson correlations (PROC CORR of SAS) among all parameters were calculated for the whole period considering OPT and CTR cows.

RESULTS

HEALTH STATUS

Approximately 40% of animals showed at least one severe clinical health problem during the experiment: 3 metritis, 1 retained placenta, 1 mastitis, 1 milk fever in CTR group (5 cows) and 2 metritis and 2 retained placenta in OPT group (3 cows). All these cows received antibiotic and/or anti-inflammatory treatments, thus they have been excluded from the statistical analysis. The frequency of severe health problems seems similar in the two groups, thus their origin should not be due to the

treatment. The inclusion of these animals in the analysis could have been cause of high data variability, especially for blood parameters of inflammation. In particular the excluded cows showed very high levels of haptoglobin and bilirubin around the clinical symptoms, followed by a reduction after drug treatments. Therefore, results will be discussed on 6 animals for each group which showed no clinical problems or just with mild and short-lived troubles (not affecting plasma indexes of inflammation), and which did not receive any drug treatment. Average milk production and body condition score are not significantly affected by the exclusion of ill cows.

FEEDING

Individual dry matter intake was not recorded, but the mean feed intake of dry cows (about 12.5 kg/d) and of lactating cows (about 23.5 kg/d) observed during the trial were near the requirements suggested by NRC (2001). In the weeks closest to calving intakes were likely much lower. The diets fed during the experiment are shown in detail in Table 1.

PERFORMANCE

No significant differences were found in milk yield: the mean in the first month of lactation was 37.5±3.1 in CTR *vs* 37.9±3.5 kg/d in OPT. Somatic cell count remained very low in all the cows, demonstrating a healthy mammary status during the trial (80±68 in CTR *vs* 30±10 cells/μl in OPT). No relevant differences between groups were detected in the milk lactose and protein content. Milk fat was numerically lower in OPT group until the 28 DIM (3.57±0.62 *vs* 4.04±0.97 % of CTR) and statistically different at 7 DIM (3.92±0.69 *vs* 4.99±0.51 % in CTR; $P<0.05$).

At the beginning of the experiment (-28 day before calving), mean BCS was close to the expected value for that period (2.5-3.0 points; ADAS, 1986). After calving, BCS showed in both groups a drop. However, the fall was numerically greater in

CTR, resulting in a marked decrease from 3 days before the calving to 42 DIM (-0.63±0.17 and -0.49±0.23 points in CTR and OPT respectively; NS).

Rectal temperatures did not show any difference between the two groups, with a general mean value of 38.9°C from 14 days before calving to 14 DIM. No cows showed fever (temperature higher than 39.5°C).

BLOOD PARAMETERS

Energy and protein metabolism indexes

Glucose trend was characterized by the typical decrease after calving. Levels (Figure 1) were steady and similar in both groups until 21 days before calving (on average 4.15±0.21 mmol/L). From 14 days before calving CTR group started decreasing, while OPT remained steady until 3 days before calving and then fell. At 3 DIM the difference between groups was the greatest (3.85±0.27 in OPT *vs* 3.18±0.73 mmol/L in CTR; $P<0.05$), but subsequently disappeared.

Before ω3 FA administration, BHB and NEFA levels were similar in the two groups. Approaching the calving, NEFA level (Figure 1) increased in both groups, but more markedly in CTR. The maximum difference of NEFA between groups was reached 3 days before calving (0.38±0.19 in OPT *vs* 0.77±0.38 mmol/L in CTR; $P<0.01$), then the difference gradually disappeared. At -10 DIM, BHB showed a very strong increase in CTR group, but not in OPT. BHB in CTR achieved a peak at 3 DIM (1.62±1.48 *vs* 0.57±0.10 mmol/L in OPT), while in OPT peaked at 10 DIM (0.94±0.78 mmol/L). Despite the large numerical differences between the groups, BHB did not show any statistically significant difference. This result may be due to the large data variability caused by the presence, immediately after calving, of some subclinical cases of ketosis (cows with at least one value ≥1.2 mmol/L; Goff *et al.*, 1997): 3 cows in CTR and one in OPT.

Creatinine gradually increased before calving in CTR while remained quite stable in OPT (104.6±10.6 in OPT *vs* 118.6±10.7 mmol/L in CTR at 7 days before

calving; P<0.05). After calving creatinine decreased in both groups, but quicker in CTR and statistical differences between groups disappeared from 7 DIM.

Inflammatory status indexes

Among +APP, haptoglobin (Figure 2) showed in both groups low and steady values until calving (<0.1 g/L); then the typical peak occurred at 3 DIM (0.56±0.42 in OPT *vs* 0.69±0.31 g/L in CTR; NS). OPT levels had a quicker recovery to pre-calving values and remained lower than in CTR during the whole lactation period, but no significant differences were observed. Ceruloplasmin trend was similar between the two groups during pregnancy (2.56±0.32 in OPT *vs* 2.59±0.58 μmol/L in CTR, mean of the last month of pregnancy; NS). After calving CTR showed a numerically higher level of ceruloplasmin, and the difference *vs* OPT progressively increased (3.03±0.36 in OPT *vs* 3.27±0.42 μmol/L in CTR at 7 DIM; NS). Globulins showed numerically higher levels in CTR starting from 7 days before calving with increasing difference to 42 DIM (NS), while ROM did not show any difference.

Among –APP, albumins levels (Figure 3) were similar before the beginning of the ω3 FA administration in the two groups. During transition period the general trends were similar between groups: gradual decrease during the last 3 weeks of pregnancy and first DIM and then progressive raise. Nevertheless, in CTR the reduction was quicker and the recovery slower than in OPT, implying numerically lower levels than OPT in the first month of lactation. Cholesterol level (Figure 2) was similar in the groups before ω3 FA supplementation. Ten days before calving, cholesterol level became statistically higher in OPT (P<0.05) *vs* CTR. After calving the difference between groups increased, showing statistically significance until 35 DIM (5.58±0.65 in OPT *vs* 4.50±0.50 mmol/L in CTR; P<0.05). Bilirubin may also be considered as an index of the protein systems synthesized by the liver, since its clearance relies on some of these proteins. Bilirubin showed a similar pattern of changes in both groups, nevertheless, its level in OPT was lower during the whole experiment. Around calving (from 10 days before calving to +3 DIM) the difference

vs CTR increased and it became statistically significant, mainly immediately after calving (6.21±4.11 in OPT *vs* 10.11±5.52 mmol/L in CTR; P<0.05 at 3 DIM). Also retinol (index of Retinol Binding Protein) displayed the typical drop at calving time in all the cows, followed by a recovery. OPT group showed a numerically quicker increase after calving (NS).

LFI and LAI indexes

LFI is an aggregate index of the liver functionality and considers the changes of some negative acute phase reactants. In OPT group, LFI showed a mean value of 1.69±2.18, while in CTR group had a negative mean value: -1.33±3.23 (P<0.10). Despite the high variability, OPT had only one cow characterized by a negative value of LFI index, whereas CTR showed only two cows with a positive LFI value. LAI index confirms the above-mentioned results (0.17±0.79 in OPT *vs* -0.17±0.47 in CTR), but the difference between the groups appeared to be lower.

Other parameters

Plasma tocopherol level (Figure 3) was similar before the experiments in the two groups. In OPT, tocopherol showed a slight rise after the beginning of the ω3 FA administration, while markedly decreased in CTR, turning out significantly different (2.72±0.59 in OPT *vs* 1.53±0.64 μg/mL in CTR; P<0.001 at 3 days before calving). After calving tocopherol fell down also in OPT group, and the difference disappeared at the 3rd DIM. Subsequently, tocopherol level increased faster in ω3-supplemented cows and the difference between groups rose from 7 to 14 DIM (2.27±0.58 in OPT *vs* 1.56±0.16 μg/mL in CTR; P<0.05 at 14 DIM). At 21 DIM, end of the ω3 administration, the difference between the two groups was no more significant, but the level remained numerically higher in OPT until 28 DIM. Considering all cows and the whole period, plasma tocopherol resulted well correlated with cholesterol (r=0.62; P<0.01).

Plasma fatty acids

In Table 3 are presented the changes of plasma FA during the experiment. As expected, plasma levels of the most important ω3 FA (EPA and DHA; Figure 4) increased during their dietary administration and turned out higher in OPT than in CTR for about the whole administration period, while were similar before. In detail, EPA reached the highest level before calving (1.08 *vs* 0.53 % of plasma FA in CTR at 3 days before calving), while DHA maintained a higher level for all the supplementation period ($P<0.001$ until 21 DIM) and a tendency 14 days after the suspension of treatment (0.12 *vs* 0.05 % of plasma FA in CTR, at 42 DIM, $P<0.10$). Moreover, also docosapentaenoic acid (DPA, 22:5 ω3) – interconverted between EPA and DHA (Bénistant *et al.*, 1996) – was higher in OPT group during the ω3 administration, but it reached a statistical evidence only at 21 DIM (0.52 *vs* 0.74 % of plasma FA in CTR, $P<0.05$). Anyway, the ω3 fatty acid present in the largest amount (73% of all ω3) in plasma was the α-linolenic acid (ALA, 18:3 ω3): 3.89 % of all plasma FA on average for the whole period and all cows. ALA (Table 3) was similar between groups at 21 days before calving (pre-treatment data item); after the beginning of the administration, it reached numerically higher levels in the OPT group, keeping the difference for the whole following period (NS). Three days before calving, ALA level in plasma showed a marked increase in both groups (4.96±0.14 in OPT *vs* 4.71±1.01 % of plasma FA in CTR, NS). After calving ALA decreased to levels lower than in the dry period. Considering all cows and the whole period, ALA was correlated with cholesterol ($r=0.67$; $P<0.01$) and thus with lipoproteins. DHA/ALA ratio (Agostoni *et al.*, 2008), an index of the whole ω3 FA pathway, was equal in the two groups before and after treatment. With the beginning of the ω3 administration, DHA/ALA increased in OPT group until 21 DIM, when it reached a statistically significant difference (0.09±0.01 *vs* 0.03±0.01 in CTR; $P<0.001$). Summarizing all data on FA and taking in account the total plasma ω3 Highly Unsaturated Fatty Acids (HUFA), which include FA with number of $C\geq20$ (EPA, DPA, DHA) out of all the determined FA, OPT showed similar values to CTR before (21 days before calving) and after (+42) the FA administration, but

higher levels during treatment period (peak at 3 days before calving: 2.03±0.19 in OPT *vs* 1.15±0.32 % of all FA in CTR; P<0.01). Interestingly, the rate of ω3 HUFA on the total HUFA rose in both groups from -21 to -3 days to calving, but more markedly in OPT. OPT level continued to rise until the end of ω3 administration (+21 DIM), reaching a significant difference *vs* CTR (26.67 *vs* 19.55% of HUFA; P<0.01). At 42 DIM no differences were detected between groups.

The rate of ω6 HUFA on the total HUFA showed an opposite outcome in respect to ω3 HUFA, but only with numerical differences between groups. The overall trend in all the cows showed the highest value 21 days before calving (7.75±0.68 % of all FA), a progressive prepartum drop and steady values until 42 DIM (5.08±0.70 %). The two most important ω6 HUFA, DGLA (diomo-γ-linolenic acid) and ARA (arachidonic acid), followed a similar trend: a prepartum decrease, and a recovery after calving. Despite the similar trend, ARA level was numerically higher in CTR *vs* OPT at 42 DIM. Indeed, DGLA showed higher levels in OPT group since before the beginning of the experiment (P<0.05 at 21 days before calving) and kept a numerical difference for all the following period.

DISCUSSION

Our research mainly deals with plasma parameters related to metabolism and inflammation; unfortunately, the trend and variability of these parameters (and likely also others) are markedly modified by serious health problems. Hence, in order to reduce this kind of interference, cows suffering clinical problems (likely not ascribable to the treatment) were cut off (4 per group).

OPT cows received 112.5 g/d of fat in addition to the diet. However, we did not check the individual feed intake and consequently it is not possible to determine the real differences in feed and energy intake among the cows. Thus our results may be influenced either by FA composition either by the energy intake, which is not only related to the supplement.

In our experiment, the use of algae-derived oil caused a slight reduction of milk fat content during the 1st month of lactation. This could be due to a partial release of unsaturated FA into the rumen caused by an inadequate protection, and consequently an incomplete biohydrogenation and trans FA production. As well known, the absorption of some trans FA in the gut cause the inhibition of milk fat synthesis in mammary gland (Bauman *et al.*, 2000). This is in agreement with the results of Mattos *et al.* (2004), that administered to periparturient cows unprotected ω3 FA (128 g/d) and observed a lower milk fat content compared to a control group (4.35 *vs* 5.20 %). Nevertheless, the lower milk fat content in OPT could be partly due to the less strong lipolysis, as suggested by the lower levels of NEFA and the numerically smaller reduction of BCS in the first month of lactation, that supplied a lower amount of precursors for mammary fat synthesis.

Despite the possible slight reduction of the ω3 FA availability in gut caused by this last event, we observed a general plasma increase of total ω3 FA in OPT group ($P<0.01$), especially before calving, confirming their absorption and their inclusion into the phospholipids of plasma lipoproteins. On the contrary, the CTR group showed a slight reduction at calving, in agreement with the trend previously shown by Bertoni *et al.* (2006).

Within plasma ω3 FA, ALA was the most abundant, and similar in both groups; in fact the content of supplement is lower (Table 2) than the intake with feed. The significant correlation between ALA and cholesterol (an index of lipoproteins) during the experimental period confirms previous data (Bertoni *et al.*, 2006). The well-known low level of ALA in adipose tissue (Christie, 1981; Seidlin, 1995), suggests that blood changes of this fatty acid depend mainly on phospholipids and not on triglyceride release from adipose tissue.

EPA and DHA were the two ω3 FA supplemented in the largest and similar amount (Table 2): their plasma level increased during the administration period, but in different extent. DHA showed the greatest and prolonged raise (Figure 4), in accordance with data collected on non-lactating beef cows by Burns *et al.* (2003). Otherwise, EPA resulted increased only before calving and in OPT. It is difficult to

explain the decline of EPA alone at the beginning of lactation; in fact, previous data (Castañeda-Gutiérrez *et al.*, 2007; Mattos *et al.*, 2004) report as EPA and DHA are present in milk in a similar extent, and also their transfer rate from plasma to milk is low and similar. Despite the lack of data, this trend might be attributed to the EPA conversion into DHA through the Sprecher's shunt (Sprecher, 2000), and the activation of this mechanism after calving might be related to the important metabolic-endocrine variations occurring at the onset of lactation. For these two FA the correlations with NEFA were not significant, while with cholesterol they turned out much lower than ALA (NS). This suggests that also EPA and DHA are not affected by lipomobilization. Moreover, we can argue that the reduction of EPA and DHA concentration in the plasma during post-calving ω3 supplementation could be due to the dilution of plasma lipid with NEFA from reserve triglycerides, which do not contain important amount of these ω3 FA (Bertoni *et al.*, 2006a).

Other important changes in the fatty acid profile concern the raise of plasma ω3-HUFA on total HUFA, about 50% higher in comparison to the pre-administration level. The value of this index is much higher (15-21% in CTR and 18-26% in OPT) than in a previous trial carried on during mid-lactation (10-12%; Trevisi *et al.*, 2009). This suggests a higher level of plasma ω3 HUFA in the current trial, likely due to different dietary intakes, both for the basic ration and for the higher supplementation.

Almost all cows undergo a subclinical inflammatory status around calving, as well reported previously (Bionaz *et al.*, 2007; Bertoni *et al.*, 2008; Sordillo *et al.*, 2009). In this experiment, despite we cut off subjects with serious clinical symptoms, CTR cows suffered more severe subclinical inflammatory conditions than OPT cows. In fact, both groups were characterized by a similar marked increase of +APP (i.e. haptoglobin and ceruloplasmin) after calving, but CTR displayed a delayed decrease of the +APP levels after the peak, sign of a slower return to normal conditions. Although not always statistically significant, the differences between groups in parameters directly (e.g. albumin, lipoproteins as total cholesterol, Retinol Binding Protein as vitamin A) or indirectly (e.g. bilirubin) related to –APP are consistent. In

detail, cholesterol (marker of lipoprotein) and retinol (marker of its carrier, Retinol Binding Protein) increased more slowly in CTR after calving; bilirubin (whose excretory enzymes are synthesized by liver; Kamisako *et al.*, 2000) was significantly higher in CTR. Altogether, the data above mentioned confirm the greater deviation of usual hepatic synthesis of proteins in CTR compared to OPT group (e.g. slightly more +APP and much less –APP). Thus, in our experiment both groups suffered similar inflammations (see haptoglobin in Figure 1), whereas the response markedly differed between groups in terms of usual liver synthesis (Figures 2 and 3 for recovery of cholesterol and albumins), as confirmed by the better values of Liver Functionality Index observed in OPT cows, due to a quicker rise of –APP after the onset of the lactation. The marked rise of ω3 HUFA observed in OPT cows may be related to a less severe response to inflammation after calving. EPA and DHA are the main ω3-HUFA taking an action in anti-inflammatory activity, exerting a double beneficial effect in animal physiology. First, they are the main replacers of ARA in phospholipids (Calder, 2001) and are precursors for several molecules characterized by a weak pro-inflammatory action (i.e. prostaglandins, leukotrienes, thromboxane). These types of mediators contribute to decrease the production of pro-inflammatory cytokines (Gruys *et al.*, 2005), which is regulated by ARA products (Rola-Pleszczynski *et al.*, 1992). Second, EPA and DHA are also involved in the production of more mediators (i.e. protectins and resolvins) of inflammation resolution phase (Serhan *et al.*, 2005). To confirm this framework, Visioli *et al.* (2003) observed in human a relationship among the high level of plasma ω3, a reduced inflammation and a lower incidence of clinical and subclinical diseases. Thus, the increased plasma levels of EPA and DHA in this experiment let us infer their involvement in the reduction of inflammatory response around calving.

Although milk yield was very similar, OPT cows showed a lower recourse to the body reserves of fat (BCS drop was 0.49 in OPT *vs* 0.63 in CTR, confirmed by the significantly lower NEFA levels, Figure 1) and protein (lower reduction of creatinine; Finco, 1997) in the first 6 weeks of lactation). This metabolic status suggests that CTR compared to OPT cows underwent a more severe risk of ketosis,

as proposed by Goff *et al.* (1997) when BHB exceeds 1.2 mmol/L, and, perhaps, of liver lipidosis (Bobe *et al.*, 2004). CTR cows did not show any clinical ketosis (but more subclinical cases than OPT), but they mobilized more reserves with a similar milk yield and showed a less favorable metabolic status than OPT cows. In OPT, both the higher intake of ω3 FA and perhaps the slightly higher intake of total energy (due to the supplement or to an higher feed consumption) may have contributed to improve the metabolic status.

Finally, among the other parameters assessed in plasma, tocopherol is an interesting index involved in oxidative processes and enhancement of mastitis resistance (Sordillo *et al.*, 2009). Its trend in OPT did not show a drop before calving, marking a difference from CTR group which showed the usual reduction around calving (Goff *et al.*, 1990; Bionaz *et al.*, 2007). This is likely justified by its abundant presence in the formula of the administered supplement (which have increased by 1.5 folds the daily intake in dry and by 3 folds in lactation period). Tocopherol is used as antioxidant to protect ω3 FA from peroxidation during the storage of Optimate; thus, a relevant amount of this vitamin may be also absorbed in the gut as confirmed by our data. Tocopherol exerts its protective action on ω3 FA until they are incorporated in tissues (Scislowski *et al.*, 2005) and, in addition, can contribute to the reduction of pro-inflammatory cytokines synthesis, as shown by van Tits *et al.* (2000). Nevertheless, the reduction of tocopherol observed after calving in both groups may be explained by a lower synthesis of carrier proteins (e. g. lipoproteins) in response or in relationship with the inflammation also observed in both groups. The good correlation between cholesterol and tocopherol (r=0.62) confirm this possibility. The shorter and less severe inflammation observed in OPT may be partly justified by the higher availability of vitamin E, but the less severe inflammation due to ω3 may have also contributed to the quicker recovery of lipoproteins and plasma vitamin E itself.

CONCLUSIONS

The supplementation of algae-derived ω3 FA contained in Optimate caused an increase in the body availability of ω3 FA (in particular the index ω3 HUFA on total HUFA) and vitamin E, particularly before calving. Furthermore, the inflammatory situation was similar between groups, as shown by the +APP levels in early post-calving. Nevertheless, the body response was less severe in OPT, which showed a significant quicker recovery of –APP to normal values, the latter well summarized in the Liver Functionality Index. Besides the higher levels of plasma ω3 FA, also vitamin E levels may explain the reduced inflammatory response of OPT group. From the practical point of view, this kind of ω3 supplementation in the transition period leads to a different inflammatory condition that could justify a lower lipomobilization, which implies a lower ketosis risk, maintaining a similar milk yield.

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Table 1 - Nutritional value of the diets fed as TMR for close-up and lactating cows

COMPONENTS [% of DM]	Close-up dry cows	Lactating Cows
Grass hay	49.63	7.37
Alfalfa hay	-	11.64
Wheat straw	16.50	-
Corn silage	25.60	32.90
Corn meal (67%) and corn flakes (34	-	24.85
Cottonseed	-	8.68
Soybean meal and mineral and vitamin supplementation	8.30	-
Commercial concentrate	-	14.56
DMI [kg/d]	12.50	23.62
CHEMICAL COMPOSITION		
NEL [Mcal/kg of DM]	1.29	1.58
CP [% of DM]	12.44	16.11
NDF [% of DM]	56.57	33.88
Starch + sugar [% of DM]	12.58	26.83
Ether extract [% of DM]	1.91	4.79

Table 2 - Detailed fatty acids composition (percentage of the total fat and mg/100 g) of ω3 supplement (Optimate, Agritech, Tipperary, Ireland) and daily amount of each fatty acid received by each treated cow with the supplement. The total daily intake of fat supplement was 112.5 g/cow and included non-lipophilic compounds of fat molecules (e.g. glycerol, choline, sphyngosine).

FATTY ACID	%	mg/100 g	g/cow/d
Total saturated	34.09	12896.64	38.35
Total monousaturated	39.01	14712.22	43.89
18:2 ω-6	4.64	1755.93	1.81
20:3 ω-6	0.10	38.53	0.04
20:4 ω-6 (ARA)	0.45	169.46	0.18
22:5 ω-6	0.16	59.55	0.06
others	0.48	184.35	0.19
Total ω-6 PUFA	5.83	2207.82	2.27
18:3 ω-3	1.21	456.72	1.36
20:5 ω-3	6.74	2548.94	7.58
22:5 ω-3	2.42	916.06	2.72
22:6 ω-3	6.67	2524.85	7.50
others	2.56	968.60	2.88
Total ω-3 PUFA	19.60	7415.17	22.05
Others	1.45	550.42	1.63
OVERALL TOTAL	100.00	37782.27	112.48

Table 3 - Main plasma fatty acids variations (as percentage of total fatty acids) in periparturient cows fed (OPT, n=3) or not (CTR, n=3) with 22 g/d of ω3 during the three weeks before and after calving assessed at -21 (pre- ω3 treatment); -3 (prepartum); +21 (ω3 treatment end) and +42 (after ω3 treatment) DIM. Data are expressed as % of total fatty acids Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001.

Fatty acid	group	-21	-3	21	42
EPA [%]	OPT	0.72	1.08	0.71	0.49
	CTR	0.71	0.53	0.78	0.58
	P	NS	<0,01	NS	NS
DHA [%]	OPT	0.10	0.30	0.31	0.12
	CTR	0.08	0.08	0.06	0.05
	P	NS	<0,001	<0,001	<0,1
DPA [%]	OPT	0.67	0.65	0.74	0.53
	CTR	0.63	0.53	0.52	0.53
	P	NS	NS	<0,05	NS
ALA [μg/mL]	OPT	90.78	73.72	80.88	106.33
	CTR	54.39	60.65	56.12	82.76
	P	<0,05	NS	NS	NS

Figure 1 - Glucose and NEFA plasma levels (mean ± SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as + P<0.10; *P<0.05; **P<0.01; ***P<0.001.

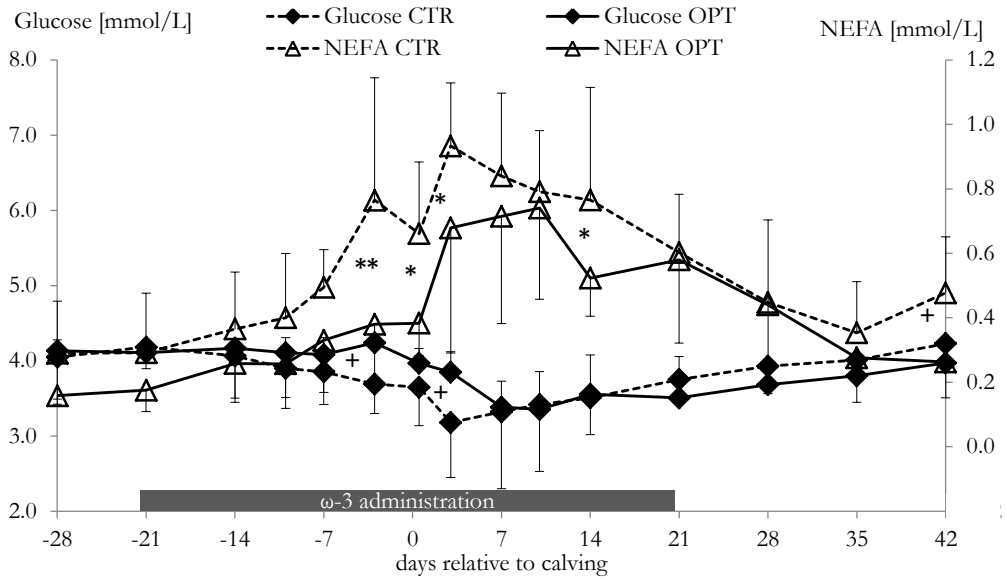


Figure 2 - Cholesterol and haptoglobin plasma levels (mean ± SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001.

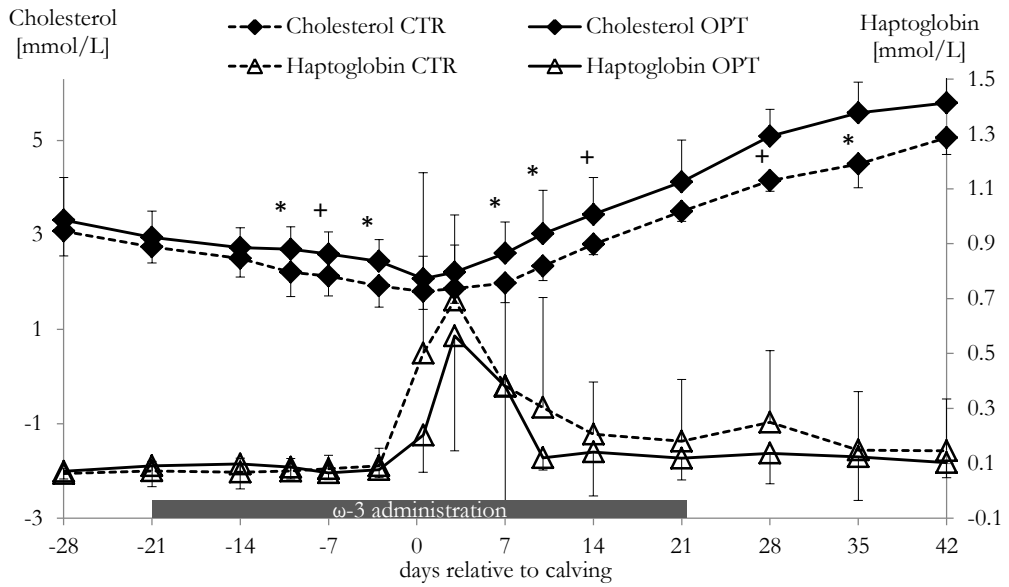


Figure 3 - Albumins and tocopherol plasma levels (mean ± SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001

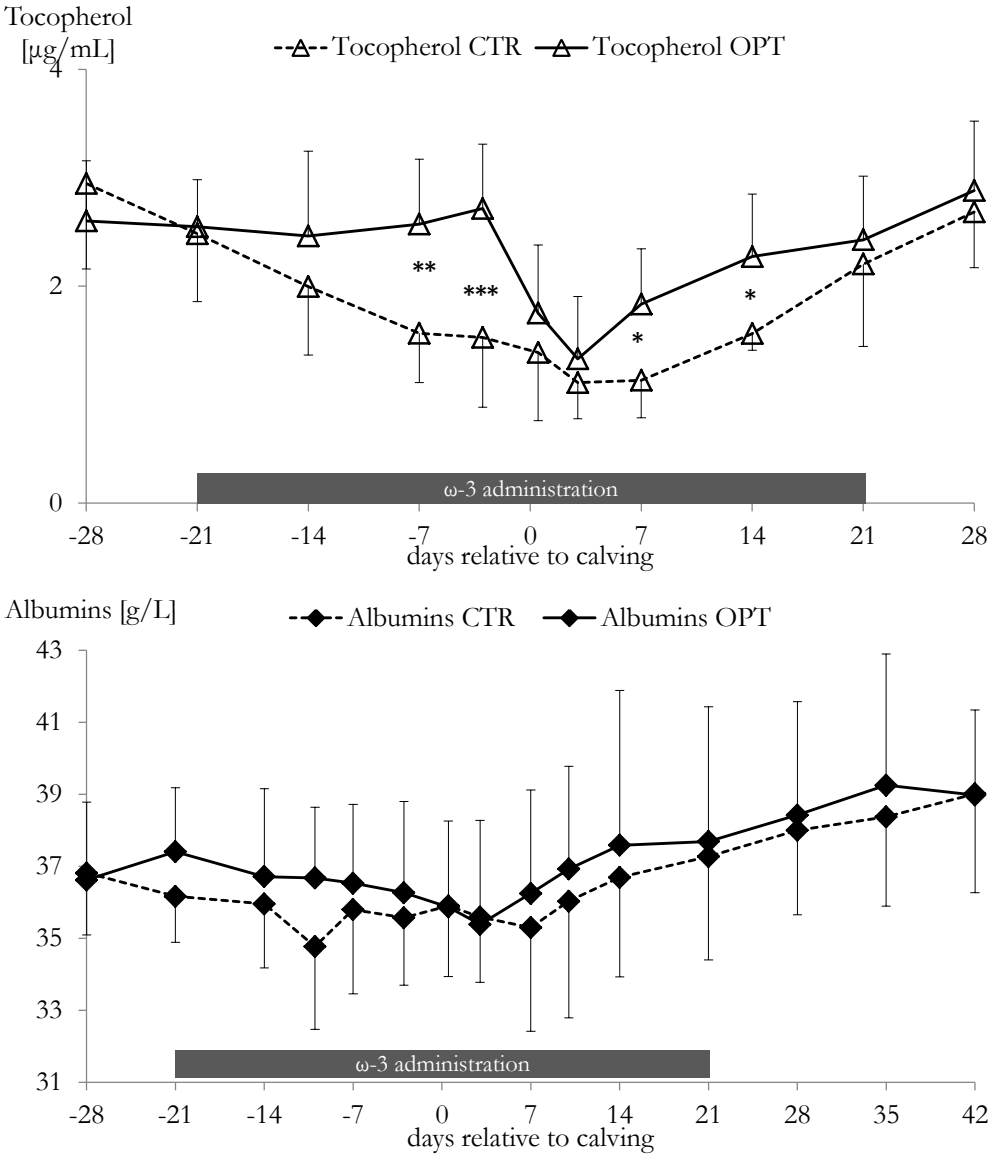
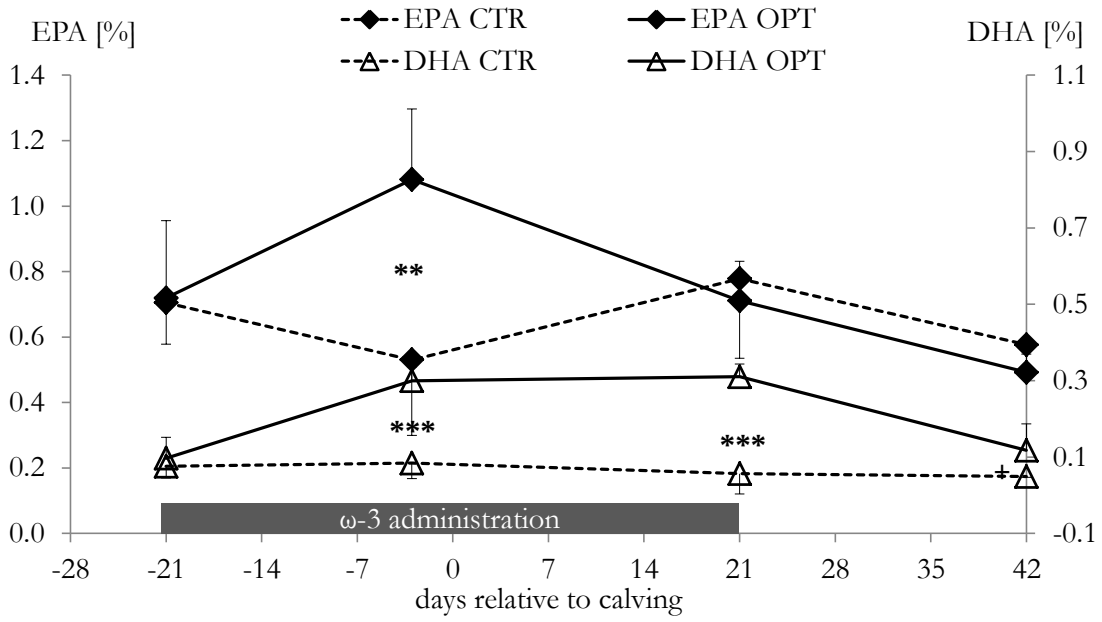


Figure 4 - EPA and DHA plasma levels (as % of total fatty acids) in periparturient cows fed (n=3, continuous line) or not (n=3, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001.



CHAPTER 3

Pre-calving administration of ω3 fatty acids alone or in combination with acetylsalicylic acid in periparturient dairy cows: effects on inflammation, performances and fertility

INTRODUCTION AND AIM

The ω3 fatty acids (FA) are told to be effective in the modulation of inflammation, as these essential fatty acids are key molecules in the metabolism of eicosanoids, mediators of inflammation (Calder, 2006; Mori and Beilin, 2004; Teitelbaum, 2001). The mechanism involves the cyclooxygenase enzymes (COX), which initiate a cascade converting arachidonic acid (ω6 fatty acid) in a number of molecules called eicosanoids which exert different actions in the inflammatory process. A prolonged administration of ω3 FA cause their accumulation in tissues (von Schacky et al., 1985) and this allows later their replacement for the arachidonic acid, causing the production of less pro-inflammatory eicosanoids (3-serie prostaglandins and thromboxane; Mori and Beilin, 2004), or other molecules involved in the resolution phase of inflammation (resolvins and protectins; Serhan and Savill, 2005). Interestingly, Groeger et al. (2010) demonstrated the formation of electrophilic oxo-derivatives (EFOX) from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through COX-2 in activated macrophages, enhanced with the modulation of this pathway by acetylsalicylic acid. These compounds have anti-inflammatory effect exerted through an agonist action on the gene expression of the peroxisome proliferator-activated receptor-γ (PPARγ) and through the inhibition of pro-inflammatory cytokine and nitric oxide production. Acetylsalicylic acid is widely used in case of acute inflammation for its potent anti-inflammatory effect exerted through the inhibition of both cyclooxygenase 1 and 2 (Vane, 2003). This action is also effective at low dose in murine models (Cyrus, 2002) and is useful to prevent inflammation in patients affected by cardio vascular diseases (Patrono et al., 2005).

There are some evidences in human that a synergic action of ω3 FA and acetylsalicylic acid at low dose may be exploited to control and to reduce the production of pro-inflammatory eicosanoids (Engström et al., 2001). In the past, we treated cows with acetylsalicylic acid after calving with good results on milk yield, fertility and metabolic status (Trevisi and Bertoni, 2008). However, the effects of

non-therapeutic doses of acetylsalicylic acid joint to ω3 FA supplementation on livestock was not investigated before. In particular, we are not aware of experiments in dairy cows based on the daily pre-calving administration of acetylsalicylic acid and ω3 FA.

Thus, our aim was to assess the effects of the synergic action of ω3 FA and acetylsalicylic acid administered before calving to reinforce the natural systems of inflammation resolution in the transition period of dairy cows.

MATERIALS AND METHODS

This study complied with Italian and European rules on animal experimentation and ethics.

BARN CHARACTERISTICS, ANIMALS AND TREATMENTS

The trial took place in the Università Cattolica experimental barn (CERZOO) located in the Northern Italy (Piacenza) during autumn-winter season and involved 26 multiparous Friesian dairy cows reared in loose stall with cubicles and milked twice a day (12 hours gap). Dry and lactating cows were fed with two different total mixed ration (TMR) diets described in Table 1. Three homogeneous groups were formed according to body condition, calving period, production potential, parity, mammary gland health, body weight. The first group received a combination of ω3 fatty acids and acetylsalicylic acid (n = 9; OMAS): the fish-derived oil administration started about 21 days before calving and lasted through the day before calving. The dose administered was 100 g/cow/d of commercial product (Orovital Cod; Ascor Chimici Srl, Capocolle di Bertinoro, FC, Italy), corresponding to 75 g/cow/d of fatty acids. According to the composition of the product (Table 2), the cows received daily 12.0 g of ω3 fatty acids (among them 4.6 g of EPA and 6.0 g of DHA); ω6 fatty acids were administered with the product in the extent of 1.6 g/d. It was fed once a day immediately before the TMR distribution, and it was mixed with about 0.5 kg of fresh cows TMR. There was no possibility of competition among

cows to ingest the supplement. The last 7 days of pregnancy OMAS cows received also a daily intramuscular injection of lysine acetylsalicylate (Lysal, Farmaceutici Gellini Srl, Peschiera Borromeo, MI, Italy) in the extent of 0.036 mg/kg BW (corresponding to 6.0 mg/kg BW of acetylsalicylic acid). The second group (n = 9; OM) only received the same ω3 fatty acids supplement of OMAS group. The last group (n = 8; CTR) received, for the same period, an administration of palm oil (75 g/d) to balance the energy intake of the other two groups (Greenfat 3G, Or Sell Srl, Limidi di Soliera, MO, Italy). The whole detailed FA composition of the product is described in Table 3.

CLINICAL CHECKS

During the experiment, animals health conditions were checked every day by general inspections and monitored by a computerized Afimilk system (S.A.E. Afikim, Kibbutz Afikim, Israel), based on automatic recording of activity and milk production through a leg transponder. In addition, rectal temperature was measured the day after calving and twice a week from 14 day before to 14 days after calving. Cows were also submitted to a thorough gynecological examination at about 10 and 30 days in milk (DIM), or when required by a suspect of pathology. Moreover, each cow was submitted to the following assessments: 1) Body condition scoring, using a 5-point scale (ADAS, 1986), starting about 35 d before the expected calving date and, then, every 14 days to 35 DIM. 2) Milk yield and its conductivity, measured and recorded by the Afimilk computer-controlled automated system at every milking. 3) Milk samples at 7, 14, 28 DIM, from the morning milking, in order to assess fat, protein and lactose content (MilkoScan FT 120, Foss Electric, Hillerød, Denmark), and somatic cell count (SCC; Fossomatic 180, Foss Electric). 4) Blood samples, collected approximately (±3 days) at -28, -21 (pre-treatment), -14, -10, -7, -3 (before calving), 1 (day after calving), 3, 7, 10, 14, 21, 28, 35 (post-treatment) days from calving. Every sample was collected in the morning before feeding, from a jugular vein and in two vacuum tubes (Vacuette, Greiner Bio-One GmbH, Kremsmunster, Austria), one containing lithium-heparin as anticoagulant, and the other silicon (no

anticoagulant). Lithium-heparin tubes were cooled immediately after collection in an ice-water bath until their arrival in laboratory. After a small aliquot of blood was taken to determine packed cell volume (centrifugation at 12000 RPM for 11 minutes), tubes were centrifuged at 3520 × g for 16 minutes at 4°C; plasma samples were divided in 5 aliquots, stored at -20°C (4) or -80°C (1). In accordance with the methods described in Bionaz *et al.* (2007) on these samples were determined: I) Inflammatory response indexes: positive acute phase proteins (+APP; haptoglobin, ceruloplasmin) and negative acute phase proteins (-APP; albumin, cholesterol as lipoprotein index). II) Liver indexes: total bilirubin, aspartate amino-tranferase (GOT), γ-glutamil transferase (GGT), alkaline phosphatase (ALP), paraoxonase (PON), activated paraoxonase, sorbitol dehydrogenase (SDH). III) Energy metabolism indexes: glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB). IV) Protein metabolism indexes: urea, creatinine. V) Oxidative stress and related parameters: total reactive oxygen metabolites (ROM), total nitric oxide metabolites (NO_x), nitrites (NO_2), nitrates (NO_3), myeloperoxidase (MPO), thiol groups (SHp), total antioxidants. VI) Minerals (Ca, P, Mg, Na, K, Cl, Zn). VII) Vitamins: retinol (index of its carrier protein), tocopherol, β-carotene. VIII) Other parameters (total proteins, globulins).

Glucose, total protein, albumin, total cholesterol, total bilirubin, creatinine, urea, Ca, P, Mg, GOT, GGT and ALP were detected at 37°C by a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA) using commercial kits purchased by Instrumentation Laboratory (IL Test), as previously described by Bionaz *et al.* (2007). Globulins were calculated as the difference between total protein and albumin. Electrolytes (Na^+, K^+, and Cl^-) were detected by the potentiometer method (Ion Selective Electrode connected to ILAB 600). Zn and NEFA were determined by commercial kits (Wako Chemicals GmbH, Neuss, Germany). Haptoglobin, BHB, and ceruloplasmin were analyzed using methods described by Bertoni *et al.* (1998), adapted to ILAB 600 conditions. ROM were measured using a method patented by Diacron International S.r.l. (Grosseto, Italy) and expressed as mg of hydrogen peroxide per 100 mL of plasma. Plasma PON

activity was assessed by adapting the method of Ferré *et al.* (2002) to the ILAB 600, as described by Bionaz *et al.* (2007). The activated PON activity was determined with the same method, but the solution was composed by 1 mmol/L of paraoxon, 1 mmol/L di $CaCl_2$ and 1 mol/L di NaCl in a glycine buffer solution 0.05 mmol/L. The activity of MPO was determined through a colorimetric method based on the reaction of MPO contained in the plasma sample with the hydrogen peroxide, which forms H2O and O-. The O-dianisidine dihydrochloride, donor of electron, reacts with the O^-, releasing H_2O and a colored compound. Thiol groups are detected using a colorimetric method by Diacron International S.r.l. (Grosseto, Italy). The principle of the test is based on the formation of a colored complex, as a product of the reaction between sulfhydryl groups and the 5,5-ditiobis-2-nitrobenzoic acid in a buffer solution (pH 7.6). The intensity of the color detected is directly related to the concentration of thiol groups. The concentration of nitric oxide can be assessed as a sum of nitrites (NO_2^-) and nitrates (NO_3^-). The assay procedure is composed by two steps: enzymatic reduction of NO_3^- to NO2- (manual; nitrate reductase) and the determination of the NO_2^- (automatic). The enzymatic reduction is an end-point reaction lasting about 3 hours in water bath at 37°C between a reducing solution and the sample. After the enzymatic reduction, the total NO_2^- are detected (NO_2^- + NO_3^-), which correspond to the NO_x. The determination is colorimetric (Griess test). The analysis is a spectrophotometric measure at 546 nm of a colored complex derived from the reaction between NO_2^- and a cromogen (naphthyl-etylen-diamine), properly buffered. The detection of the only NO_2 consists in the automatic analysis, without the preliminary enzymatic reduction and a with a difference in the calibration. The determination of SDH was carried out on serum samples through a kit provided by Catachem Inc. (Bridgeport, CT, USA, enzymatic test). The test is based on the reversible oxidation-reduction reaction between sorbitol and fructose, catalyzed by SDH, with concomitant oxidation of NADH to NAD^+. The absorbance decrease is monitored at 340 nm and is directly proportional to SDH activity in serum samples.

The working conditions of ILAB600 are summarized in appendix 2.

Plasma retinol, tocopherol and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Spherisorb ODS-2.3 μm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL); a UV detector set at 325 nm (for retinol) or 290 nm (for tocopherol); and 80:20 methanol:tetrahydrofurane as the mobile phase. Total antioxidants were determined through the oxygen radical absorbance capacity (ORAC) assay, which is based on the free radical damage to a fluorescent probe (fluorescein), resulting in a downward change of radical damage. This analysis was performed with a multi-detection microplate reader equipped with a dual reagent injector and with a software for kinetic data collection and analysis (BioTek Synergy 2, Winooski, VT, USA).

DATA HANDLING AND STATISTICAL ANALYSIS

All data in this paper are presented in the form mean ± standard deviation.

Repeated measures analysis of variance. Data were submitted to repeated measures variance analysis using a mixed model (MIXED procedure, SAS Inst. Inc., Cary, NC; Littell *et al.*, 1998). Before analysis the normality of distribution was verified for each parameter through skewness and kurtosis calculation according to the Shapiro test (SAS Inst. Inc.). When necessary, data were normalized through logarithmic, quadratic, inverse or root-square transformations. The layout of our statistical model can be summarized as follows:

$$Y_{iklm} = \mu + G_i + T_k + GT_{ik} + B_{l(im)} + e_{iklmn}$$

where Y_{ijklm} = m^{th} observation of the l^{th} cow B_l within the i^{th} treatment G_i, at the k^{th} time to calving T_k; μ = total average; G_i = effect of the i^{th} treatment (3 treatments: OM, OMAS and CTR); T_k = effect of the k^{th} time to calving (the number of levels being defined as a function of pregnancy phase and actual variable); GT_{ik} = effect of the interaction between the i^{th} treatment and the k^{th} time to calving; $B_{l(im)}$ = effect of the l^{th} cow within the i^{th} treatment; e_{iklmn} = random effect or error.

The analysis was carried out using 3 covariance structures: Autoregressive, Compound symmetry, and Spatial Power. These were ranked according to their AIC (Akaike's Information Criterion; Akaike, 1974), choosing as better the lowest one

(Littell *et al.*, 1998). For each treatment, least squares means were computed, and preplanned pairwise comparisons (PDIFF option, SAS Inst. Inc.) were carried out when the F-test of one of the main factors (time, treatment, treatment × time) was significant at $P<0.10$. Statistical significance was designated as $P<0.05$, tendencies were declared at $P<0.10$.

One-way variance analysis. The differences among groups of some parameters (pregnancy length, number of artificial inseminations, days open) were statistically evaluated through a one-way analysis of variance (GLM procedure, SAS Inst. Inc., Cary, NC) considering as a fixed factor the group. The layout of our statistical model can be summarized as follows:

$$Y_{ij} = \mu + G_i + e_{ij}$$

where: Y_{ij} = i^{th} observation of the group with j^{th} parameter; μ = total average; G_i = effect of the i^{th} value (i = group); e_{ij} = random effect or error.

PIRI index application. Cows were divided in tertiles based on their score ofPost-calving Inflammatory Response Index (PIRI), described in detail in the first chapter of this thesis: high PIRI (HI-PIRI), intermediate PIRI (IN-PIRI) and low PIRI (LO-PIRI). This index combines the plasma concentrations of cholesterol, haptoglobin, paraoxonase and reactive oxygen metabolites assayed 7 days after calving, in order to give an idea of the inflammatory status and of the inflammatory response that affect the cow in the immediate post-calving. The cows falling in the lowest tertile are characterized by an important inflammatory status at calving and by an inadequate response, while in the highest tertile are gathered the cows that suffered a mild inflammation (HI-PIRI). Among the three tertiles were carried out the same model of statistical analysis already performed among the groups of treatment (repeated measures and one-way variance analysis).

X^2 test was carried out to assess a possible association between inflammation (expressed by PIRI index) and treatment.

FSI index application. The Fertility Status Index was calculated for each group according to Esslemont and Eddy (1977). This index is calculated for a group of cows and takes into account the number of pregnant cows on the total cows, the

pregnant cows at 1st AI, the average number of AI per pregnancy and the average days open.

RESULTS

A) EFFECTS OF Ω3 FA AND ACETYLSALICYLIC ACID TREATMENTS

The planned days of supplement administration slightly differed because of the calving date variability. OM group received ω3 FA for 19±5 days, while OMAS cows for 25±5. The OMAS group received acetylsalicylic acid for 8±2 days and the CTR group received the administration of saturated fatty acids for 22±7 days.

The length of pregnancy was evaluated: OM cows calved in advance in respect to the other groups (277±6 days of pregnancy); CTR groups calved in 281±6 days of pregnancy, while OMAS calved after 284±5 days of pregnancy. The difference between OM and OMAS in days of pregnancy turned out statistically significant ($P<0.02$).

Animal health

Most of the cows involved in the experiment did not show any disease during their transition period. Some of them were affected by mild troubles which did not require any kind of intervention (e.g. one-day high temperature or diarrhea). However, some cows were affected by severe pathologies immediately after calving (Table 5). In CTR group two cows showed lameness and metritis. In OM group one only cow was affected by retained placenta and one by diarrhea. In OMAS group three cows suffered several problems: one retained placenta, one severe ketosis and one both retained placenta and ketosis. The cows that suffered retained placenta were treated with oxytetracyclin Tablets in uterus, and in one case with serotonin + ergometrine and an anti-inflammatory drug. Cows with severe ketosis were administered glucose in jugular vein for 2-3 days according to the severity of the pathology.

Rectal temperature trend did not show any statistical difference among groups. However, a numerically lower temperature (0.2°C) has been found between OMAS and the other two groups 3 days before calving (38.83±0.18 in OMAS, 39.03±0.14 in OM, 39.02±0.32 in CTR). The reported cases of fever showed temperatures higher than 39.5°C for more days. Episodic temperatures >39.5°C without any other clinical symptom are not reported here as fever cases.

BCS (Figure 2) showed in all the three groups the typical post-calving drop: it started a week before calving and kept on through all the checked period. OM group showed a tendency to have a smaller decrease of BCS compared to CTR: 0.33±0.19 *vs* 0.57±0.27 points of body condition from 7 days before calving to 35 DIM; $P<0.10$. OMAS group lost 0.51±0.27 points.

Performances

Milk yield and quality. Milk yield (Figure 1) was not statistically different among groups: CTR cows produced 1158±100 kg in the first 28 DIM, while OM gave 1115±258 and OMAS 1075±115 kg. The assessed parameters of milk quality did not show any statistical difference.

Fertility. Five cows were culled due to infertility during the lactation following the experiment: 1 in CTR, 1 in OM and 3 in OMAS groups. CTR cows showed an overall better reproductive performance than the other two groups (Table 7): days open were 95±46 and the number of inseminations was 1.7±1.1. For OM cows the days open were 120±47 and the number of inseminations was 2.3±1.2. The OMAS group was characterized by the worst values: 158±55 days open and 3.3±1.5 inseminations. The differences between CTR and OMAS were significant for days open ($P<0.03$) and for number of inseminations ($P<0.03$).

The values of FSI index confirmed an optimal situation in CTR (83.9), good in OM (44.5) and bad in OMAS (-4.5).

Blood parameters

Energy metabolism. Glucose (Figure 4) showed in all groups a peak after calving followed by a drop in the following days. However the glycaemia in OM group recovered pre-calving levels before than the other groups, and at 7 DIM was higher than in the other groups (4.07±0.43 mmol/L in OM, 3.42±0.95 mmol/L in OMAS and 3.52±0.46 mmol/L in CTR; $P<0.001$ OM *vs* OMAS and $P<0.01$ OM *vs* CTR).

BHB trend (Figure 12) displayed post calving a high peak in OMAS at 7 DIM (1.63±1.58 mmol/L). In OM and CTR groups the peak occurred at 1 (0.81±0.27 mmol/L) and 3 DIM (0.89±0.62 mmol/L). At 7 DIM, BHB level was higher in OMAS than in the other two groups ($P<0.01$ *vs* OM and $P<0.05$ *vs* CTR). At 10 DIM OMAS group tended to maintain higher levels of BHB than OM (0.53±0.14 mmol/L in OM *vs* 1.02±0.92 mmol/L in OMAS; $P<0.10$) and CTR (NS), while a tendency to be lower was detected in OM *vs* CTR (0.44±0.10 *vs* 0.68±0.33 mmol/L at 21 DIM; $P<0.10$). NEFA levels (Figure 11) were characterized by similar trends, with higher levels in OMAS and lower in OM after calving, but not confirmed by statistics. The peak occurred in OMAS at 7 DIM (0.98±0.50 mmol/L), while it was reached at 3 DIM in OM (0.68±0.42 mmol/L) and CTR (0.73±0.44 mmol/L).

Inflammatory status. Haptoglobin (Figure 10) maintained low values (<0.2 g/L) in all the three groups until 7 days before calving. Three days before calving it started to increase in OMAS group and reached a peak at 7 DIM (1.17±0.90 g/L in OMAS; 0.29±0.30 g/L in CTR, 0.40±0.34 g/L in OM $P<0.01$ CTR *vs* OMAS and $P<0.05$ OM *vs* OMAS). The difference continued to be significant between CTR and OMAS at 10 and 14 DIM ($P<0.05$). CTR and OM groups reached the peak already at 3 DIM (0.49±0.43 g/L in CTR, 0.70±0.38 g/L in OM), and after decreased. At 21 DIM the levels were similar among the three groups.

Cholesterol (as index of lipoprotein, Figure 5) had a significantly higher level already at the beginning of the experiment in OM than in the other two groups (3.55±0.71 mmol/L in OM, 2.79±0.79 mmol/L in CTR and 2.69±0.41 mmol/L in OMAS 28 days before calving; $P<0.01$ *vs* CTR and OMAS). Cholesterol confirmed significantly higher levels in OM for the whole period of the experiment. The level

in OMAS, similar to CTR 28 days before calving, decreased more markedly and reached the greatest difference at 1 DIM (1.95±0.46 mmol/L in OMAS, 1.70±0.34 mmol/L in CTR; *NS*). Later, OMAS showed an increasing trend less marked than CTR, and the values tended to be lower at 10 DIM (2.03±0.65 mmol/L in OMAS, 2.36±0.44 mmol/L in CTR; $P<0.10$).

Albumin levels (Figure 6) were similar before calving, while after calving the increase resulted delayed in OMAS *vs* CTR and OM (34.58±3.17 g/L in OMAS, 36.86±2.57 g/L in CTR, 36.58±1.79 g/L in OM; $P<0.01$ OMAS *vs* OM and CTR at 10 DIM).

Retinol (as index of retinol binding protein, Figure 14) plasma level decreased approaching calving in all the groups. After calving, it showed a quick raise in CTR and OM, while in OMAS it was slower. The greatest differences were reached at 10 DIM (33.79±19.63 μg/100 mL in OMAS, 47.31±15.22 μg/100 mL in OM, 51.56±19.98 μg/100 mL in CTR; $P<0.01$ OMAS *vs* CTR and $P<0.05$ OMAS *vs* OM). Later the differences decreased and at 21 DIM the levels were similar.

Paraoxonase plasma levels (Figure 13) were slightly lower in OMAS before calving in respect to OM and CTR (NS). After calving this difference increased and at 14 DIM paraoxonase concentrations were 124.23±24.72 U/mL in CTR, 126.43±26.18 U/ mL in OM and 94.22±28.85 U/ mL in OMAS; $P<0.05$ OMAS *vs* CTR and $P<0.01$ OMAS *vs* OM.

Bilirubin (whose clearance enzymes are synthesized by the liver, Figure 8) plasma trend showed an overall increasing trend in late pregnancy, reaching a peak 3 days after calving in all groups (6.72±2.62 mmol/L in CTR, 6.61±3.68 mmol/L in OM and 7.61±4.99 mmol/L in OMAS) and a decrease in the following 3 weeks after calving. Despite no significant differences were found, OMAS showed higher values in comparison to OPT and CTR.

Other parameters. GGT plasma level (FigureFigure 7) showed lower values in OMAS than in the other experimental groups (19.02±3.37 U/L in OMAS *vs* 26.91±6.61 U/L in CTR; $P<0.01$) already 28 days before calving. The difference was significant until 7 DIM, then levels were similar. ALP (Figure 9) showed in

OMAS group a numerically lower level in late pregnancy than in CTR and OM and peaked in both groups the day after calving (54.44±27.80 U/L in OMAS *vs* 60.01±23.13 U/L in CTR; *NS*). SDH (Figure 15) had a similar trend, keeping lower levels in OMAS from 28 days before calving to 14 DIM. The day after calving levels were 8.23±1.92 U/L in OMAS and 11.43±5.01 U/L in CTR.

B) DIFFERENCES AFTER DIVISION OF COWS ACCORDING TO THE PIRI INDEX

In order to assess the frequency of severe inflammatory conditions around calving in different treatments, cows were assigned a score of PIRI index and later divided in tertiles according to these values (Table 4). Data will be shown and discussed mainly comparing the two extreme tertiles (high, HI-PIRI and low, LO-PIRI), while the intermediate tertile (IN-PIRI) will be mentioned only in case it shows particularly interesting values. Furthermore, only important and interesting parameters will be reported in the text.

Animal health

As a result of PIRI index application, cows which suffered most of the health problems have been gathered in the lowest tertile (Table 6). No health diseases can be found in the highest tertile and in the intermediate except one case of diarrhea.

Rectal temperatures did not show any significant difference among the tertiles.

The body condition of the cows in the lowest tertile was significantly higher than the other two tertiles 35 days before calving (2.74±0.35 points in LO-PIRI *vs* 2.48±0.21 points in HI-PIRI; $P<0.05$; Figure 17). The difference remained significant until calving, but later it thinned because of the greater reduction in LO-PIRI: body condition loss from 14 days before calving to 35 DIM was 0.34±0.12 points in HI-PIRI *vs* 0.56±0.32 in LO-PIRI.

Performances

Milk yield and quality. Milk yield (Figure 16) was significantly different between HI-PIRI and LO-PIRI. The total average production in the first 35 days of lactation was 1530±231 kg in HI-PIRI vs 1314±193 kg in LO-PIRI ($P<0.01$). Somatic cell count was higher in LO-PIRI at 14 DIM (283±251 n/μL in LO-PIRI vs 95±105 n/μL in HI-PIRI $P<0.05$).

Fertility. HI-PIRI cows showed a shorter period of days open (Table 8): 101.3±45.9 *vs* 147.2±49.8 in LO-PIRI (*N.S.*). The number of inseminations was 2.0±1.2 in HI-PIRI *vs* 3.0±1.5 in LO-PIRI (*NS*).

The values of FSI index were 71.3 in HI-PIRI, 52.0 in IN-PIRI and -4.3 in LO-PIRI.

Blood parameters

Energy metabolism. Glucose (Figure 19), similar before calving, showed a higher peak in LO-PIRI at 1 DIM ($P<0.01$ *vs* HI-PIRI), while in the following days it greatly decreased in the same group and at 7 DIM it was significantly lower (3.26±1.04 mmol/L in LO-PIRI vs 3.89±0.26 mmol/L in HI-PIRI; $P<0.001$). In the following controlled period it partly recovered its level, but it remained numerically lower than in the other tertiles.

NEFA levels (Figure 28) were similar until 7 days before calving. Three days before calving they tended to be higher in LO-PIRI and the difference remained higher for the first 10 DIM, peaking at 7 DIM in LO-PIRI (1.04±0.51 mmol/L vs 0.45±0.17 in HI-PIRI; $P<0.001$).

BHB (Figure 29) peaked at 1 DIM in HI-PIRI, while in LO-PIRI it peaked at 7 DIM maintaining significantly higher levels from 3 to 10 DIM (at 7 DIM levels were 1.85±1.56 mmol/L in LO-PIRI vs 0.52±0.18 mmol/L in HI-PIRI; $P<0.001$).

Creatinine (Figure 30) showed in LO-PIRI increasing level during pregnancy and through the first day after calving (at 1 DIM levels were 116.42±13.73 mmol/L in

LO-PIRI and 104.09±4.68 mmol/L in HI-PIRI; P<0.01). From 3 DIM no differences were shown and the trends were decreasing.

Inflammatory status. Among the +APP, plasma concentration of haptoglobin (Figure 27) started increase three days before calving and achieved a peak in HI-PIRI at 3 DIM. In LO-PIRI peaked at 7 DIM with the highest values (1.31±0.78 g/L in LO-PIRI vs 0.22±0.12 g/L in HI-PIRI; *P*<0.001). Ceruloplasmin (Figure 22) showed a tendency to be higher in LO-PIRI 3 days before calving. At 3 DIM ceruloplasmin peaked in LO-PIRI and confirmed the tendency to be higher in this group (3.95±0.48 μmol/L in LO-PIRI vs 3.43±0.35 μmol/L in HI-PIRI; *P*<0.10). However, IN-PIRI tertile showed the highest values of ceruloplasmin already during pregnancy: 21 days before calving its plasma concentration was 3.53±0.62 μmol/L in IN-PIRI vs 2.89±0.58 μmol/L in HI-PIRI (*P*<0.05). After calving the difference was greater (4.18±0.48 μmol/L in IN-PIRI vs 3.37±0.50 μmol/L in HI-PIRI; *P*<0.01 at 14 DIM).

Among the –APP, albumin plasma concentration (Figure 23) was similar before calving and through the first 3 DIM. Starting at 7 DIM the albumin concentration in HI-PIRI increased, while in LO-PIRI decreased creating a marked gap which persisted until 21 DIM (*P*<0.001). The maximum difference was found at 10 DIM (37.62±2.03 g/L in HI-PIRI vs 33.46±2.10 g/L in LO-PIRI; P<0.001). Cholesterol (Figure 20) basal level at -28 DIM was higher in HI-PIRI than in LO-PIRI (3.74±0.55 mmol/L in HI-PIRI vs 2.60±0.60 mmol/L in LO-PIRI; *P*<0.001). Later, the difference decreased and was not significant from -3 to +3 days from calving. During lactation, cholesterol plasma concentration increased more quickly in HI-PIRI, and was higher in HI-PIRI from 7 to 35 DIM (*P*<0.001). The greatest difference was recorded at 35 DIM (5.23±0.77 mmol/L in HI-PIRI vs 3.81±0.70 mmol/L in LO-PIRI; *P*<0.001). Retinol (Figure 32) followed a similar trend: in HI-PIRI it was higher already during pregnancy (56.21±11.82 μg/100 mL in HI-PIRI vs 45.13±9.59 μg/100 mL in LO-PIRI; *P*<0.05 14 days before calving). After calving the difference was wider (51.65±16.12 μg/100 mL in HI-PIRI vs 23.52±13.84 μg/100 mL in LO-PIRI; *P*<0.001 at 7 DIM) and retinol level was

significantly higher in HI-PIRI until 35 DIM. The plasma concentration of paraoxonase (Figure 31) was significantly higher in HI-PIRI *vs* LO-PIRI for all the experiment period, with the wider difference at 10 DIM (146.85±17.45 U/mL in HI-PIRI vs 72.15±17.02 U/mL in LO-PIRI; $P<0.001$).

Bilirubin plasma concentration (Figure 26) did not show any important difference among tertiles before calving. It was characterized by a general marked increasing trend starting from 7 days before calving and peaked in both HI-PIRI and LO-PIRI tertiles the day after calving (5.78±4.24 mmol/L in HI-PIRI vs 10.08±6.16 mmol/L in LO-PIRI; $P<0.01$). However, the peak of bilirubin was significantly higher and persistent in LO-PIRI until 10 DIM ($P<0.01$ from 1 to 10 DIM *vs* HI-PIRI).

Other parameters. Urea plasma levels (Figure 21) were higher in HI-PIRI after calving than in LO-PIRI (4.01±0.60 U/mL vs 2.98±0.61 U/mL; $P<0.05$ at 14 DIM). Transaminases (GOT and GGT; Figures 24 and 25) showed similar levels between HI-PIRI and LO-PIRI before calving, while after that markedly increased in LO-PIRI. GOT turned out significantly higher at 10 and 14 DIM (118.73±32.57 U/L in LO-PIRI vs 97.76±14.82 U/L in HI-PIRI; $P<0.05$ at 10 DIM), and GGT at 14 and 21 DIM (37.93±11.60 U/L in LO-PIRI vs 25.57±5.90 U/L in HI-PIRI; $P<0.05$ at 21 DIM).

DISCUSSION

EFFECT OF THE Ω3 FA ADMINISTRATION ALONE OR IN COMBINATION WITH ACETYLSALICYLIC ACID

Energy metabolism. In spite of a similar milk yield, the administration of ω3 FA (OM) may have caused some positive effects on energy metabolism in respect to CTR cows which received palm oil, as suggested by the smaller drop of BCS in OM. The lower lipomobilization in OM cows is confirmed by the significantly lower level of BHB (Figure 12) and numerically lower levels of NEFA (Figure 11) compared to CTR and to OMAS cows. This in turn agrees with the level of plasma glucose after calving, resulted higher in OM than in the two other groups. The better energy

status is likely due to a higher level of DMI in OM cows (not directly measured). Our previous work (second chapter of the thesis) and data of Ballou et al. (2009) confirm the better post-calving energy metabolism in cows fed a diet enriched in ω3 FA. Conversely, Mattos et al. (2004), found lower plasma concentrations of glucose and higher of BHB in cows fed ω3 FA, while no differences were shown in NEFA *vs* cows fed olive oil. However, in this last trial, fish oil was administered in a large amount, which likely caused a strong reduction in DMI and several other problems reported by the authors.

Inflammatory status and response to inflammation. Several works describe improvements in metabolism and inflammatory status after the administration of ω3 FA in human (Calder, 2006; Clària et al., 2011; Mori and Beilin, 2004; Teitelbaum, 2001). At the same time it is well known the action of acetylsalicylic acid during inflammation, either in human and in livestock (Vane, 1971). Trevisi and Bertoni (2008) administered acetylsalicylic acid to dairy cows in the post-calving, showing positive effects on milk yield, fertility, health condition and inflammatory status. Serhan et al. (2002) demonstrated in murine models the effectiveness of the combination of low dose acetylsalicylic acid and ω3 FA in the inflammation resolution through the production of resolvins. In humans the combination of ω3 FA and acetylsalicylic acid was previously tested with some improvements in the eicosanoid pattern reducing the production of the pro-inflammatory types (Engström et al., 2001), but with some negative effects on bleeding time (Mueller et al., 1991; Larson et al., 2008). We are not aware of experiments based on the pre-calving daily administration of acetylsalicylic acid in dairy cows.

Our aim was to verify the possible synergic action of ω3 FA together with a low dose of acetylsalicylic acid to reinforce the natural systems of inflammation resolution in the last days of pregnancy of dairy cows, when important inflammatory processes are shown to take place (Bionaz et al., 2007; Trevisi, et al., 2009). Data concerning the inflammatory status show that all the groups underwent an inflammatory condition around calving, as demonstrated by the raise of positive acute phase proteins (e.g. haptoglobin; Figure 10) and in agreement with Bertoni et

al. (2008) and Trevisi et al. (2010). However, the levels of haptoglobin show a more critical situation in the OMAS group cows, in fact the post-calving increase was significantly higher than in CTR and in OM. Furthermore, the trends of –APP support the possibility that all OMAS cows (with or without clinical symptoms) suffered more severe and prolonged negative consequences to inflammation in comparison to CTR cows; indeed, the release from liver of the usual proteins (e.g. –APP) resulted impaired in the first weeks of lactation, likely due to the higher production of +APP. In detail, plasma cholesterol (index of lipoprotein synthesis; Figure 5) showed lower levels in OMAS than in CTR during the first month of lactation. Albumin and paraoxonase (Figures 6 and 13) showed similar trends between groups, but OMAS showed lower levels after calving due to a slower recovery from minimum values. Plasma retinol trend (as index of Retinol Binding Protein synthesis; Figure 14) was also characterized by a slower recovery in the OMAS group and kept significantly lower levels in respect to CTR in the first two weeks after calving. The trends of –APP in OM group were similar to CTR, but noteworthy is cholesterol, which showed higher levels than the other two groups for the whole period of the experiment. The severe negative consequences to inflammation in OMAS group cows could have been a contributory cause in the occurrence of the worse energy balance, the lower milk yield in the first month of lactation and the more negative reproductive indices. Further clues of a possible altered hepatic functionality are given by the lower plasma levels of GGT ($P<0.01$; Figure 7) as well as SDH (*NS*; Figure 15) and ALP (*NS*; Figure 9) observed in OMAS cows in comparison to CTR already before the treatment and thus also before calving.

The marked inflammatory processes observed in OMAS groups are in agreement with the higher incidence of clinical diseases (33% of cows; Table 5), mainly RP and ketosis, and consequent drug treatments (33% of cows needed antibiotic and/or anti-inflammatory treatments in the first week of lactation). Therefore, it is interesting to point up as all cows of OMAS group recovered later than other groups a satisfactory metabolic and health status. Nevertheless, at the end of the

first month, OMAS cows reached a good milk yield (close to CTR cows) and did not show any further clinical health problem during their lactation assignable to the treatment. Also interesting is the peculiar pattern of changes of some –APP in the last weeks of pregnancy. In particular, in OMAS group, retinol, bilirubin, PON, tocopherol, β-carotene, SDH and GGT tended to be lower or to decrease some time before the beginning of inflammation (identified by the haptoglobin raise and by the zinc drop). This makes suspect that, for some unknown reason, the OMAS cows were more susceptible to inflammation regardless of the treatment. A possible cause could be the higher BCS, despite within normal values.

Fertility. CTR cows showed optimal reproductive performance (101±46 days open and 2.0±1.2 AIs; Table 7) confirmed by the high level of FSI index (84 points), better than the ideal level suggested by Esslemont and Eddy (1977) (80 points). OM had acceptable values of days open (120±47 days) and number of artificial inseminations (2.3±1.2 AIs) with a FSI = 44 points, better than the average values found in the area (179 days open and 2.8 AIs; A.I.A., 2010). The cows of OMAS group showed the worst situation in this experiment (158±55 days open and 3.3±1.5 AIs; FSI = -5 points), but anyway better than the area averages. Several works which deal with the post-calving administration of ω3 FA report improvements in fertility performances. Petit and Twagiramungu (2006) and Petit et al. (2002) showed an improvement in gestation rate of lactating dairy cows fed flaxseed (rich in linolenic acid) during lactation, partly attributed to a lower production of $PGF_{2\alpha}$. Positive effects on fertility may be attributed to a higher concentration of ω3 FA showed in endometrium after the oral administration of fish meal in non-lactating beef cows by Burns et al. (2003) and in lactating dairy cows by Heravi Moussavi et al. (2007). In our experiment, the ω3 FA administered pre-calving did not improve fertility in comparison with cows that received palm oil. However, the bad fertility performances found in OMAS group may be attributed to the more severe inflammatory status which suffered these cows (Bertoni et al., 2009), partly regardless of the treatment.

Possible implications in prostaglandin metabolism. Results showed as OMAS cows suffered immediately after calving some issues related to the reproductive apparatus, since the group on average delayed the calving 3 of days in comparison to CTR cows and of 7 days in comparison to OM and the 25% of cows suffered retained placenta. It is reported that some problems related to parturition, uterus motility and fetal membranes expulsion may be related to the action of the acetylsalicylic acid. This molecule exerts an inhibitory effect on cyclooxygenases (Vane, 2003), causing also a decrease in the production of $PGF_{2\alpha}$, an important component of the prostaglandin family derived from arachidonic acid (Stahringer et al., 1999). In addition, the administration of ω3 FA causes their replacement in phospholipids for arachidonic acid (Simopoulos, 2008), contributing to reduce the production of prostaglandins (including $PGF_{2\alpha}$). Several researches demonstrated the relationship between a low plasma level of $PGF_{2\alpha}$ and troubles in the operations related to labor and uterus motility. In particular, it was connected to RP (Chassagne and Barnouin, 1992; Takagi et al., 2002) and uterine involution (Lindell et al., 1982; Thompson et al., 1987). Despite we did not determine the plasma level of $PGF_{2\alpha}$, it is possible to speculate their drop in OMAS group cows after the acetylsalicylic acid and ω3 FA administration in OMAS cows. This synergic effect may have been a contributory cause in the etiology of RP and much more in the higher frequency and severity of inflammation which has been suggested to have a role in the prolonged pregnancy noticed in OMAS group cows.

IDENTIFICATION OF THE SUBJECTS AT RISK THROUGH A BLOOD PROFILE AT 7 DIM

In the first chapter of this thesis we described how a combination of some plasma inflammatory indices (cholesterol, haptoglobin, paraoxonase and reactive oxygen metabolites) assayed at 7 DIM allows a good prediction of the coming performance of the cows (Post-calving Inflammatory Response Index, PIRI). Here we apply this index to this set of data with the aim to give a support to its effectiveness.

Basing on this index we divided cows in tertiles with the aim to compare cows with the best (higher tertile) and the worst (lower tertile) inflammatory status at 7 DIM. In the Table 4 are reported the average values of PIRI for every group. Data of plasma parameters strongly confirm that the cows in the lowest tertile suffered a more severe inflammatory condition than the cows in the highest tertile. In particular LO-PIRI, in respect to HI-PIRI cows, had significantly higher levels of haptoglobin and ceruloplasmin (Figures 27 and 22) for the first week of lactation; on the contrary, –APP (cholesterol, albumin, paraoxonase, retinol; Figures 20, 6, 13, 14) levels were contemporaneously reduced (and bilirubin increased; Figure 26). Furthermore, the recovery of satisfactory plasma levels was reached much later in LO-PIRI. This trend of delayed –APP recovery was already observed in cows with a more severe inflammatory status as highlighted by the LAI and LFI indices (Trevisi et al., 2011). This higher occurrence of inflammation is also confirmed by the lower milk yield in the first month of lactation, while surprisingly the energy metabolism was characterized by worse indices in LO-PIRI cows (lower glucose, higher NEFA and BHB; Figures 19, 28, 29) which make suppose a more negative energy balance than in HI-PIRI cows.

Interestingly, Table 4 shows that the 67% of OMAS cows is gathered in the lowest tertile, confirming the more frequent occurrence of inflammatory problems in the cows of this group. On the contrary, the presence of the 55% of OM cows in the best tertile compared with 33% of CTR cows, makes guess that OM cows had a better health and inflammatory status. Chi-square test confirms the association ($P<0.10$) between the treatment and the level of inflammation (PIRI index).

According to these results, PIRI seems to be a useful index to split cows – one week after calving – in "good" subjects (characterized by a good clinical and subclinical health status, by a better response to inflammation and by better performances) and in "bad" subjects. This could allow a prompt intervention on the subjects that suffer more issues in the immediate post-calving and seems to be more operable than LAI and LFI, which give a response only after the first month of lactation (Trevisi et al., 2011). Interestingly, one only blood sample is needed at 7

DIM, which makes easy the calculation of the index and so opens to the possibility of its field application. Furthermore, a possible application could be the attempt to improve as soon as possible the reproductive functionality that has been confirmed to be worse in LO-PIRI cows (FSI index = 6.2 in LO-PIRI *vs* 65.1 in HI-PIRI).

CONCLUSION

The attempt to attenuate inflammatory processes at calving through the use of low-dose NSAIDs in advance in combination with ω3 FA did not exert any positive effect. In fact, such a treatment caused a lengthening of pregnancy and an increase in the frequency of retained placenta. On the contrary, the results of this experiment confirm the usefulness of an ω3 FA administration in the peripartum period of dairy cows. The improvements are found in the slight reduction of inflammatory status after calving (higher frequency of cows treated with ω3 FA among cows with a low response to inflammation), and mainly in the energy metabolism immediately after calving, likely justified by a quicker raise of feed intake.

After these results it is possible to suggest the administration of ω3 FA during the whole transition period (from three weeks before calving through three weeks after), while NSAIDs should be given only starting 24 hours after calving and in any case after the delivery of the placenta to avoid the interference in the prostaglandin metabolism, of major importance during these phases.

The application of the Post-calving Inflammatory Response Index highlighted the possibility to treat in advance subjects that do not show any clinical health problem, but which likely would benefit from a support, since their subclinical problems may have later resulted in some troubles (liver and reproductive systems).

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Table 1 - Nutritional value of the diets fed as TMR for close-up and lactating cows

COMPONENTS [% of DM]	Dry cows	Lactating cows
Corn silage	25.0	33.5
Corn grain ground	-	17.2
Alfalfa hay	-	15.3
Soybean meal	7.3	9.2
Cottonseed	-	7.7
Corn flakes	-	7.4
Grass hay	51.6	3.5
Sunflower seed	-	2.4
Mineral and vitamin supplementation[§]	1.0	2.1
Corn semolina glutinated	-	1.7
Wheat straw	15.3	-
CHEMICAL COMPOSITION		
NEL [Mcal/kg of DM]	1.4	1.5
CP [% of DM]	12.5	16.0
NDF [% of DM]	54.9	33.7
Starch + sugar [% of DM]	12.5	29.8
Ether extract [% of DM]	2.7	4.5

[§] 42.9% Ca_2PO_4; 28.6% urea; 14.3% MgO; 7.1% NaCl; 7.1% mineral and vitamin supplement (1500000 IU/kg vitamin A; 150000 IU/kg vitamin D; 7000 IU/kg vitamin E; 10 mg/kg Co; 70 mg/kg I; 1100 mg/kg Mn; 500 mg/kg Cu; 23 mg/kg Se; 4000 mg/kg Zn)

Water was added to each ration to reach the 52% of DM

Table 2 - Amount of fatty acids [g/d] received daily by cows of OM and OMAS group from 21 days before calving to the last day of pregnancy

FATTY ACID		g/d
C14:0	Myristic acid	3.06
C16:0	Palmitic acid	5.60
C16:1 ω-7	Palmitoleic acid	9.94
C18:0	Stearic acid	0.88
C18:1 ω-9	Oleic acid	11.34
C18:1 ω-7	Cis-vaccenic acid	3.14
C18:2 ω-6	Linoleic acid	1.05
C18:3 ω-3	Linolenic acid	0.37
C18:4 ω-3	Stearidonic acid	1.01
C20:0	Arachidic acid	0.07
C20:1 ω-11	Icosenoic acid	1.77
C20:1 ω-9	Eicosenoic acid	8.09
C20:2 ω-6		0.15
C20:4 ω-6		0.31
C20:3		0.09
C20:5 ω-3	**Eicosapentaenoic acid**	**4.57**
C22:0	Behenic acid	0.04
C22:1 ω-9	Erucic acid	0.69
C22:6 ω-3	**Docosahexaenoic acid**	**6.06**
C24:1	Nervonic acid	0.43
	Others	15.83
	TOTAL	**74.51**
	ω-3 total	12.02
	ω-6 total	1.51

Table 3 - Composition of the 74.5 g/d of fat received daily by cows of CTR group from 21 days before calving to the last day of pregnancy

FATTY ACID		%
C14:0	Myristic acid	2
C16:0	Palmitic acid	60 max
C18:0	Stearic acid	45 max

Table 4 - Distribution of cows per tertile of PIRI and group; average PIRI score per tertile

	LO-PIRI	IN-PIRI	HI-PIRI	Σ
CTR	1	4	3	8
OM	2	2	5	9
OMAS	6	2	1	8
Σ	9	8	9	26
$X^2 = 8.18$	$P < 0.10$			

	LO-PIRI	IN-PIRI	HI-PIRI
Average PIRI score	9.15	20.48	28.49
STD	4.32	2.81	3.31

Table 5 - Count of clinical health problems found in each group of cows

	DIARRHEA	LAMENESS	RP	METRITIS	KETOSIS
CTR		1		1	
OM	1		1		
OMAS			2		2

Table 6 - Count of clinical health problems found in each tertile of PIRI

	DIARRHEA	LAMENESS	RP	METRITIS	KETOSIS
HI-PIRI					
IN-PIRI	1				
LO-PIRI		1	3	1	2

Table 7 - Fertility data divided for the three groups of treatment and calculation of FSI index

GROUP	N. OF COWS	PREGNANT		PREGNANT AT 1st AI			N. AI / PREGNANCY	DAYS OPEN		FSI INDEX
		n.	%	n.	% of total cows	% of pregnant cows		mean	sd	
CTR	8	7	88	4	50	57	1.7	95.1	46.5	83.9
OM	9	8	89	3	33	38	2.3	120.3	47.0	44.5
OMAS	9	6	67	1	11	17	3.3	157.7	55.1	-4.7

Table 8 - Fertility data divided for the tertiles based on PIRI index and calculation of FSI index

TERTILE	N. OF COWS	PREGNANT		PREGNANT AT 1st AI			N. AI / PREGNANCY	DAYS OPEN		FSI INDEX
		n.	%	n.	% of total cows	% of pregnant cows		mean	sd	
HI-PIRI	9	7	78	3	33	43	2.0	101.3	45.9	65.1
IN-PIRI	8	8	100	4	50	50	2.3	122.8	59.1	49.4
LO-PIRI	9	6	67	1	11	17	3.0	147.2	49.8	6.2

Figure 1 - Milk yield [kg/d] of dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (OMAS group).

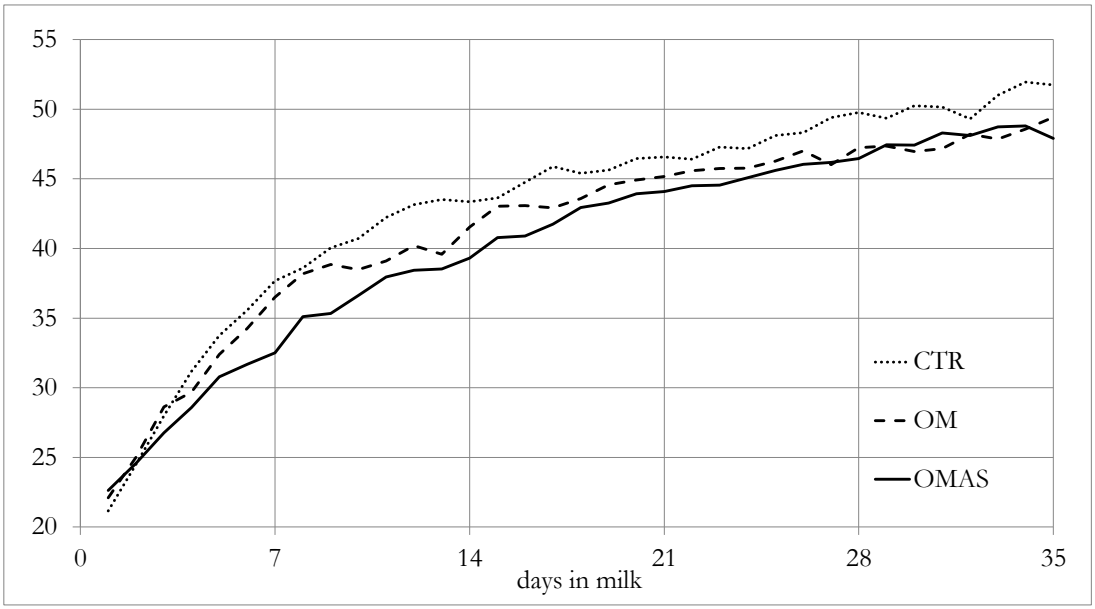


Figure 2 - Trend of body condition score [score] of dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (OMAS group).

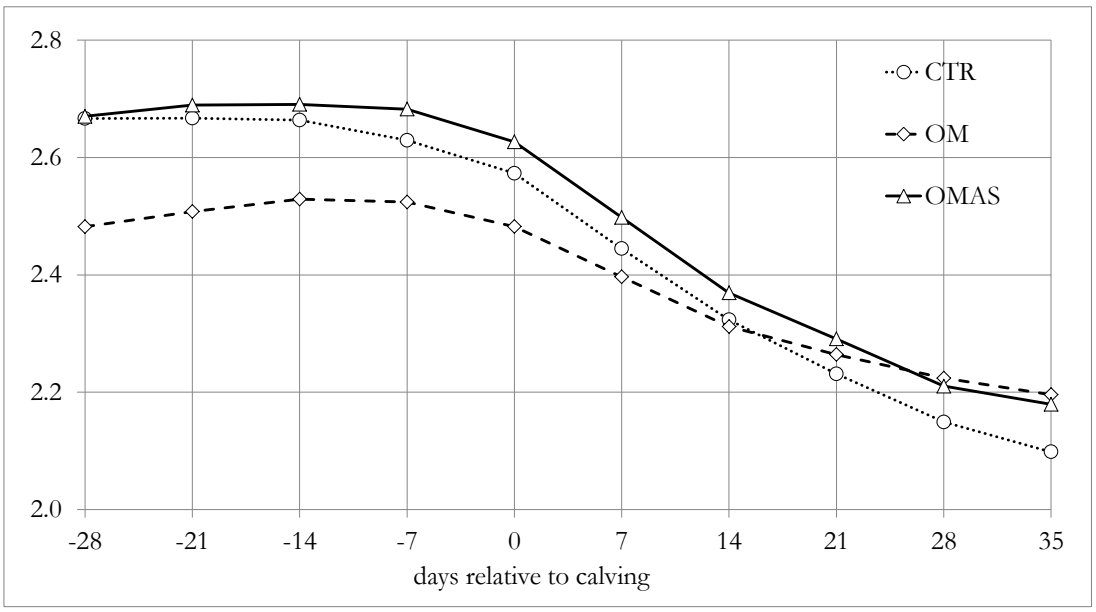


Figure 3 - Trend of rectal temperature [°C] of dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (OMAS group).

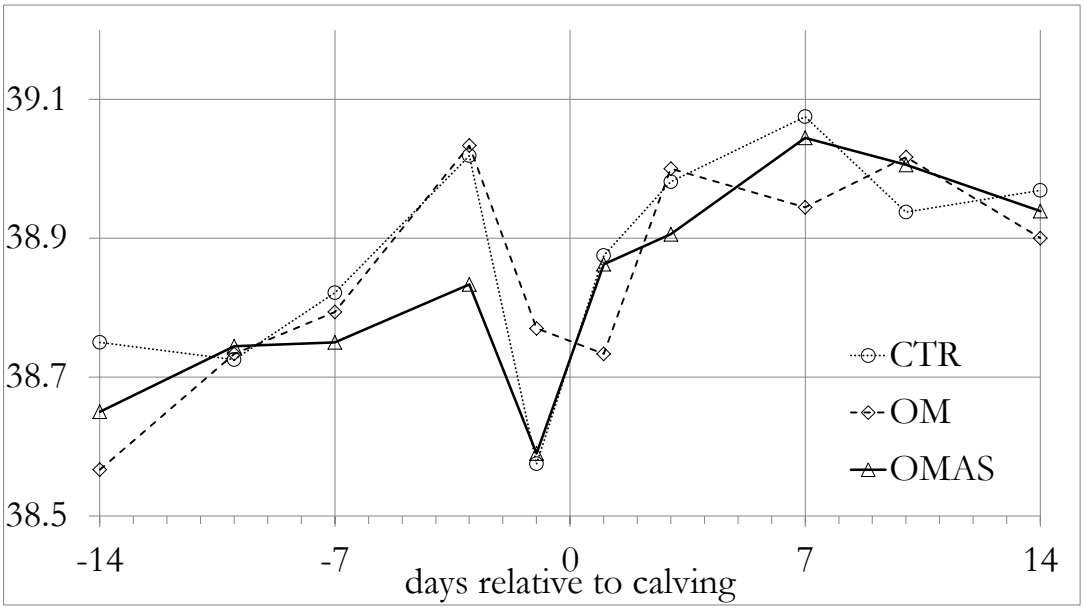
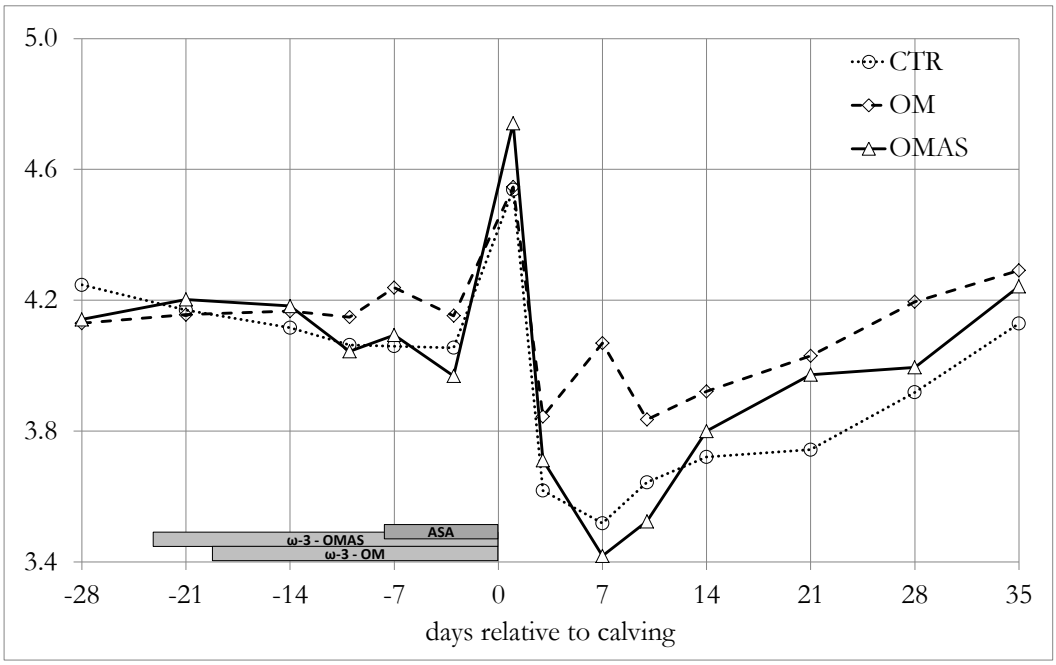


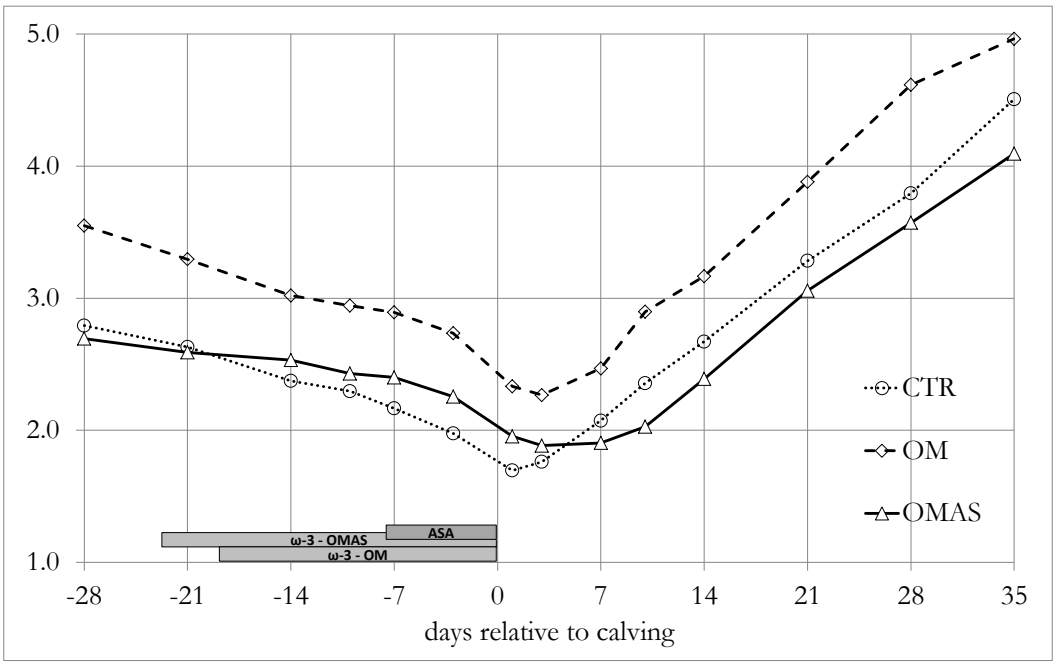
Figure 4 - Pattern of changes of glucose [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM									**					
CTR *vs* OMAS														
OM *vs* OMAS									***	+				

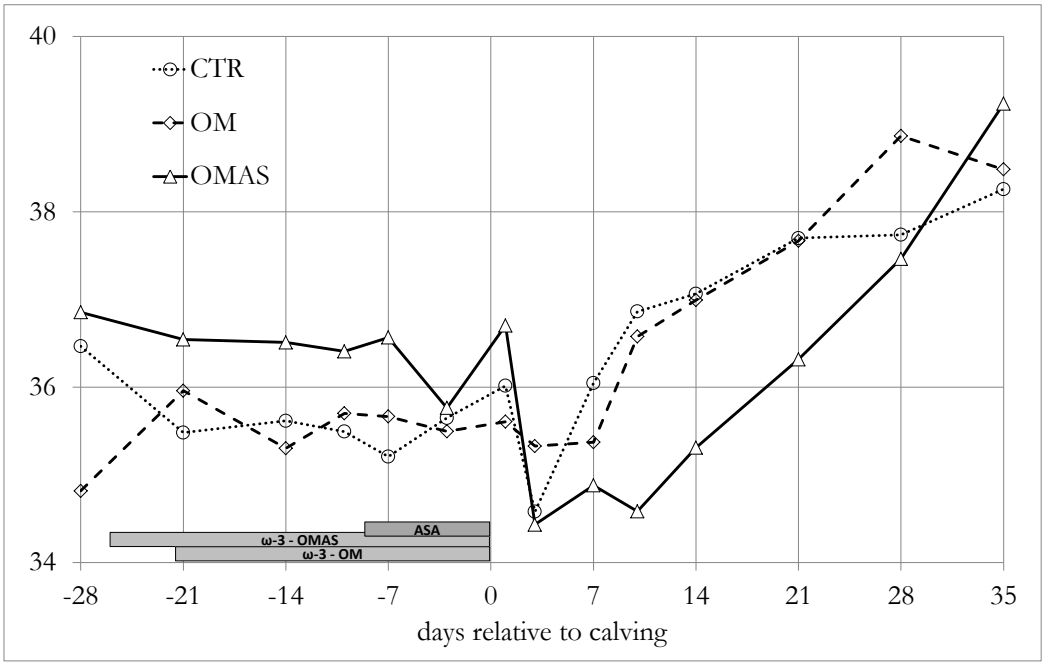
Figure 5 - Pattern of changes of cholesterol [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM	**	*	*	**	**	***	***	**	+	*	+	+	*	
CTR *vs* OMAS										+				
OM *vs* OMAS	**	*	+	*	*	*	*	*	**	***	***	**	**	*

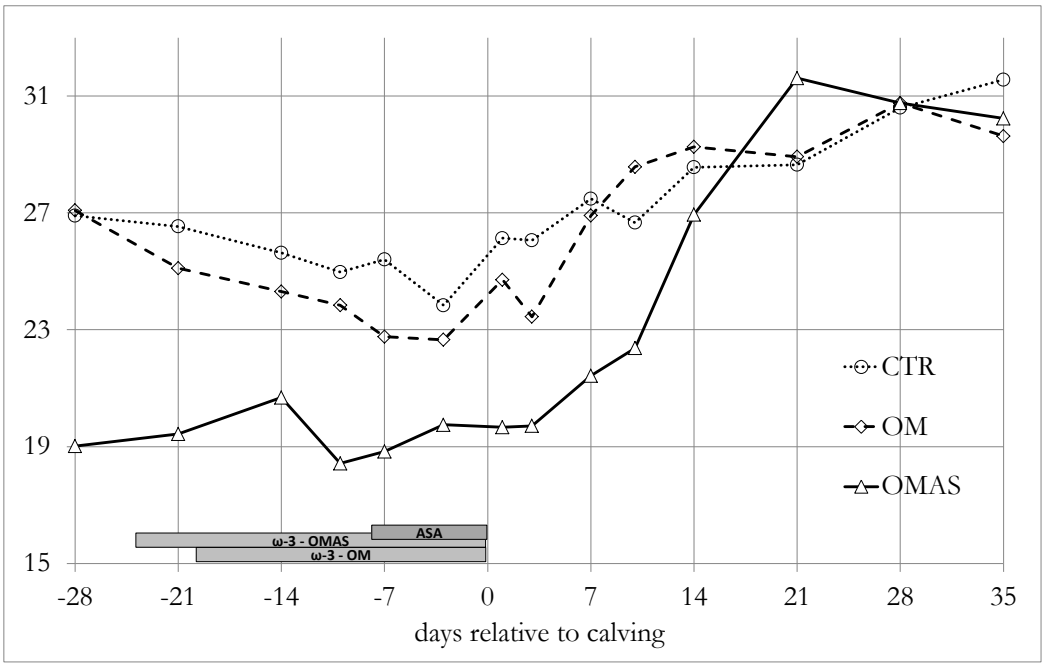
Figure 6 - Pattern of changes of albumin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS										*	+			
OM *vs* OMAS	+									*	+			

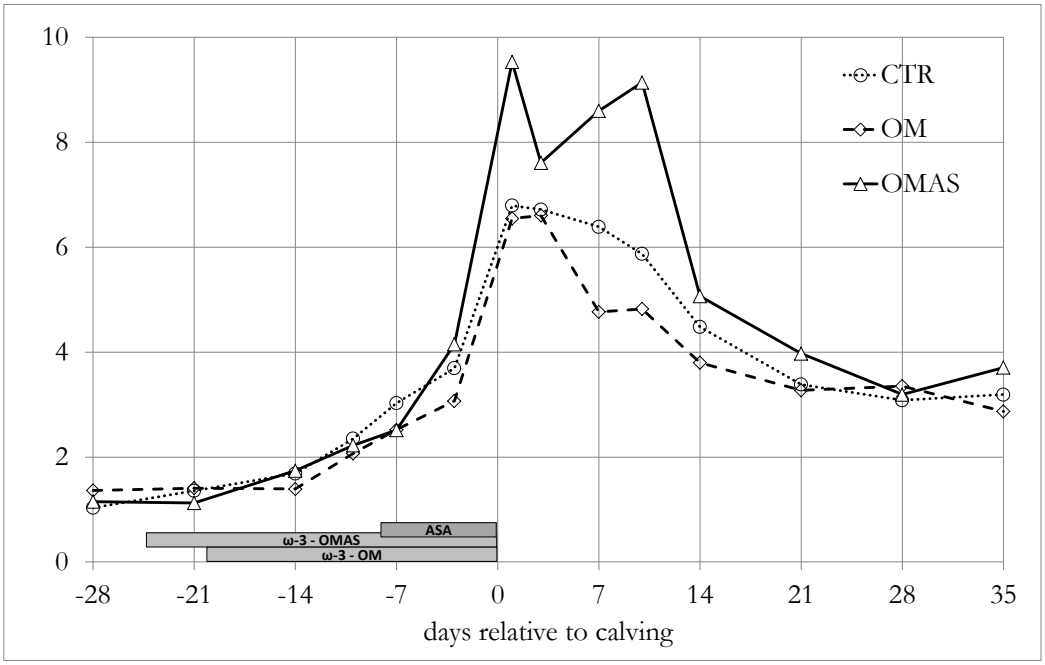
Figure 7 - Pattern of changes of GGT [U/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS	**	**	*	**	**	*	**	**	*					
OM *vs* OMAS	*	*		*	+		+							

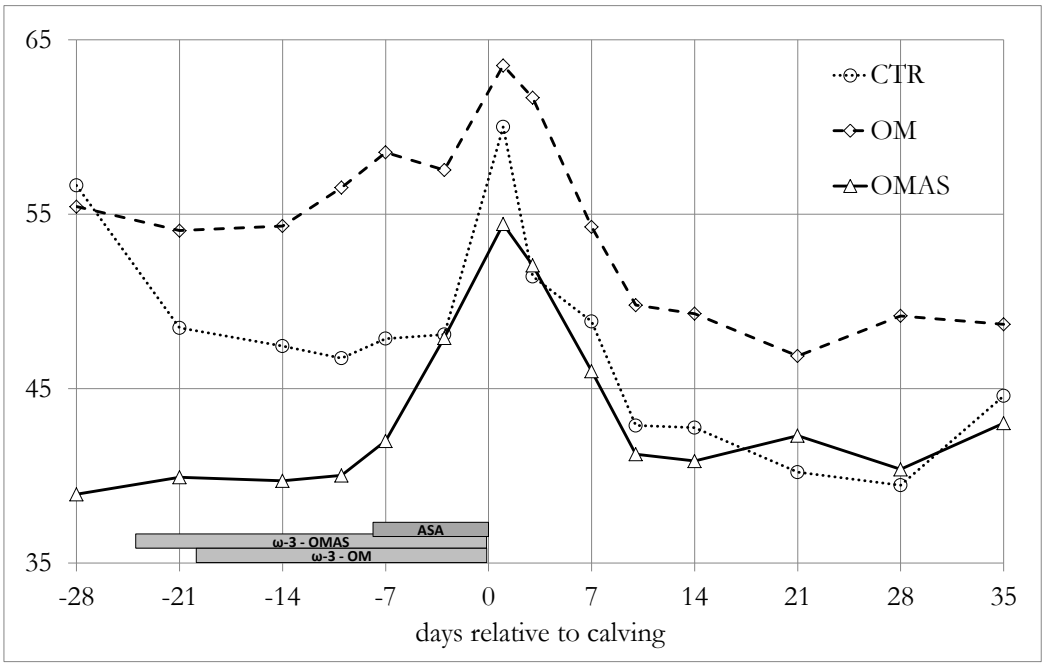
Figure 8 - Pattern of changes of bilirubin [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS														
OM *vs* OMAS														

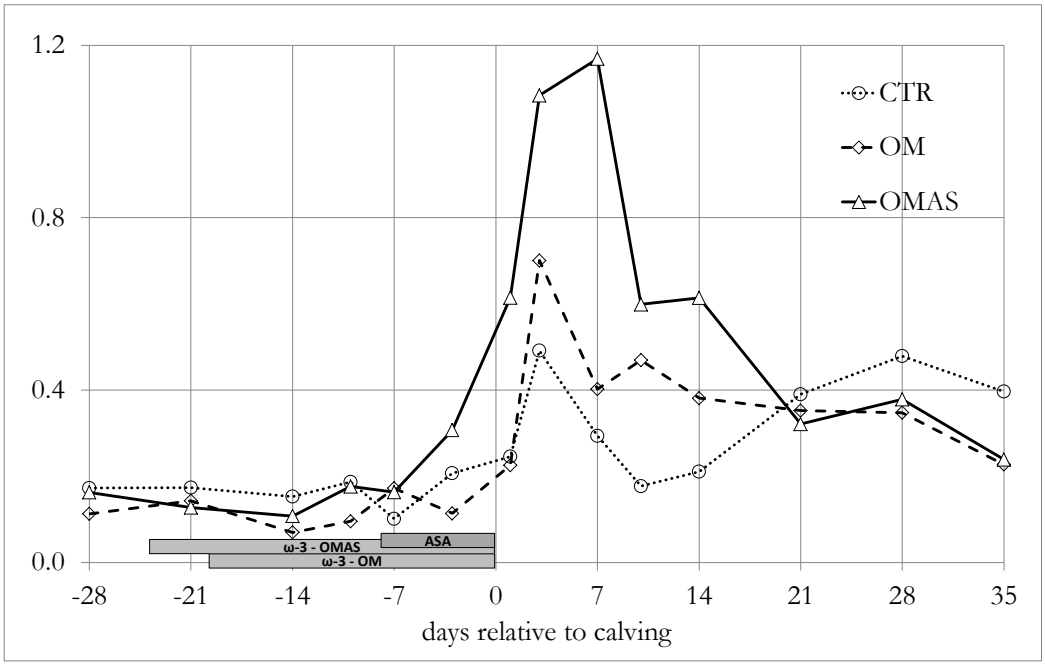
Figure 9 - Pattern of changes of ALP [U/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS														
OM *vs* OMAS														

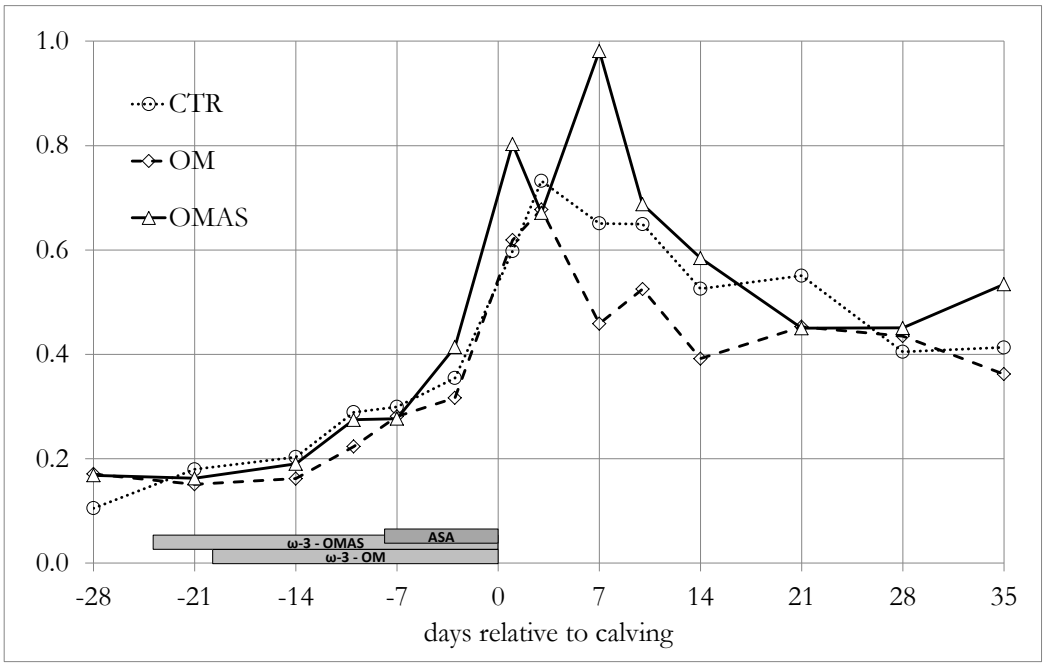
Figure 10 - Pattern of changes of haptoglobin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM										+				
CTR *vs* OMAS								*	**	*	*			
OM *vs* OMAS									*					

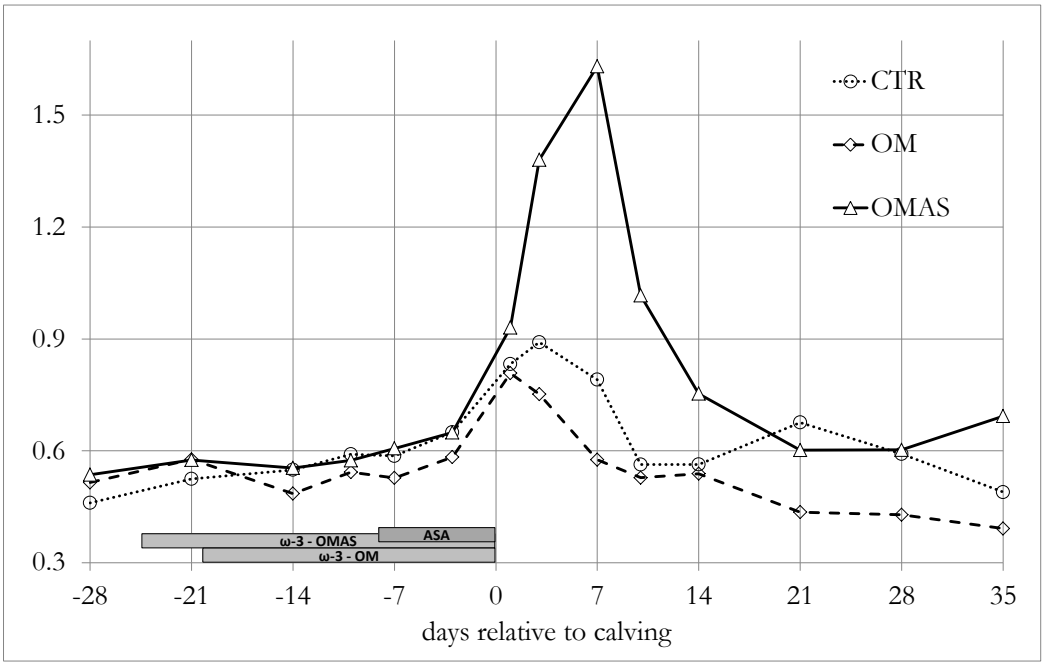
Figure 11 - Pattern of changes of NEFA [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS														
OM *vs* OMAS														

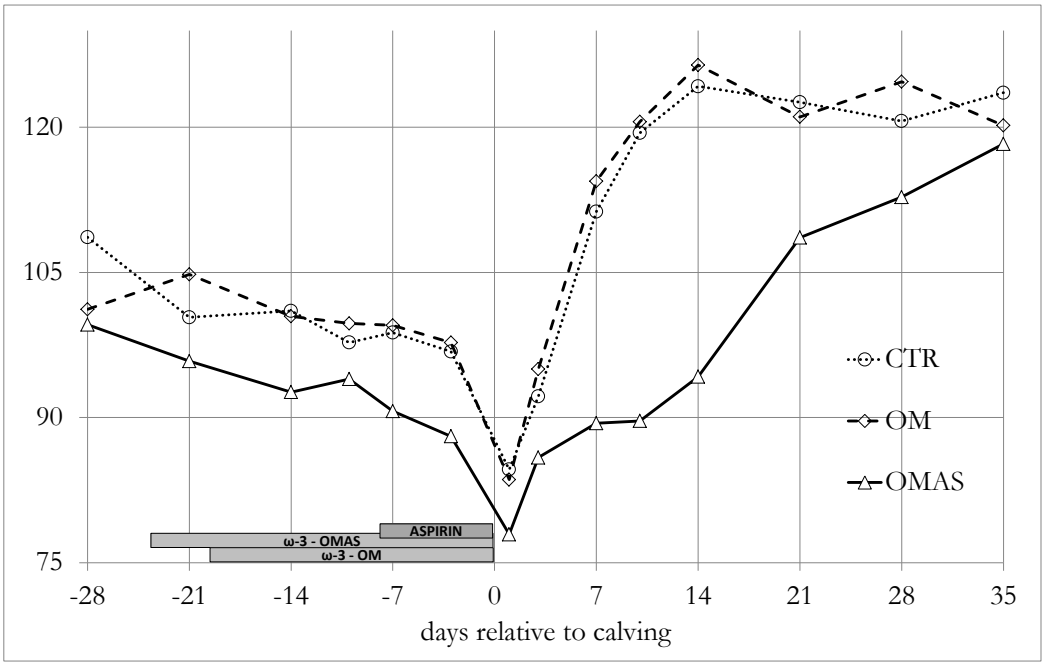
Figure 12 - Pattern of changes of β-hydroxybutyric acid [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM			+									+		
CTR *vs* OMAS									*					
OM *vs* OMAS			+					+	**	+				+

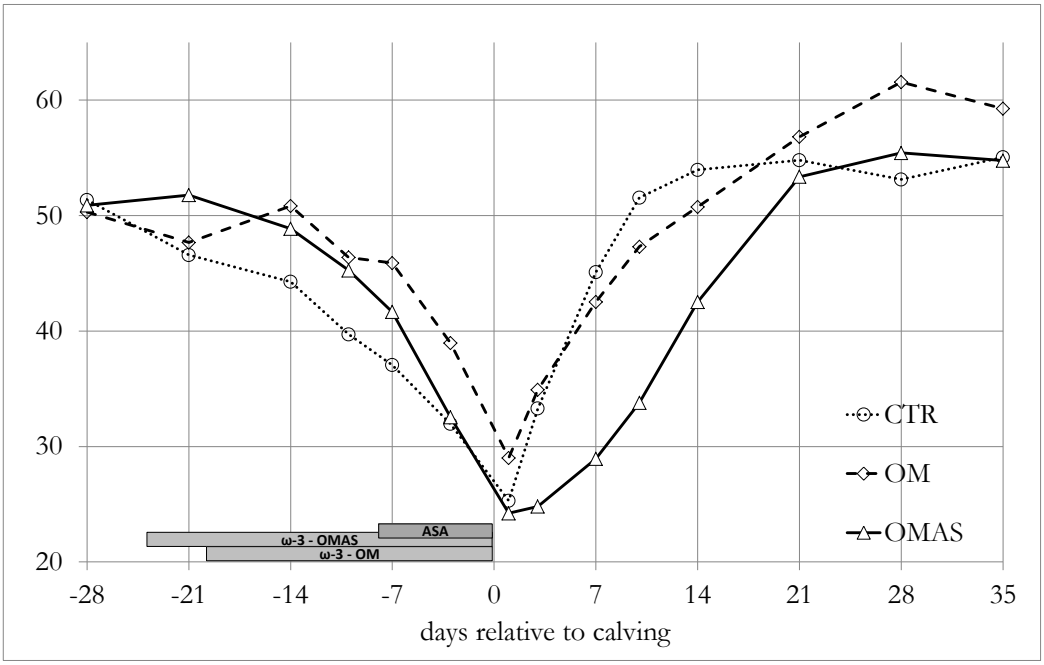
Figure 13 - Pattern of changes of paraoxonase [U/mL] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS									+	*	*			
OM *vs* OMAS									*	**	**			

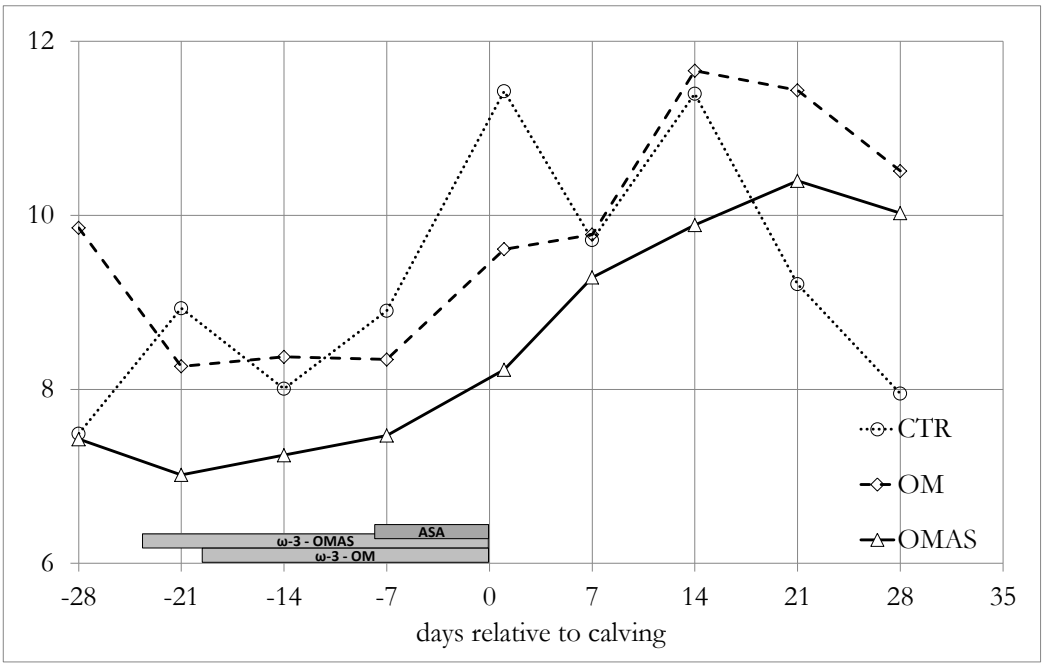
Figure 14 - Pattern of changes of retinol [µg/100 mL] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS									*	**	+			
OM *vs* OMAS									*	*				

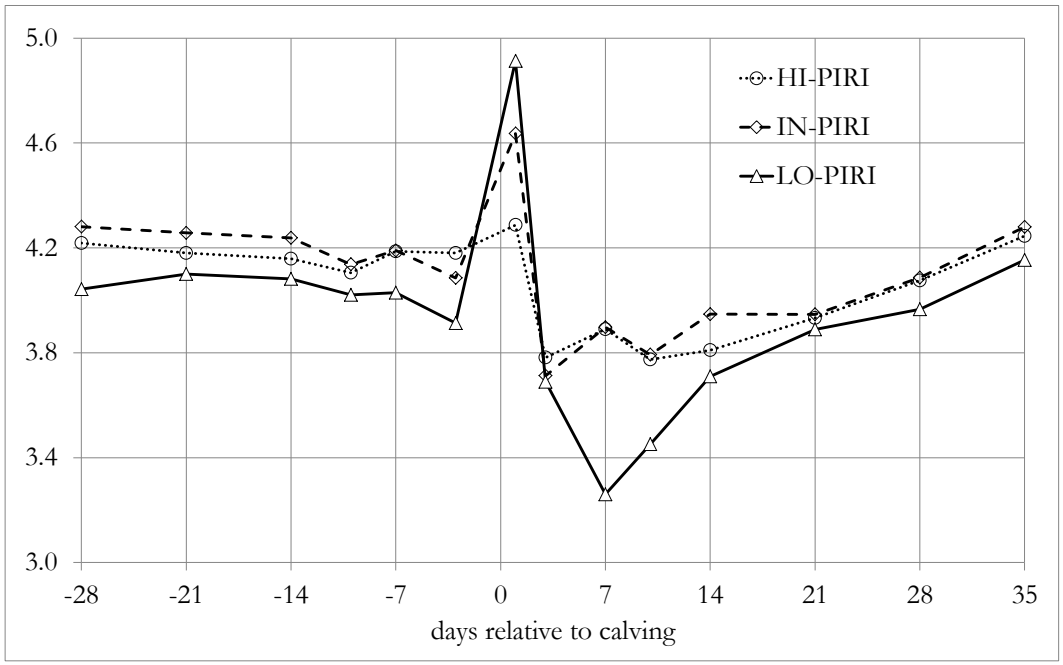
Figure 15 - Pattern of changes of SDH [U/L] in plasma collected in the morning before feeding from periparturient dairy cows that received before calving a supplement of palm oil (CTR group), ω-3 fatty acids alone (OM group) or in combination with an i.m. injection of acetylsalicylic acid (ASA; OMAS group). The average administration period for each group is showed by the horizontal bars.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
CTR *vs* OM														
CTR *vs* OMAS														
OM *vs* OMAS														

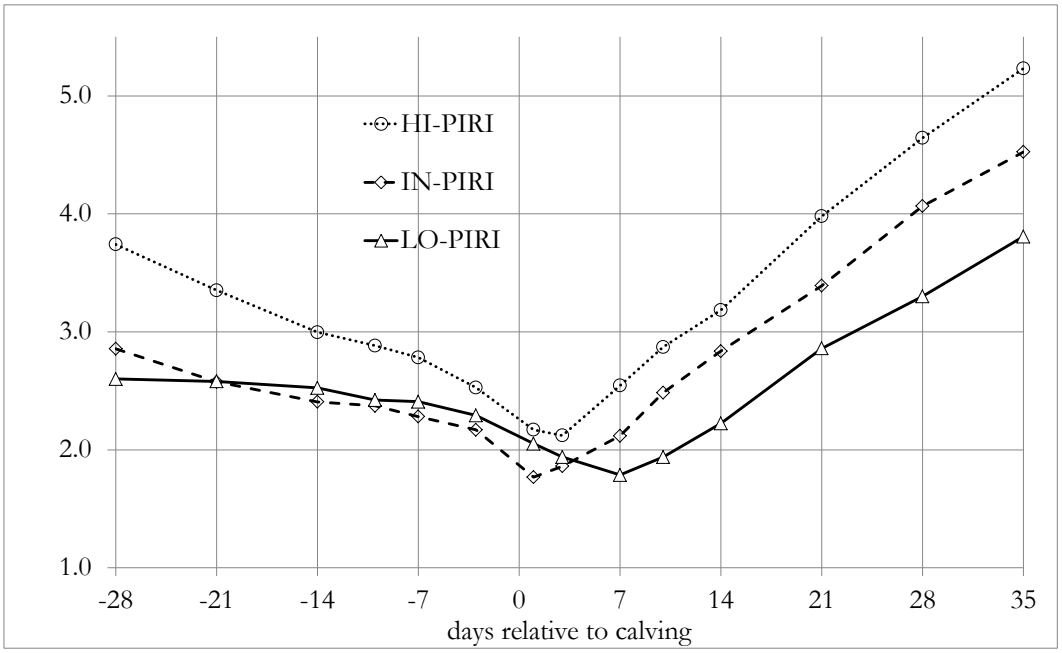
Figure 19 - Pattern of changes of glucose [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI							**		***	+				
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI									***	+				

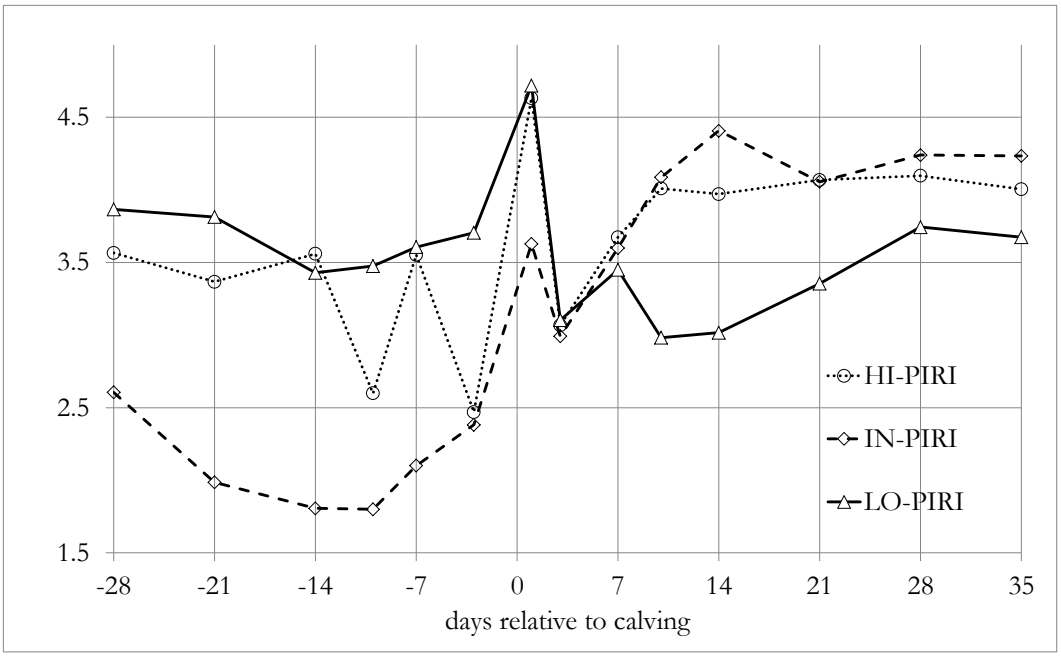
Figure 20 - Pattern of changes of cholesterol [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	***	**	*	*	+				***	***	***	***	***	***
HI-PIRI vs IN-PIRI	**	**	*	*	*	+	**		*	+		+		
IN-PIRI vs LO-PIRI							+		*	**	**	+	*	*

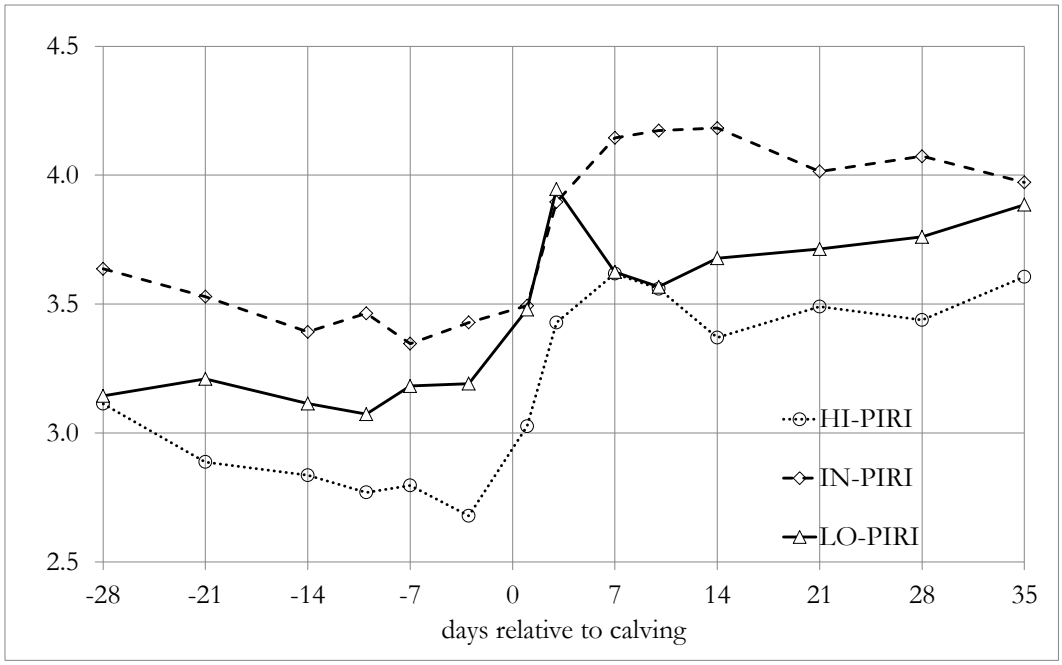
Figure 21 - Pattern of changes of urea [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI				*		**				*	*	+		
HI-PIRI vs IN-PIRI	*	**	***	+	**		*							
IN-PIRI vs LO-PIRI	**	***	***	***	***	**	*			*	**			

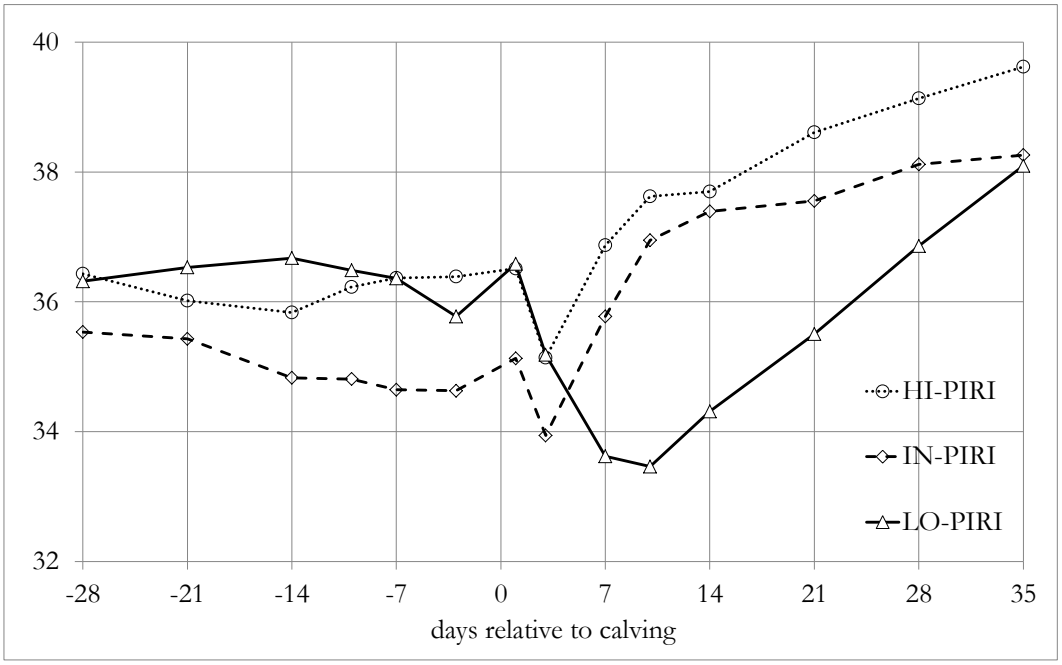
Figure 22 - Pattern of changes of ceruloplasmin [μmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						+		+						
HI-PIRI vs IN-PIRI	+	*	+	*	+	*			+	*	**	+	*	
IN-PIRI vs LO-PIRI									+	*	+			

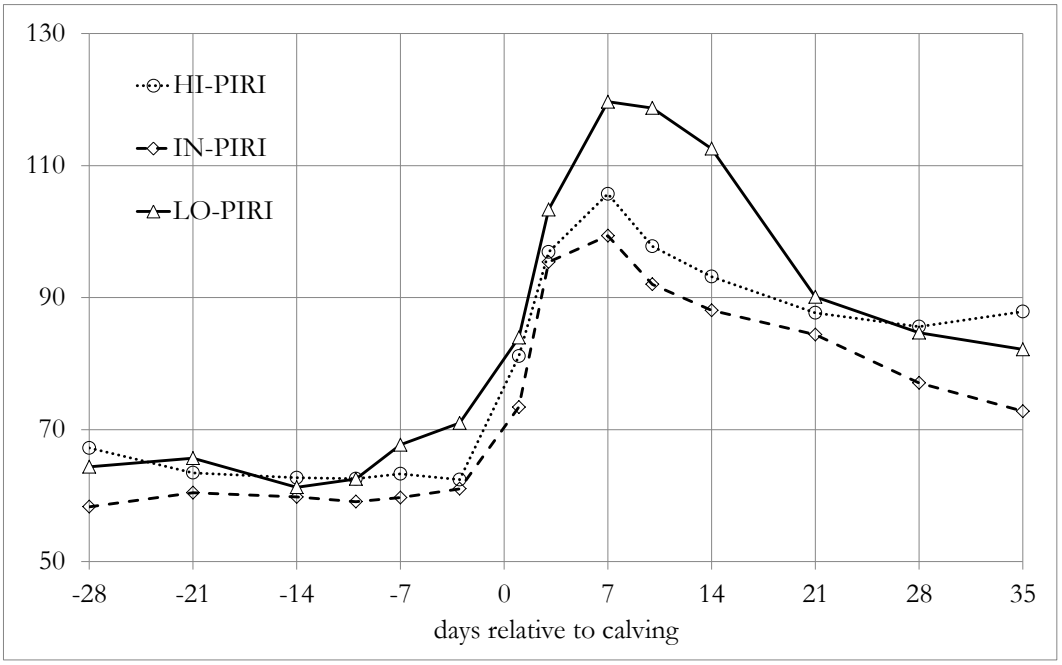
Figure 23 - Pattern of changes of albumin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI									***	***	***	***	**	+
HI-PIRI vs IN-PIRI					+	+								
IN-PIRI vs LO-PIRI			*	+	+				*	***	***	*		

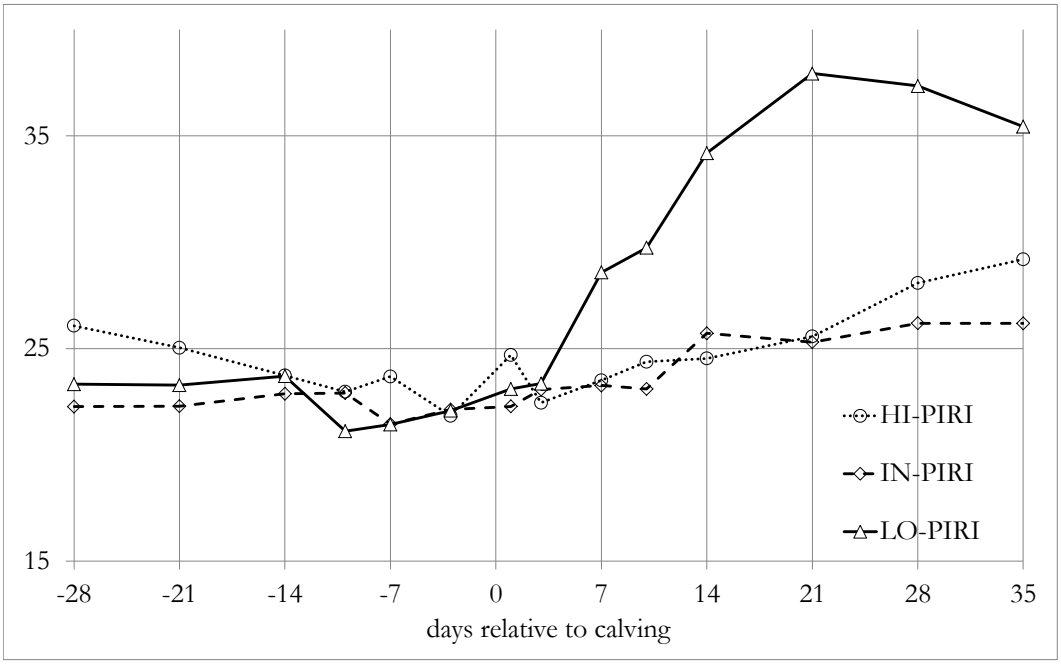
Figure 24 - Pattern of changes of GOT [U/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						+				*	*			
HI-PIRI vs IN-PIRI														*
IN-PIRI vs LO-PIRI						*	+		*	**	**			

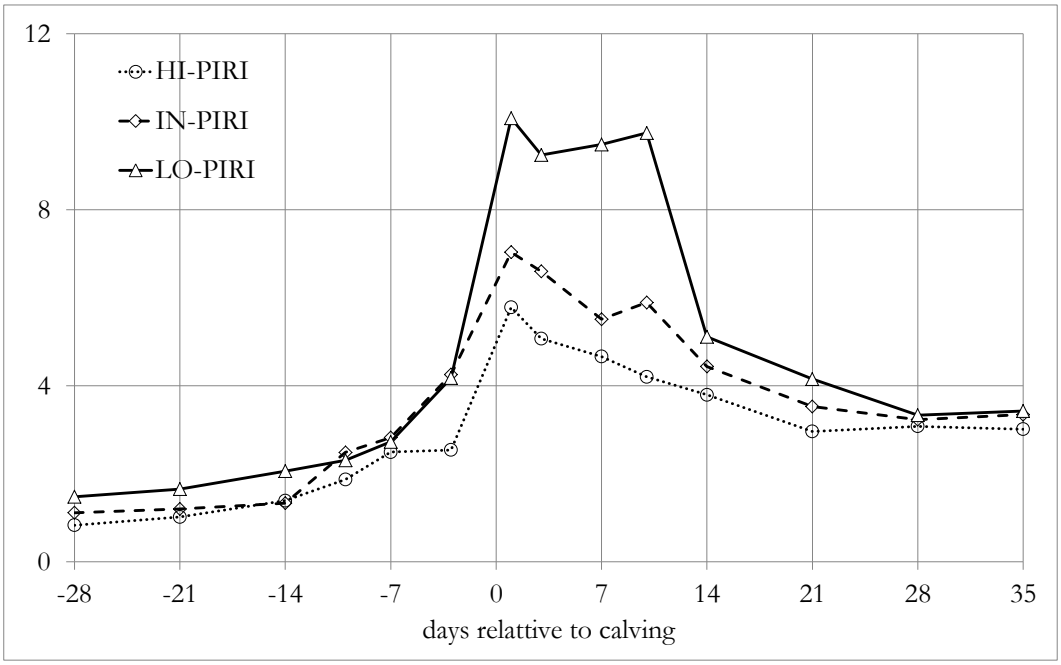
Figure 25 - Pattern of changes of GGT [U/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI											*	*	+	
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI												*	+	+

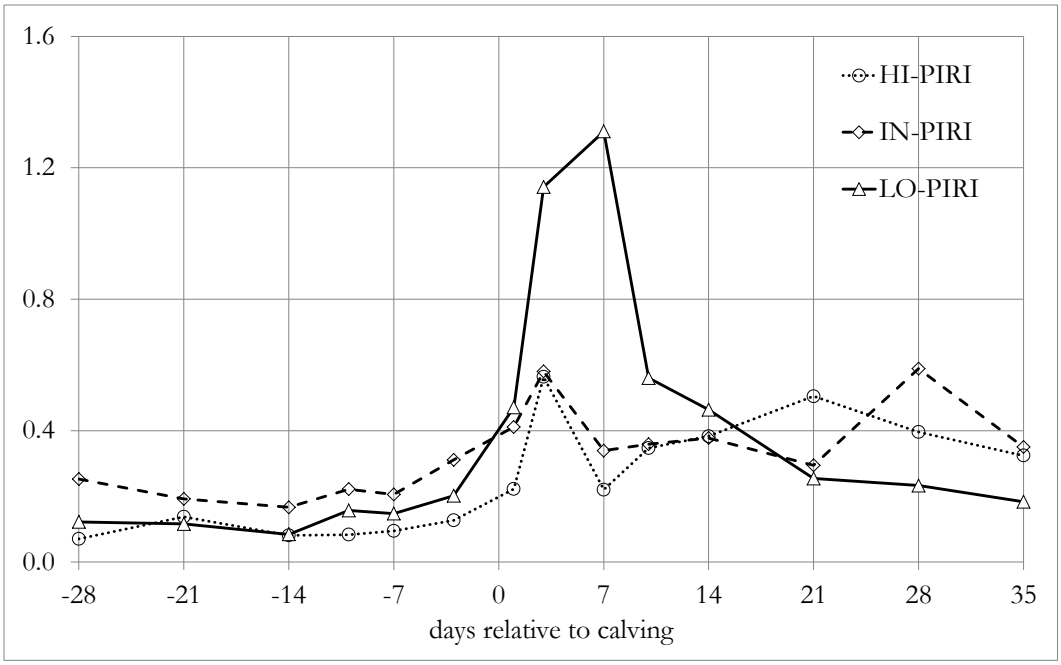
Figure 26 - Pattern of changes of bilirubin [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI		*				+	*	*	*	*				
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI			+											

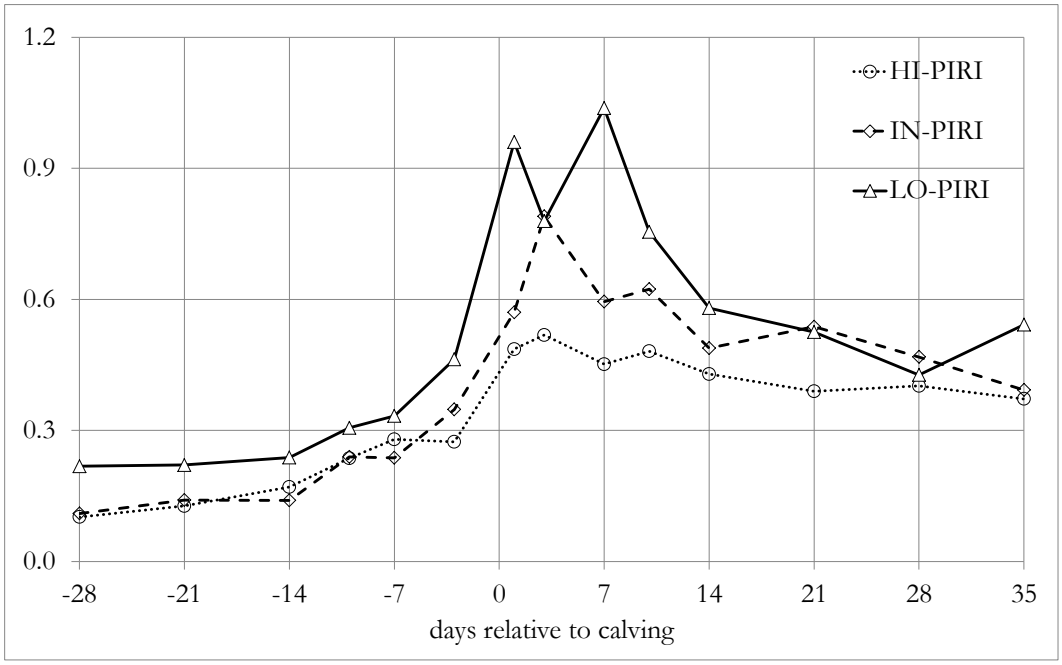
Figure 27 - Pattern of changes of haptoglobin [g/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI								*	***				*	
HI-PIRI vs IN-PIRI				+										
IN-PIRI vs LO-PIRI								+	***				+	

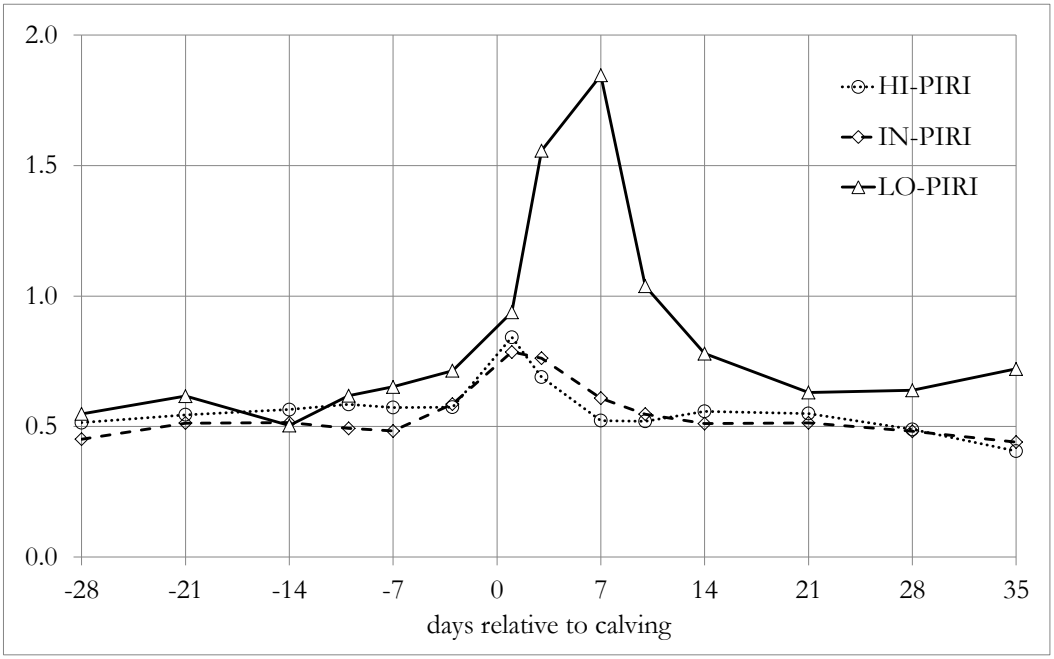
Figure 28 - Pattern of changes of non-esterified fatty acids [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI						+	**	+	***	+				
HI-PIRI vs IN-PIRI								+						
IN-PIRI vs LO-PIRI							*		**					

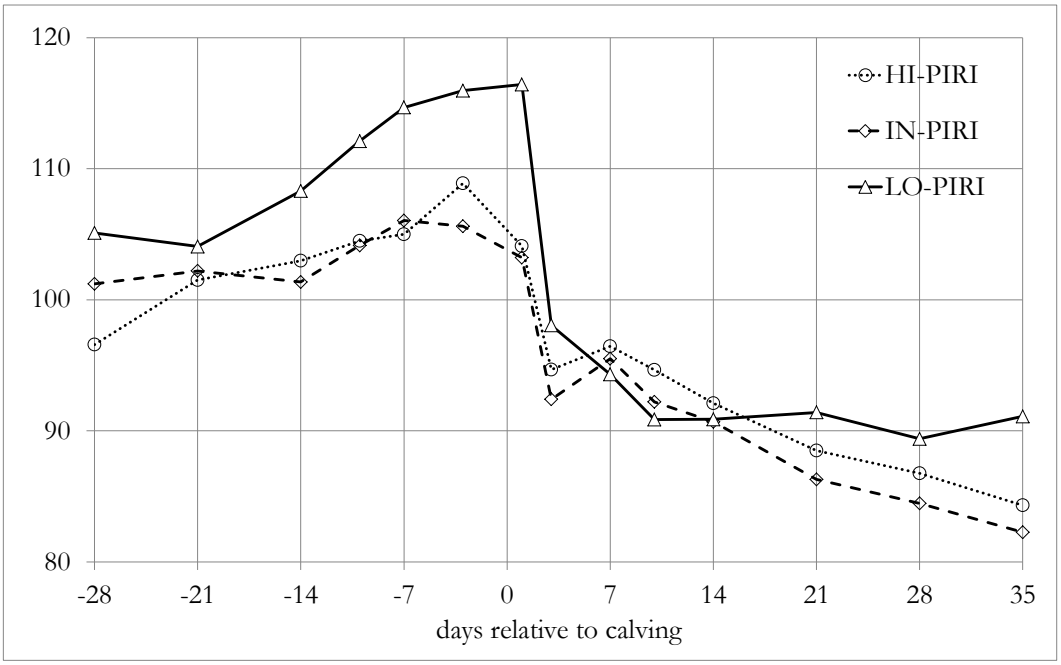
Figure 29 - Pattern of changes of β-hydroxybutyric acid [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI			+					**	***	*				+
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI								**	***	+				

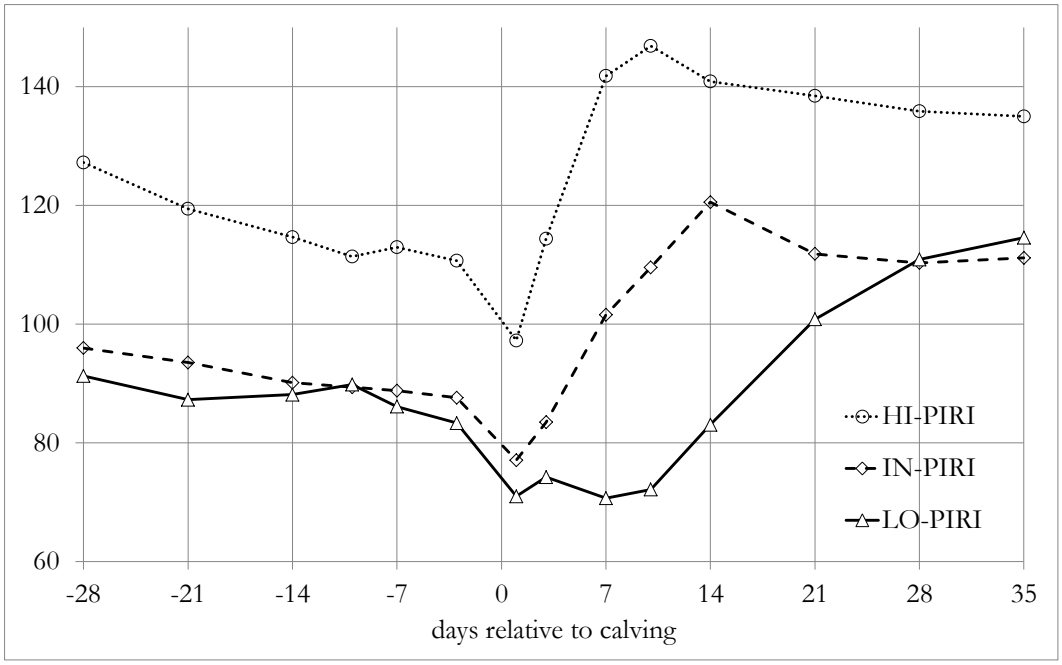
Figure 30 - Pattern of changes of creatinine [mmol/L] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI				+	*		**							
HI-PIRI vs IN-PIRI														
IN-PIRI vs LO-PIRI				+	+	*	**							+

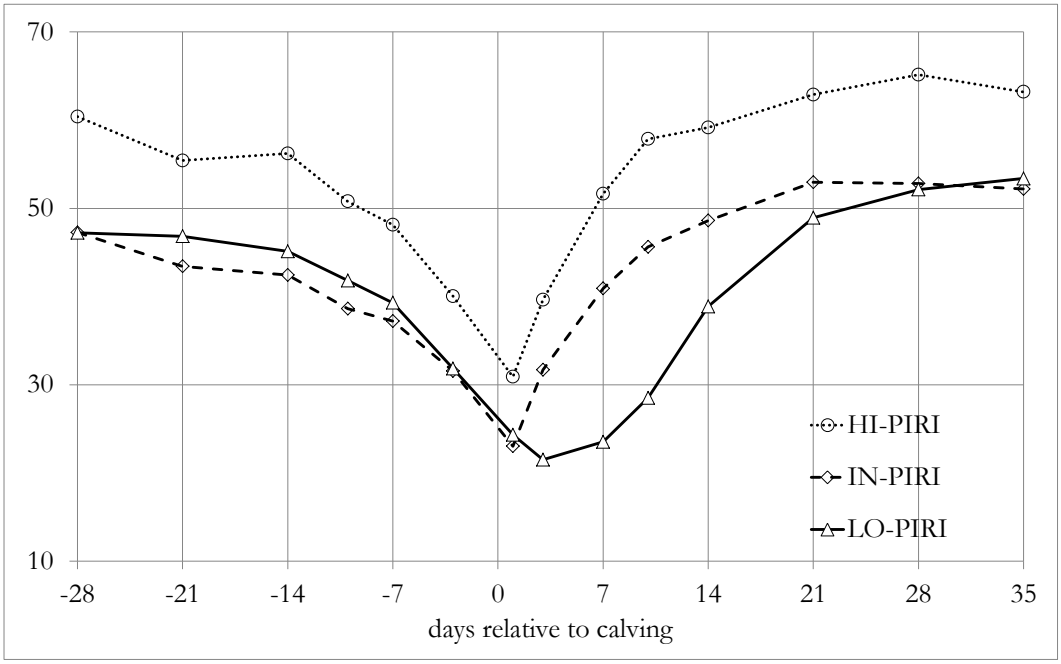
Figure 31 - Pattern of changes of paraoxonase [U/mL] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	***	***	**	**	**	**	**	***	***	***	***	***	**	*
HI-PIRI vs IN-PIRI	***	**		*	**	**	*	***	***	***	*	**	**	**
IN-PIRI vs LO-PIRI									***	***	***			

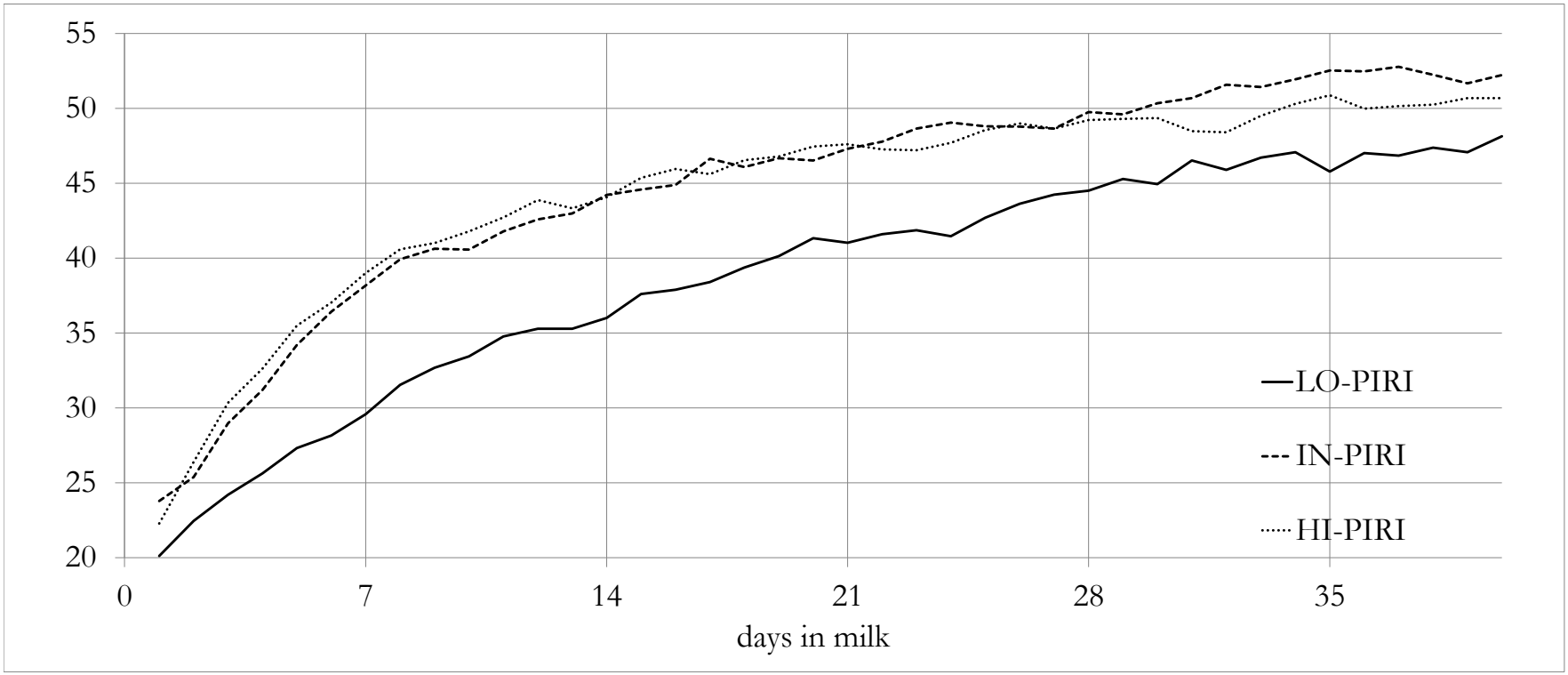
Figure 32 - Pattern of changes of retinol [μg/100 mL] in plasma collected in the morning before feeding from periparturient dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-10	-7	-3	1	3	7	10	14	21	28	35
HI-PIRI vs LO-PIRI	+		*					**	***	***	***	*	*	+
HI-PIRI vs IN-PIRI	+	*	*	*	+				+	*	+	+	*	+
IN-PIRI vs LO-PIRI								+	**	**	+			

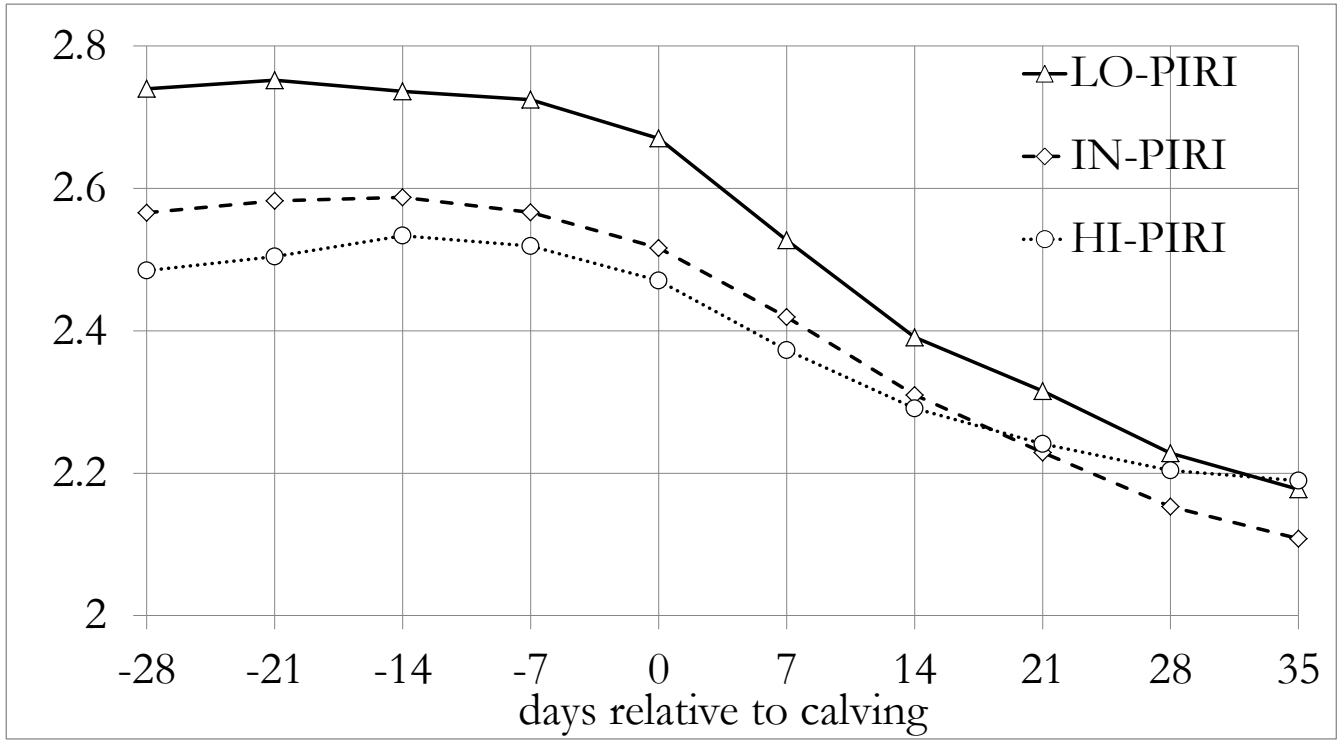
Figure 16 - Milk yield [kg/d] of dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
HI-PIRI vs LO-PIRI			*	*	**	**	***	**	**	**	**	**	**	**	**	**	**	*	*	*	*	*	+	*	*	+		+							+
HI-PIRI vs IN-PIRI																																			
IN-PIRI vs LO-PIRI			+	+	*	**	**	**	**	*	*	*	**	**	*	*	**	*	*	+	*	*	*	**	*	+		+		+		*	+	+	*

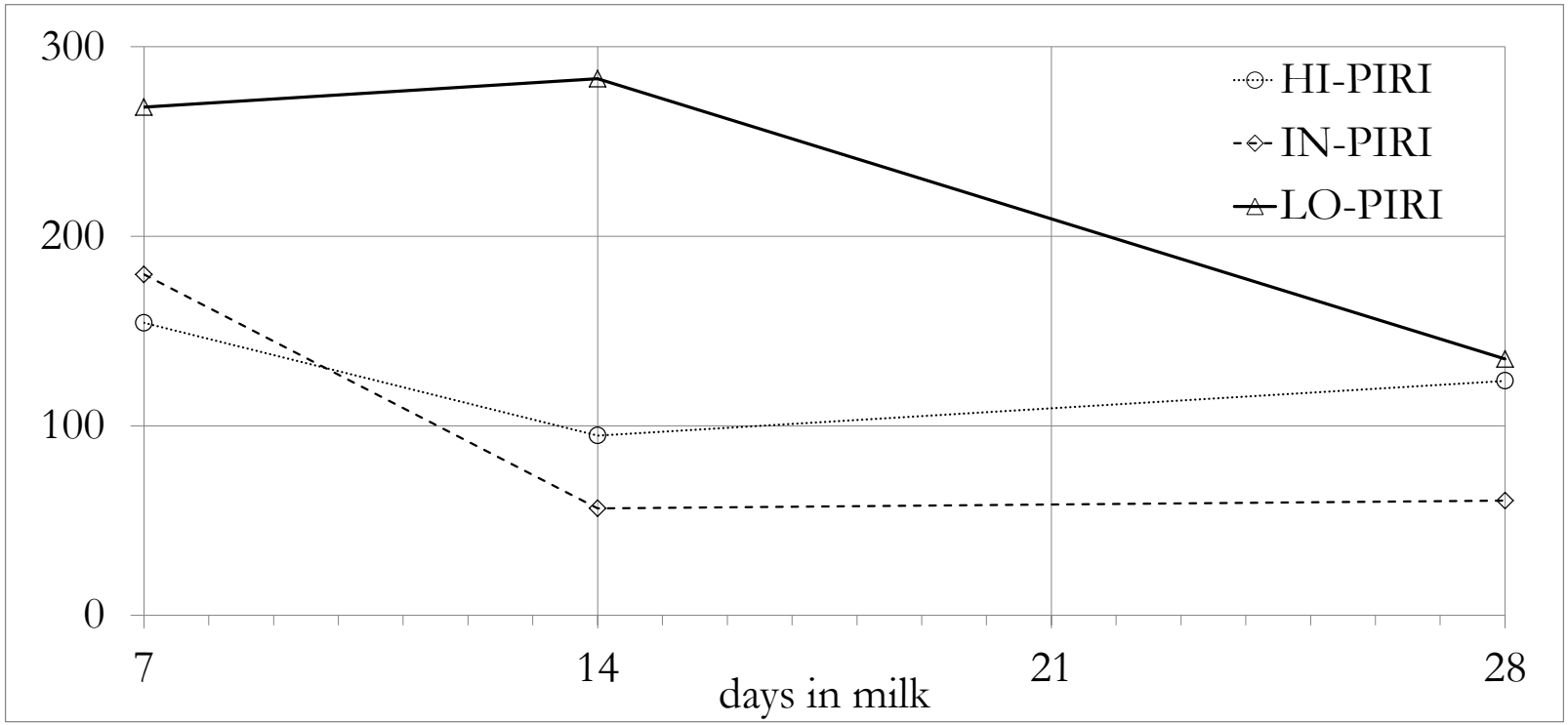
Figure 17 - Trend of body condition score [points] of dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	-28	-21	-14	-7	0	7	14	21	28	35
HI-PIRI vs LO-PIRI	*	*	+	+	+					
HI-PIRI vs IN-PIRI										
IN-PIRI vs LO-PIRI										

Figure 18 - Trend of somatic cell count [n/μL] of dairy cows divided in tertiles according to their score of Post-calving Inflammatory Response Index (PIRI). HI-PIRI = higher values; IN-PIRI = intermediate values; LO-PIRI = lower values.



Statistically significant differences among groups. + P<0.10; * P<0.05; ** P<0.01; *** P<0.001

DIM	7	14	28
HI-PIRI vs LO-PIRI		*	
HI-PIRI vs IN-PIRI			
IN-PIRI vs LO-PIRI		**	

Appendix 1 - Abbreviations

AIC	Akaike's Information Criterion	LX	Lipoxins
ALA	α-linolenic acid	MPO	Myeloperoxidase
ALP	Alkaline phosphatase	NAPI	Nutritional and Acute Phase Indicator
API	Acute Phase Index	NDF	Neutral detergent fiber
+APP	Positive acute phase protein	NEFA	Non-esterified fatty acids
-APP	Negative acute phase protein	NEL	Net energy of lactation
APR	Acute phase response	NFkB	Nuclear factor kB
ARA	Arachidonic acid	NSAID	Non-steroidal anti-inflammatory drug
ASA	Acetylsalicylic acid	ORAC	Oxygen radical absorbance capacity
BCS	Body condition score	PCA	Principal component analysis
BHB	β-hydroxybutyric acid	PG	Prostaglandin
BW	Body weight	PGHS	Prostaglandin endoperoxide synthase
CLA	Conjugated linoleic acid	PINI	Prognostic Inflammatory and Nutritional Index
COX	Cyclooxigenase	PIRI	Post-calving Inflammatory Response Index
CP	Crude protein	PON	Paraoxonase
DA	Displacement of abomasum	PPAR	Peroxisome proliferator-activated receptor
DGLA	Dihomo-γ-linoleic acid	PUFA	Polyunsaturated fatty acids
DHA	Docosahexaenoic acid	RBP	Retinol binding protein
DIM	Days in milk	ROM	Reactive oxygen metabolites
DMI	Dry matter intake	ROS	Reactive oxygen species
EFOX	Electrophilic oxo-derivatives	RP	Retained placenta
EPA	Eicosapentaenoic acid	Rv	Resolvin
FA	Fatty acid	SCC	Somatic cell count
FSI	Fertility Status Index	SD	Standard deviation
GGT	γ-glutamyl transpeptidase	SDH	Sorbitol dehydrogenase
GH	Growth hormone	SHp	Thiol groups
GOT	Glutamic oxaloacetic transaminase	TG	Triglycerides
HUFA	Highly unsaturated fatty acids	TGF	Transforming growth factor
IFN	Interferon	TMR	Total mixed ration
IL	Interleukin	TNF	Tumor necrosis factor
LAI	Liver Activity Index	TX	Thromboxane
LFI	Liver Functionality Index	VFA	Volatile fatty acid
LOX	Lypooxygenase	VLDL	Very-low density lipoprotein
LT	Leukotrienes		

Appendix 2 - ILAB600 photometric test parameters

Test	Methodology	Wavelength (nm)	Sample volume (mL)	Reagents			
				Reagent 1 (mL)	Diluent (H_2O) (mL)	Reagent 2 (mL)	Diluent (H_2O) (mL)
Glucose	Endpoint	510 - 600	9	150	75		
Cholesterol	Endpoint	510 - 700	5	100	150		
Urea	Endpoint	340	8	270	30		
Calcium	Endpoint	570 - 660	9	180	0	180	0
Phosphorus	Endpoint	340 - 375	6	150	20	150	20
Magnesium	Rate	340 - 405	6	240	10	60	10
Na, K, Cl	ISE device *						
Zinc	Endpoint	546 - 660	30	150	30		
Ceruloplasmin	Endpoint	546 - 660	25	140	40		
Total protein	Endpoint	546 - 700	6	200	0		
Albumins	Endpoint	600 - 700	5	230	0		
AST/GOT	Rate	340	14	70	75	35	0
GGT	Rate	405	16	175	10		
Bilirubin	Endpoint	546	40	105	35	75	0
Alkaline phosphatase	Rate	405	6	170	30		
Haptoglobin	Endpoint **	450	12	60	5	170	5
NEFA	Endpoint	546 - 660	4	140	40	70	20
BOHB	Endpoint	340	7	280	10		
Creatinine	Endpoint	510 - 570	12	90	0	90	0
Paraoxonase	Endpoint	405	8	125	125		
Activated paraoxonase	Endpoint	405	8	125	125		
Reactive oxygen metabolites	Rate	510	6	150	30		
Thiol groups	Endpoint	405-600	10	205	0		
NO_2	Endpoint	546	40	160	0		
NOx	Endpoint	546	40	160	0		
Myeloperoxidase	Rate	450	4	250	0		
Sorbitol dehydrogenase	Rate	340	18	215	0		

* The ILAB600 performs ISE measurements by an Indirect Method, since the samples are diluted before the measurement. The sample/diluent ratio is 1:21,7

** Final result of each samples is corrected by subtracting the corresponding sample blank