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). 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-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 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 receptor 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 pens (housing both CTR and OPT cows) 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

Table 1. Ingredients and main chemical and nutritional traits of the diets fed as TMR for close-up and lactating cows.

	Dry cows	Lactating cows
Components, % DM		
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 DM	1.29	1.58
CP, % DM	12.44	16.11
NDF, % DM	56.57	33.88
Starch + sugar, % DM	12.58	26.83
Ether extract, % DM	1.91	4.79

DM, dry matter; NEL, net energy lactation; CP, crude protein; NDF, neutral detergent fibre; °42.9% Ca_2PO_4; 28.6% urea; 14.3% MgO; 7.1% NaCl; 7.1% mineral and vitamin supplement (1,500,000 U/kg vitamin A; 150,000 U/kg vitamin D; 7000 U/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); #58,800 U/kg vitamin A; 5200 U/kg vitamin D; 102 U/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.

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 $3520 \times g$ 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 accordance with the method previously described by Visioli et al. (2003) at -21, -3, 21 and 42 days from calving and expressed as % of total FA measured.

Data handling and statistical analysis

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

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 elaboration. 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 elaboration 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.

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 produc-

Table 2. Detailed fatty acids composition (percentage on 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	32.22
Total monounsaturated	39.01	14712.22	36.86
18:2 ω6	4.64	1755.93	4.38
20:3 ω6	0.10	38.53	0.09
20:4 ω6 (ARA)	0.45	169.46	0.43
22:5 ω6	0.16	59.55	0.15
others	0.48	184.35	0.45
Total ω6 PUFA	5.83	2207.82	5.51
18:3 ω3 (ALA)	1.21	456.72	1.14
20:5 ω3 (EPA)	6.74	2548.94	6.37
22:5 ω3 (DPA)	2.42	916.06	2.29
22:6 ω3 (DHA)	6.67	2524.85	6.30
others	2.56	968.60	2.42
Total ω3 PUFA	19.60	7415.17	18.52
Others	1.45	550.42	1.37
Overall total	100.00	37782.27	94.48

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ALA, α-linolenic acid; ARA, arachidonic acid; PUFA, polyunsaturated fatty acids.

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

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

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

where Y_{ijklm} = m^{th} observation of the lth cow B_l within the ith treatment G_i, at the kth time to calving T_k; μ = total average; G_i = effect of the ith treatment (2 treatments: OPT and CTR); Tk = effect of the kth 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 ith treatment and the kth time to calving; $B_{l(im)}$ = fixed effect of the lth cow within the ith 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.*, 2006a) 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 =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

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 on 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 calv-

ing to 42 DIM (-0.63±0.17 and -0.49±0.23 points in CTR and OPT respectively; P<0.28).

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
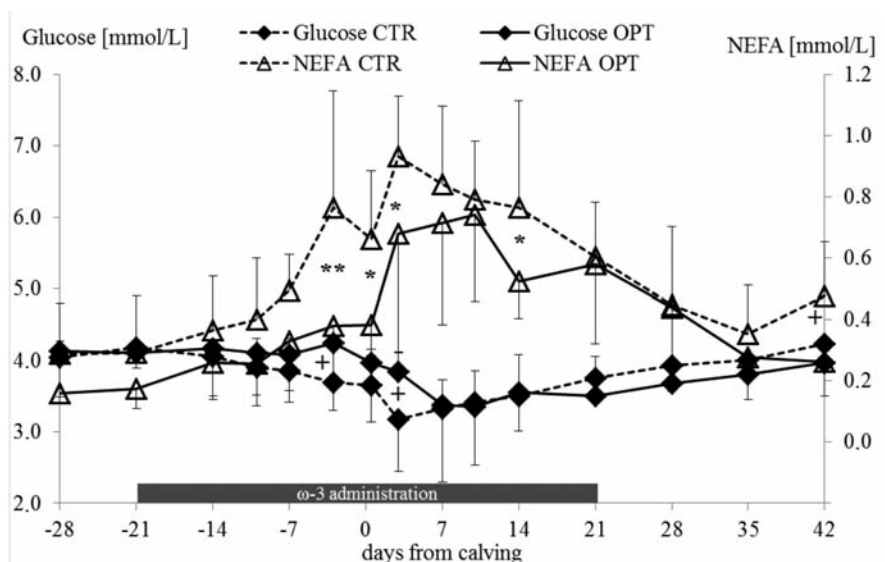

Figure 1. Glucose and NEFA plasma levels (mean±SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of 3 during the three weeks before and after calving. Statistically significant differences are marked as + P<0.10; *P<0.05; **P<0.01; ***P<0.001.

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; P<0.71). 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; P<0.53). 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; P<0.53). Globulins showed numerically higher levels in CTR starting from 7 days before calving with increasing difference to 42 DIM (P<0.32), 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 pro-
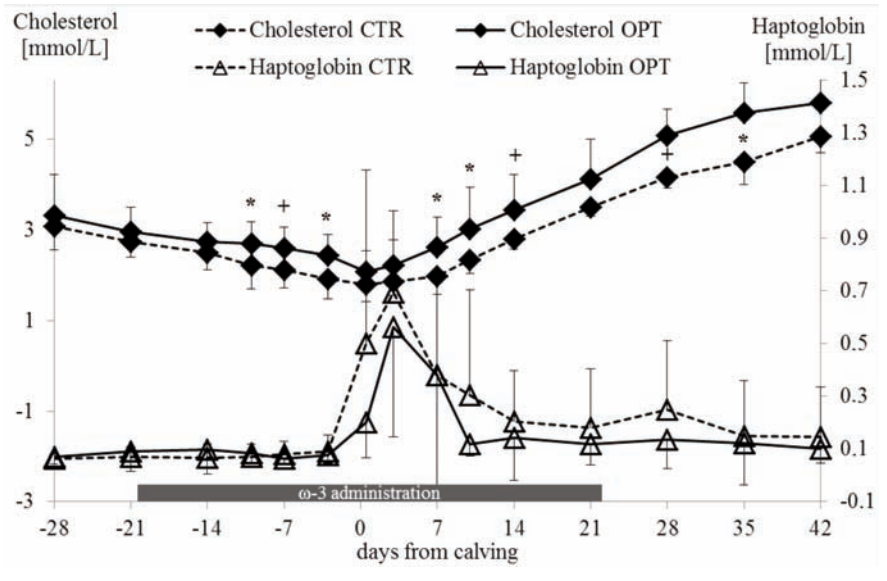

Figure 2. Cholesterol and haptoglobin plasma levels (mean ± SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001.
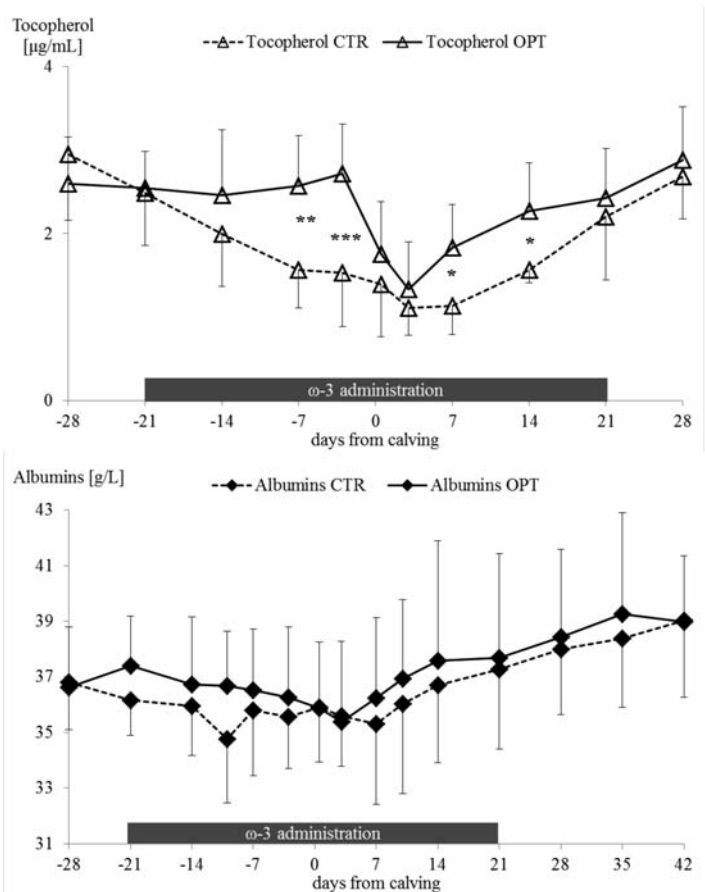

Figure 3. Albumins and tocopherol plasma levels (mean ± SD) in periparturient cows fed (n=6, continuous line) or not (n=6, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P<0.001.

tein 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 (P<0.41).

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<0.09). 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 evi-

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\DIM	-21	-3	21	42
20:5 ω3	OPT	0.72	1.08	0.71	0.49
(EPA)	CTR	0.71	0.53	0.78	0.58
	P	ns	**	ns	ns
	SEM	0.15			
22:6 ω3	OPT	0.10	0.30	0.31	0.12
(DHA)	CTR	0.08	0.08	0.06	0.05
	P	ns	***	***	+
	SEM	0.04			
22:5 ω3	OPT	0.67	0.65	0.74	0.53
(DPA)	CTR	0.63	0.53	0.52	0.53
	P	ns	ns	*	ns
	SEM	0.08			
18:3 ω3	OPT	3.83	4.96	3.41	3.77
(ALA)	CTR	4.01	4.71	3.06	3.40
	P	ns	ns	ns	ns
	SEM	0.45	-	-	-

DIM, days in milk; OPT, optimate treatment group; CTR, control group; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ALA, α-linolenic acid; ns, not significant.
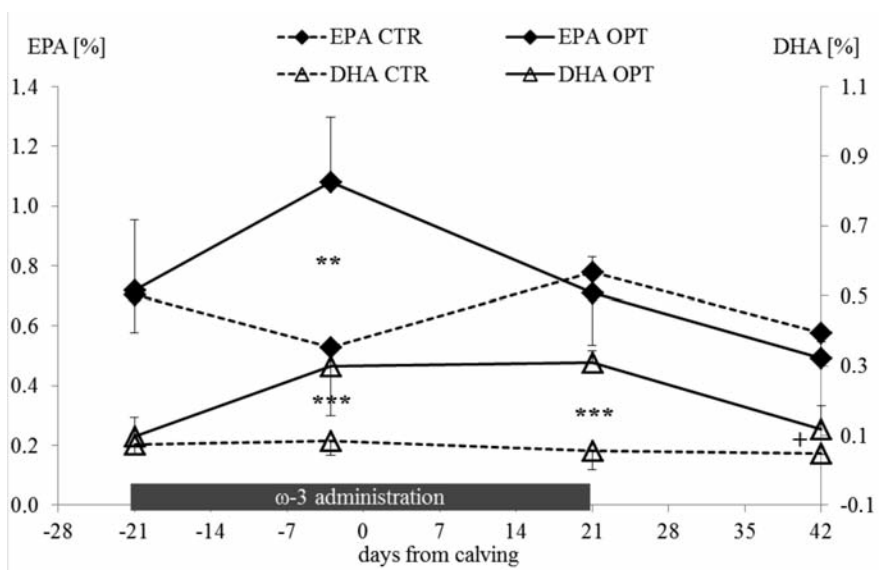

Figure 4. EPA and DHA plasma levels (as percentage of total fatty acids) in periparturient cows fed (n=3, continuous line) or not (n=3, dotted line) with 22 g/d of ω3 during the three weeks before and after calving. Statistically significant differences are marked as +P<0.10; *P<0.05; **P<0.01; ***P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

dence 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 (P<0.26). 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, P<0.26). 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≥20 (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. The level continued to rise in OPT 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*. (2006a).

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*., 2006a). 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*., 2006b).

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 recovery of the +APP, 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, albumin was lower in CTR before and after calving; 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 3 folds the daily intake in dry and by 1.5 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 lower inflammatory condition that could justify a lower lipomobilization, which implies a lower ketosis risk, maintaining a similar milk yield.

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