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NUTRITION AND STRESS: A field study on the effects of diet on stress-related responses in sheep

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GENERAL INTRODUCTION

Stress, Nutrition and Hypothalamic-Pituitary-Adrenal (HPA) axis

Animal welfare can be affected by different condition of stress, and kinds of "stressors": environmental, such as ambient temperature; and physiologicals, such as post partum period, with the starting of the lactation that influences the health status of lactating animals, increasing their susceptibility to the diseases development and reducing the production performances of the animal.

The physiological stress response consists in an activation of the sympathetic nervous system (SNS), a parasympathetic withdrawal, and increased activity of the HPA axis, that separately activates a response (Kunz-Ebrecht et al., 2003). Coupled with endocrine system the immune system interacts via activating different pathways, (Felten and Felten, 1994); as a consequence stress influences also the immune functions (Maier and Watkins, 1998).

Glucose and insulin plasma levels depend from dietary energy in dairy cows (Andersen et al., 2004), in condition of stress it is important the availability of glucose for central nervous system, indeed the glucocorticoids have a central role in energy mobilization during stress (Sapolsky et al., 2002). In ruminants the increase of the HPA-axis activity is connected with acute stress and causes the mobilization of the energy to cope with stress; moreover, the corticotrophin-releasing factor (CRF) system, at level of central nervous system (CNS), is involved in the regulation of feed intake together with behavioural resposes to stress (Koob, 1999). Hypercortisolemia caused by stress can alter the number of circulating lymphocytes (Dhabnar et al., 2009), and change the number and percentage of peripheral blood lymphocytes together with T-lymphocyte subsets, wich have an effect on animal immunocompetence (Caroprese et al., 2010).

The understanding of the nutrient requirements of dairy sheep at various stages of lactation and in different physiological conditions in essential for combining various feed ingredients to meet dairy animals needs.

Nutrition and Heat stress

Recent developments in housing and management practices of farm animals under heat stress reflect the increase in moral concerns about animal welfare; few papers discussed the interrelationships between heat stress and animal welfare, while much more it was been studied regarding the interaction between heat stress and livestock productivity in relation to the management systems (Silanikove, 2000). Moreover, the effect of heat stress is substantial in the subtropical-Mediterranean zones, and in farm animals raised in central and western Spain, or in the southern areas of France, Italy and Greece, are exposed annually for 3-5 months to considerable heat stress. Growth, milk production and reproduction are impaired under heat stress as a result of the drastic changes in biological functions caused by stress (Habeeb et al., 1992; Silanikove, 1992). However, these responses are also indications for poor welfare (Broom and Johnson, 1993).

The changes in the biological functions of sheep due to exposure to heat stress include the depression in feed intake and utilization, disturbances in the metabolism of water, protein, energy and mineral balances, enzymatic reactions, hormonal secretions and blood metabolites (Habeeb et al., 1992; Marai et al., 2000, 2003, 2004, 2006a).

Sheep convert fibrous, low quality feedstuffs into meat (protein), milk and other products in a better manner than cattle (Hafez, 1987). However, moderate heat stress reduces intakes and growth in young sheep consuming a high feed intake of medium quality roughage diets; this does not affect the relative

responses to supplementation providing principally fermentable Metabolic Energy (ME) or a similar amount of fermentable ME and additional metabolizable protein (Dixon et al., 1999). Supplementation of brown seaweed (Tasco) to post-harvest fescue hay was found to enhance the immune system and protect the wether lambs against prolonged heat-induced oxidative stress (Saker et al., 2004). Exposure to high ambient temperatures augments the efforts to dissipate body heat, resulting in an increase of respiration rate, body temperature and consumption of water, and a decline in feed intake. Heat increment is defined as energy expenditures associated with the digestion and assimilation of food; for this the digestion and the metabolism of nutrient creates heat (Baldwin et al., 1980). A higher heat increment is caused by the specific dynamic action that accompanies the metabolism of feed which is highest in the case of poor quality, fibrous feedstuffs (Marai et al., 2001). It is possible to formulate diets by exploiting different efficiencies of nutrient utilization to decrease the heat increment. (West, 1999) Factors such as water deprivation, nutritional imbalance and nutritional deficiency may exacerbate the impact of heat stress. Sheep, however, recorded a lower sensitivity to heat stress, when compared to cattle, at a maintenance feed level. The provision of shade shelter is suggested as a practical measure applicable under extensive conditions (Silanikove-Nissim, 2000). Feeding excessive quantities of nutrients, such as crude protein, can contribute to reduced efficiency of energy utilization, potentially increasing the level of stress. Is it necessary understanding how dietary modification can minimize heat stress.

Heat stress can affect, moreover, the nutrition of animals by altering the dynamic characteristics of the digestion processes; recent studies on nutrient digestibility in ruminants under heat stress are contradictory: in cattle exposed to

hot environments the diet digestibility increased (Weniger and Stein 1992); in contrast, negative or no relationships between high ambient temperatures and diet digestibility have been reported in dairy cattle and small ruminants (Silanikove, 1992). Furthermore, in ewes exposed to chronically heat stress, the diet digestibility was affected in a time-dependent manner, and this change suggested an adaptive response of the digestive tract to heat stress conditions, due to changes in bacterial concentration (Bernabucci et al., 2009); when the exposition to high temperature was finished, to re-establish the function of rumen and intestinal tract, it needed around 2 weeks, even though animals normalize clinical parameters and feed intakes.

Physiological state and nutrient restriction can influence thermoregulatory responses of ewes under heat stress condition (Mohammed et al., 2008).

Nutrition and post-partum period

Dairy animals during peri-partum period need to mobilize adipose tissue to compensate the relative deficit of glucose due to the ongoing of the lactation in concomitance with negative energy balance (Hachenberg at al., 2007).

In dairy cow, this phase of the reproductive life is particularly critical in terms of health, productivity and fertility (Roche et al., 2009). In the past two decades the reproductive efficiency of dairy cows registered a decrease with a concomitant increase of production of milk per cow; to the basis of reduction of reproductive efficiency there is an increasing of disease due to variable and often prolonged state of negative energy balance in dairy cow during peri-partum and early post-partum. It is well known that nutrition has a central role in regulation of reproductive function in cattle; in particular, acute nutritional restriction involved

in anovulation and detrimental effects on dominant follicle growth (Roche et al., 2000).

Furthermore, in the peripartum period many hormonal and metabolic alterations occur: a decrease of progesterone and oestradiol level at parturition and an increase of four-fold of plasma cortisol is observed; these alterations lead the functions of polymorphonuclear leukocytes causing immonosuppression (Dosogne et al. 1999) and consequently a major susceptibility of disease.

During post-partum the oxygen consumption increases, with consequently increase of reactive oxygen species (ROS), that caused oxidative stress related disorders, such as puerperal dysfunction, and alterations in colostrums and milk quality (Rizzo et al, 2007 and 2009).

In this metabolic conditions, an adequate dietary supplementation with antioxidant vitamins, such as Vitamin E, β-carotene and vitamin B_{12} may control the oxidative disease, improve the immune system, and decrease the post-partum disease (Rizzo et al., 2013). The level of dietary supplementation with vitamins need to be understood because an excess of antioxidant may result in an increase of ROS generation, as demonstrated by Salganik (2001).

Supplementation of diet with fat, such as n3 and n-6 polyunsaturated fatty acids (PUFA) has been showed to be important modulators of immune reactions (Calder et al., 2002), and to supply additional energy requirements for growth as well as for milk production with an alteration of the endocrine profile favoring milk synthesis rather than body reserve replenishment (Drackley et al., 2003).

Linoleic acid (18:2n6) can be converted though enzymatic reactions into arachidonic acid, which is precursor of the pro-inflammatory mediators, prostaglandin E_2. The α-linolenic acid (C18:3n3), can be converted in

eicosapentaenoic acid, that is precursor, instead, of prostaglandin E_3 (PGE_3) and leukotriene B_4, that trigger less severe inflammatory reactions than PGE_2 and leukotriene B_4 (Yaqoob and Calder, 1995). Dietary supplementation with fat around parturition was demonstrated to reduce the production of inflammatory factors that could contribute to attenuate the immunesuppressive effect during parturition and could re-establish the immune function against pathogens (Lessard et al., 2004). Dairy cattle fed with whole flaxseed, rich in omega 3 fatty acids, registered a modification of both secretion of prostaglandin and reproduction (Petit et al., 2002). Furthermore, the concentration of progesterone (P_4) and PGE_2 were affected from the composition of unsaturated fatty acids supplemented in the diet, particularly improving reproductive performance, because both molecules are implicated in the regulation of uterine and systemic immune responses (Lessard et al., 2004). During transition period Caroprese et al., (2006), found that IL-6 level in plasma of dairy ewes can be used to as a reliable indicator of stress connected with lambing; in ewes with multiple lambing were characterized by impaired immune functions and consequently more subjected to invasion of pathogens.

AIM OF THE PhD THESIS

The aim of the present thesis was the study of the effects of nutrition on immune system of dairy ewes under different types of stressors.

The experiments were divided in four different trials. The first experiment was undertaken to evaluate the potential effects of phytosterols extracted from a microalga, intented to be used in sheep nutrition as feed supplements, on in vitro immunological responses of cells from dairy ewes. The second and the third trials were undertaken in order to evaluate the effects of PUFA supplementation on welfare and immune responses of sheep under heat stress. The second trial evaluated the effects of supplementation with polyunsaturated fatty acids from seaweed and flaxseed on welfare and in vivo immunological profile and HPA-axis activation during exposition of ewes to high ambient temperature. The third experiment studied the effects of supplementation with polyunsaturated fatty acids from seaweed and flaxseed on ex vivo inflammatory profile of cells from dairy ewes under high ambient temperature. The fourth trial evaluated the effects of supplementation with polyunsaturated fatty acids from flaxseed on immunological profile in dairy ewes during post partum.

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FIRST TRIAL

A mixture of phytosterols from *Dunaliella tertiolecta* affects proliferation of peripheral blood mononuclear cells and cytokine production in sheep

1. INTRODUCTION

Plants, fungi, and algae are a source of biological active compounds which have a key role in the production of nutraceutical foods and of pharmaceutical products (Lordan et al., 2011). Among the biological compounds extracted from plants, fungi, and algae, phytosterols have a key role for human nutrition. Sterol biosynthesis lead to different final products in different organisms: plants produce sitosterol, stigmasterol, campesterol and spinasterol (Salimova et al., 1999), fungi produce mainly ergosterol. Green algae are characterized by a wide range of sterols in their composition such as chondrillasterol, poriferasterol, 28-isofucosterol, ergosterol, cholesterol and others (Patterson, 1982). Phytosterols have been investigated in human studies for their role in lipid metabolism because of their cholesterol-lowering activity. This biological effect is due to the structure of phytosterols that are very similar to cholesterol (Ling et al., 1995). It is generally assumed that LDL cholesterol reduction results directly from inhibition of cholesterol absorption through displacement of cholesterol from lipidic micelles during intestinal absorption of phytosterols contained in the diet without changes in HDL cholesterol level (Methiev et al., 2008). Beside their action on LDL cholesterol, however, phytosterols have been proposed as therapeutic compounds for their immunomodulatory properties. Ergosterol and ergosterol peroxide extracted from mushrooms have been shown to suppress proliferation of human and mouse cells, when stimulated with mitogens, to inhibit the growth of some cancer cells, and to induce apoptosis of human leukemia cells (Kobori et al., 2007; Kuo et al., 2003). Ergosterol is a precursor of vitamin D_2 and can be converted to vitamin D_2 by UV irradiation (Jasinghe and Perera, 2003). Furthermore, ergosterol has the ability of influencing the expression and

production of some cytokines. It has been shown (Kuo et al., 2011) that, when tested on LPS-stimulated macrophages in vitro, ergosterol can inhibit the production of TNF-α and the expression of COX-2. Ergosterol, together with a large variety of other phytosterols, is contained also in algae and microalgae. The utilization of algae and microalgae for phytosterols purification and extraction could be an opportunity for finding new molecules of particular interest for human health or a mixture of molecules able to enhance the nutraceutical activities of single phytosterols by synergistic mechanisms. Recently the microalga *Dunaliella tertiolecta* was found to be a source of phytosterols, among which ergosterol and 7-dehydroporiferasterol were the most abundant; as a consequence its utilization for commercial production of phytosterols has been suggested (Francavilla et al., 2010). To the best of our knowledge no studies have investigated the immunological activity of a mixture of ergosterol and 7-dehydroporiferasterol on animals or human cells. Based on previous findings, the study of the suppressive effects of a mixture of phytosterols on the immunological responses of ovine PBMC could be remarkable and of practical use for reducing tissue damaging and uncontrolled immune activation resulting from cytokine release during inflammatory diseases, such as fly strike, mastitis and acute parasitoses in sheep. The evaluation of sheep PBMC responses in vitro to a mixture of phytosterols purified and extracted from *Dunaliella tertiolecta* could be preliminary to the use of microalgae extracts in sheep feeding, as feed supplements. The choice of the sheep model to study the effects of a mixture of phytosterols from *Dunaliella tertiolecta* could have a twofold advantage: firstly, the use of bioactive mixtures of proven immunosuppressive properties extracted from *Dunaliella tertiolecta* could be effective to control inflammatory diseases in sheep production systems;

secondly, the supplementation of sheep diet with bioactive mixtures could contribute to the potential enrichment of sheep dairy products in phytosterols. From this point of view studies on sheep immune response could be used to better understand human immune responses to enriched food. Nowadays, no information, however, on the possible bio-hydrogenation occurring in the rumen environment due to rumen microorganisms are available. The mixture extracted from *Dunaliella tertiolecta*, however, could be microencapsulated by spray-drying or spray-congealing, as reported in Eldem et al. (1991) to improve its intestinal absorption and to escape rumen bio-hydrogenation.

We hypothesized that a mixture of phytosterols extracted from the microalga *Dunaliella tertiolecta* might have immunomodulatory properties on sheep cells. Furthermore, it could be crucial to evaluate the dose of phytosterols at which their immunomodulatory properties become apparent for future in vivo applications in sheep feeding strategies. This study, therefore, was undertaken to evaluate the effects of a mixture of total phytosterols, or a mixture of only ergosterol and 7-dehydroporiferasterol extracted from the microalga *Dunaliella tertiolecta* on the proliferative response and cytokine production of sheep peripheral blood mononuclear stimulated cells.

1.2 MATERIALS AND METHODS

1.2.1 Separation and purification of total sterols fraction and of ergosterol / 7- dehydroporiferasterol mixture from Dunaliella tertiolecta

Total sterols were extracted and separated from *Dunaliella tertiolecta* as described by Francavilla et al. (2010). Briefly, total lipids were extracted, according to Bligh and Dyer (1959), from 0.5 g of freeze dried biomass of *D.*

tertiolecta grown in the Walne's culture medium (modified from Laing, 1991), at a salt concentration of 0.6 M NaCl. Extracted lipids were saponified and the unsaponified fraction (containing sterols not esterified) was separated and concentrated. Total sterols from unsaponified material were isolated by preparative thin layer chromatography (TLC 20 × 20 cm, silica gel 60 Å, layer thickness 500 μm) developed in one dimension in *n*-hexane /ethyl acetate 8:2 (v/v).

Silver Ion Flash Chromatography (Ag-FLC) was used for purification of ergosterol (22*E*,24*R*)-methylcholesta-5,7,22-trien-3*β*-ol) and 7-dehydroporiferasterol ((22*E*,24*R*)-ethylcholesta-5,7,22-trien-3*β*-ol) as described by Francavilla et al. (2012). The column was eluted in isocratic conditions using as eluent n-hexan- ethyl acetate 8:2 (v/v). Fractions containing only ergosterol and 7-dehydroporiferasperol were combined and weighed after the solvent was removed on a rotary evaporator (Büchi Rotavapor).

1.2.2 Analyses by gas chromatography-mass (tandem) spectrometry (GC-MS/MS)

Purified sterols fraction of *D. tertiolecta* were analyzed by gas chromatography-mass spectrometry. A Varian Saturn 2200 GC/MS/MS ion trap (Varian Analytical Instruments, Walnut Creek, CA) was used. Identification of sterols was based on the comparison of their retention times relative to authentic standards, mass spectra of authentic standards, and available spectra in NIST05 and Wiley 07 mass spectral libraries as described by Francavilla et al. (2010).

Figure 1.1 represents a ion chromatogram (GC-MS) of Total Sterol Fraction extracted from *Dunaliella tertiolecta.*

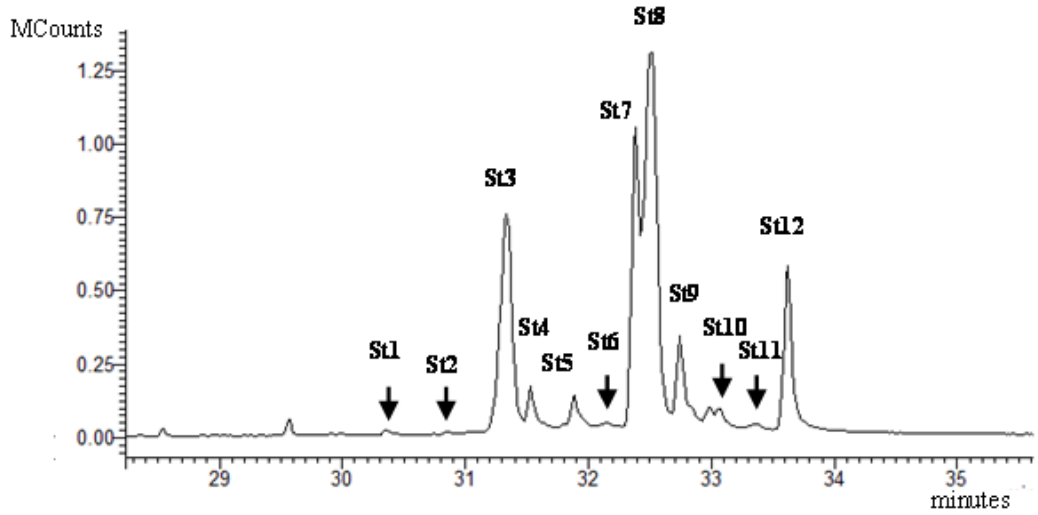

Figure 1.1 *Ion Chromatogram (GC-MS) of Total Sterol Fraction extracted from Dunaliella tertiolecta:* ***St1****=Cholesta-5-en-3β-ol[Cholesterol],* ***St2****=(22E,24R)Methylcholesta-5,7,9(11),22-tetraen3βol[9(11)Dehydroergosterol],* ***St3****=(22E,24R)-Methylcholesta-5,7,22-trien-3β-ol [Ergosterol],* ***St4=*** *(22E,24R)-Methyl-5α-cholesta-7,22-dien-3β-ol [5-Dihydroergosterol],* ***St5****=(24ξ)-Methyl-5α-cholesta-8(14)-en-3β-ol,* ***St6****=(22E,24R)-Ethylcholesta-5,7,9(11),22-tetraen-3β-ol,* ***St7****=(24S)-Methyl-5α-cholesta-7-en-3β-ol [Fungisterol],* ***St8****=(22E,24R)-Ethylcholesta-5,7,22-trien-3β-ol [7-Dehydroporiferasterol],* ***St9****=(22E,24R)-Ethyl-5α-cholesta-7,22-dien-3β-ol [Chondrillasterol],* ***St10=****(24ξ)-Ethyl-5α-cholesta-8(14)-en-3β-ol,* ***St11=****(24ξ)-Ethylcholesta-5,7-dien-3β-ol,* ***St12****=(24S)-Ethyl-5α-cholesta-7-en-3β-ol[22-Dihydrochondrillasterol].*

The molecular structures of twelve sterols purified from *Dunaliella tertiolecta* are reported in Figure 1.2.

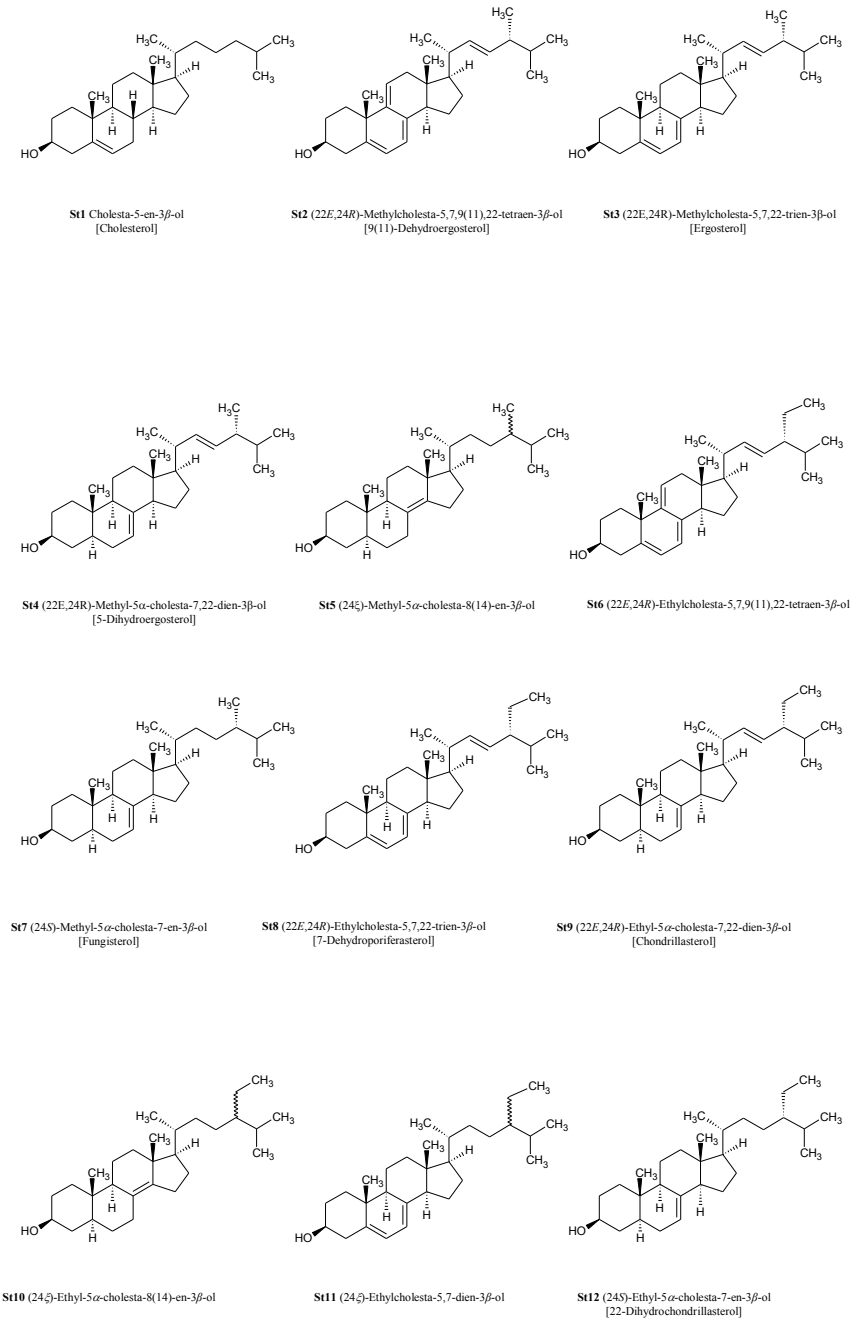

Figure 1.2 *Molecular structures of twelve sterols purified from Dunaliella tertiolecta.*

1.2.3 Isolation of PBMC

Ten Comisana sheep were randomly selected from the breeding flock at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE).

Isolation of PBMC was performed by density gradient centrifugation according to Wattegedera et al. (2004). Briefly, blood from each animal was diluted in cold PBS (pH 7.4), and centrifuged at 670g at 4°C for 20 min. The buffy coat was recovered, layered over a Ficoll gradient (1.077g/ml), and centrifuged at 1130g at 15°C for 30 min. The mononuclear cell band was recovered, washed and finally resuspended at a final concentration of 2x 10^6 cells/ml in Iscove's Modified Dulbecco's medium (Sigma-Aldrich, Italy) supplemented with FBS and gentamicin.

1.2.4 Proliferation Suppression Assay and Cytokine Quantification

Lymphocyte proliferation assays were performed adding 100 µl of cell suspension into quadruplicate wells of 96 well U-bottom plates. PBMC were treated with synthetic ergosterol (E) (Sigma-Aldrich, Milan, Italy), a mixture of eleven Algae sterols extracted and purified from *Dunaliella tertiolecta* (Algae Extract, AE), a mixture of ergosterol and 7-dehydroporiferasterol extracted and purified from *Dunaliella tertiolecta* (Purified Extract, PE), and 50 µl of ConA (Sigma-Aldrich, Italy) at a final concentration of 5µg/ml. For each treatment, 0.0mg/ml, 0.2 mg/ml, 0.4mg/ml and 0.8mg/ml were tested on PBMC to verify the effects of sterols on their proliferation. Negative Control wells contained 100 µl of PBMC suspensions without mitogen (NC). Positive Control wells contained 100 µl of PBMC suspensions with ConA (Stimulated Cells, SC). Cell viability was greater than 90-95% in all the assays, as determined by the Trypan Blue exclusion

test. The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 96 h. In order to test lymphocyte proliferation after 96 h of incubation a BrDU test was performed using a commercial kit (Roche, Milan, Italy). After 18h of incubation, BrDU incorporation during DNA synthesis was measured by reading optical density with a titer-ELISA spectrophotometer (Power Wave XS, Biotek, UK) at 450nm.

To determine the suppressive effects of Algae sterols on inflammatory responses, cytokines production by PBMC was evaluated: 100 µl of cell suspension were treated with 50 µl of LPS at a final concentration of 1µg/ml (Sigma-Aldrich, Milan, Italy), 50 µl of ConA at a final concentration of 5µg/ml (Sigma Aldrich, Milan, Italy), and 50 µl of E, AE, or PE (0.0mg/ml, 0.2 mg/ml, 0.4mg/ml and 0.8mg/ml) in 96 well U-bottom plates (Kobori et al., 2007). Control wells contained 100 µl of PBMC suspensions without LPS and mitogen. Positive Control wells contained 100 µl of PBMC suspensions with both LPS and ConA. The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for four days. After 96 h of incubation, cell suspensions were centrifuged at 300 g at 4°C for 10 min, and cell-free supernatants from each well were collected and stored at-20 °C until ELISA-test time to measure cytokine production.

1.2.5 Determination of cytokines in culture supernatant by ELISA-test

The levels of IL-6 and IL-1β in cell-free supernatants were determined by capture ELISA performed on 96-well microtiter plates, according to Caroprese et al. (2006). The presence of bindings for IL-6 and IL-1β was detected using sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP, Sigma Aldrich, Italy).

The determination of IL-10 and TNF-α in cell-free supernatants was carried out by an ELISA test according to Kwong et al. (2002) and Hope et al. (2003),

respectively. Plates were read by a titer-ELISA spectrophotometer (Power Wave XS, Biotek, UK). Culture supernatants were read for IL-1 β detection against a standard curve obtained using scalar dilution of recombinant ovine IL-1β (CAB, Australia). Data were expressed as ng of IL-1β /ml. IL-6 data were expressed as optical density (OD). The ELISA for IL-10 detection was standardized using biologically-active recombinant ovine IL-10 expressed in Chinese hamster ovary (CHO) cells using the Gln synthetase (GS) expression vector™ (Lonza, UK). Production of recombinant ruminant cytokines using this system has been described in detail elsewhere (Graham et al., 1995; Entrican et al., 1996). Recombinant ovine IL-10 was provided by the BBSRC/RERAD Immunological Toolbox; recombinant bovine TNF-α was purchased from Serotec (UK). Data were expressed as bU of IL-10 /ml and pg of TNF-α/ml.

1.2.6 Statistical Analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965) and transformed into logarithm form to normalize their frequency distribution, when necessary. Then, data were processed by ANOVA using the GLM procedure of SAS (1999).

The model utilized was: $y_{ijkl} = \mu + \alpha_i + \beta_{ij} + \gamma_k + (\alpha\gamma)_{ik} + \varepsilon_{ijkl}$

Where μ=the overall mean; α=treatment β=animal effect within treatment; γ=dose; αγ=interaction of treatment x dose and ε=error. When significant effects were found (at $P<0.05$), the Student *t*-test was used to locate significant differences between means.

1.3 RESULTS

1.3.1 Proliferative response to ConA

On average proliferation of PBMC stimulated with Con A after 96 h of incubation was significantly higher than proliferation of PBMC stimulated with Con A and treated with E, AE and PE ($P<0.001$); in particular, on average the proliferation of PBMC stimulated with Con A in the presence of E was significantly lower ($P<0.05$) than proliferation of PBMC stimulated with only Con A.

After 96 h of incubation, the cells stimulated with Con A and PE showed a significantly different proliferation rate according to the dose of PE administrated ($P<0.01$, Fig. 1.3).

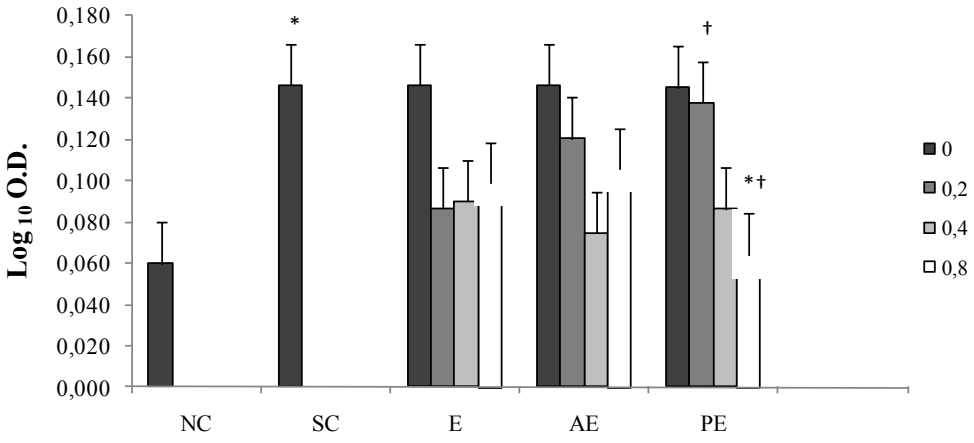

Figure 1.3 *Proliferation of sheep PBMC following in vitro stimulation (Least Squares means ± SEM). PBMC were treated with 0.0mg/ml, 0.2 mg/ml, 0.4mg/ml and 0.8mg/ml of ergosterol (E), a mixture of eleven Algae sterols extracted and purified from Dunaliella tertiolecta (Algae Extract, AE), a mixture of ergosterol and 7-dehydroporiferasterol extracted and purified from Dunaliella tertiolecta (Purified Extract, PE), and ConA. Negative Control wells contained PBMC without mitogen (NC). Positive Control wells contained PBMC with ConA (Stimulated Cells, SC). (*) Shows a significant difference ($P < 0.05$) between PBMC proliferation treated with 0.8 mg/ml PE and ConA stimulated cells (SC). (†) Shows a significant difference between PBMC proliferation treated with 0.2 mg/ml and 0.8 mg/ml PE.*

Con A-stimulated cells showed higher proliferation than cells treated with Con A and PE at a concentration 0.8 mg/ml. In addition, cells treated with Con A and PE at a concentration of 0.8 mg/ml showed a lower proliferation than cells treated with Con A and PE at a concentration of 0.2 mg/ml (P=0.07).

1.3.2 Cytokine production by PBMC

The production of TNF-α, IL-6, IL-1β at different concentrations of sterols, in supernatant by stimulated cells is shown in Table 1.1.

	Dose (mg/ml)	Treatment						Effects,P		
		NC	E	AE	PE	SC	SEM	Treatments	Dose	Treatments x Dose
TNF-α (Log pg/ml)	0	2.841	2.841	2.902	2.841	3.130				
	0.2	2.841	2.833	2.864	2.834	3.136				
	0.4	2.841	2.828	2.841	2.823	3.136				
	0.8	2.841	2.833	2.842	2.842	3.142	0.050			
	Mean	2.841b	2.834b	2.862b	2.835b	3.136a	0.023	***	NS	NS
IL-1 β (Log ng/ml)	0	0.371	0.392	0.392	0.392	0.392				
	0.2	0.371	0.362	0.396	0.364	0.392				
	0.4	0.371	0.352	0.376	0.382	0.392				
	0.8	0.364	0.351	0.381	0.375	0.392	0.030			
	Mean	0.369	0.364	0.386	0.378	0.392	0.014	NS	NS	NS
IL-6 (Log O.D.)	0	0.087	0.087	0.087	0.087	0.107				
	0.2	0.087	0.075	0.108	0.094	0.107				
	0.4	0.087	0.080	0.076	0.074	0.107				
	0.8	0.087	0.076	0.082	0.073	0.107	0.015			
	Mean	0.087b	0.080b	0.088ab	0.082b	0.107a	0.007	*	NS	NS

Means followed by different letters are significantly different at P<0,05.

NS: non significant.

*** P<0.001

*P<0.05

Table 1.1 *TNF-α, IL-1β and IL-6 production (Least Squares means ± SEM) in LPS and ConA stimulated sheep pheripheral blood mononuclear cells (PBMC). PBMC were treated with 0.0mg/mL, 0.2 mg/mL, 0.4mg/mL and 0.8mg/L of ergosterol (E), a mix of eleven Algae sterols extracted and purified from Dunaliella tertiolecta (Algae Extract, AE), a mix of ergosterol and 7-dehydroporiferasterol extracted and purified from Dunaliella tertiolecta (Purified Extract, PE), LPS and ConA. Negative Control wells contained PBMC without mitogens (NC). Positive Control wells contained PBMC with LPS and ConA (Stimulated Cells, SC).*

Cells stimulated with LPS and Con A in presence of E, AE and PE had a highly significantly lower mean production of TNF-α than cells stimulated with LPS and Con A ($P<0.001$). TNF-α produced by cells in the presence of E, AE and PE was similar to the production of TNF-α by unstimulated cells. In addition, no differences were detected among TNF-α produced by cells in the presence of different doses of E, AE and PE.

Mean values of IL-6 production by cells stimulated with LPS and Con A in the presence of both E and PE was significantly lower ($P<0.05$) than production of IL-6 by cells stimulated with LPS and Con A (0.080 ± 0.015 and 0.082 ± 0.015 vs 0.107 ± 0.015, respectively). No differences emerged for IL-6 production when cells were treated with different doses of E, PE, and AE.

Mean values of IL-1β production by cells stimulated with LPS and Con A or stimulated with LPS, Con A and different doses of E, PE, and EA did not show differences.

Cells stimulated with LPS and Con A produced on average significantly higher IL-10 in the presence of PE ($P<0.1$). Furthermore, significant differences emerged with regard to the dose of PE used in contact with cells. In fact, the IL-10 produced by cells stimulated with LPS and Con A in the presence of PE significantly increased ($P<0.05$, Figure 1.4) when the concentration of PE increased from 0.2 mg/ml to 0.4 mg/ml. IL-10 produced by cells stimulated with LPS and Con A in the presence of 0.4 mg/ml PE was higher than IL-10 produced by cells stimulated with LPS and Con A without Algae sterols or in the presence of 0.4 mg/ml E, and of 0.4 mg/ml AE.

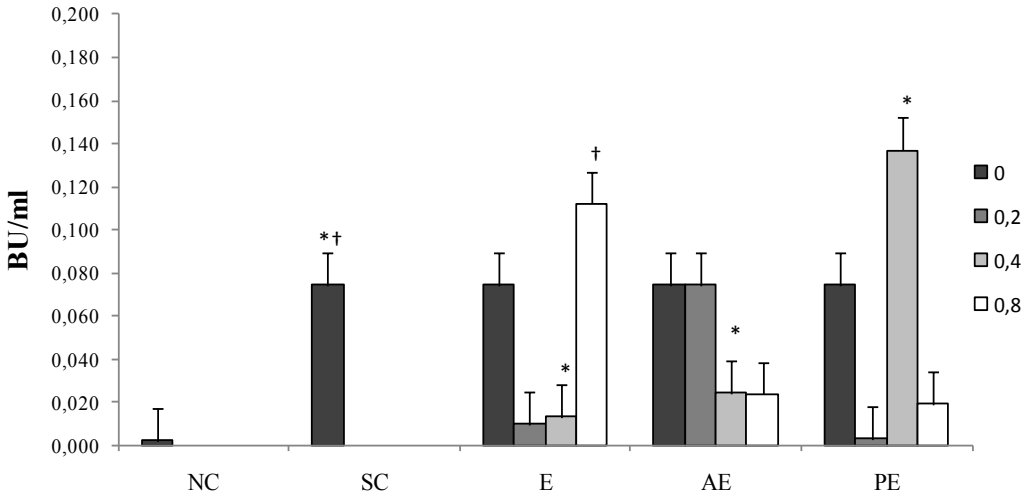

Figure 1.4 *IL-10 production (Least Squares means ± SEM), in LPS and ConA stimulated sheep peripheral blood mononuclear cells (PBMC). PBMC were treated with 0.0mg/ml, 0.2 mg/ml, 0.4mg/ml and 0.8mg/ml of ergosterol (E), a mixture of eleven Algae sterols extracted and purified from Dunaliella tertiolecta (Algae Extract, AE), a mixture of ergosterol and 7-dehydroporiferasterol extracted and purified from Dunaliella tertiolecta (Purified Extract, PE), LPS and ConA. Negative Control wells contained PBMC without mitogens (NC). Positive Control wells contained PBMC with LPS and ConA (Stimulated Cells, SC). (*) Shows significant differences (P < 0.05) between IL-10 production by PBMC treated with 0.4 mg/ml PE and 0.4 mg/ml AE, with 0.4 mg/ml PE and 0.4 mg/ml E, and 0.4 mg/ml PE and LPS and ConA stimulated cells (SC). (†) Shows a significant difference between IL-10 production by PBMC treated with 0.8 mg/ml E and LPS and ConA stimulated cells (SC).*

The production of IL-10 by cells stimulated with LPS and Con A in the presence of 0.8 mg/ml E was significantly higher than the production of IL-10 in the presence of 0.4 mg/ml E ($P<0.05$). Furthermore, IL-10 produced by the cells stimulated with LPS and Con A in the presence of 0.8 mg/ml E was higher ($P<0.05$) than IL-10 produced by cells stimulated with only LPS and Con A.

1.4 DISCUSSION

This study was undertaken to evaluate the immunomodulatory and anti-inflammatory activities of a particular mixture of phytosterols extracted from *Dunaliella tertiolecta* on sheep cells. Proven immunomodulatory properties of a mixture of phytosterols extracted from *Dunaliella tertiolecta* could validate their use to sustain animal health.

Ergosterol is the major sterol in the cell membrane of mushrooms, representing about 70% of the sterols in fungi (Kuo et al., 2011). In their studies, Kuo et al. (2011) and Kobori et al. (2007) demonstrated that ergosterol has anti-inflammatory properties on LPS-stimulated RAW 264.7 macrophages in vitro by inhibiting TNF-α production and COX-2 expression. TNF-α is a highly proinflammatory cytokine which is responsible for heightened inflammatory responses produced by a number of different cell types, including macrophages, lymphocytes, neutrophils, and epithelial cells (Angelini et al., 2005). TNF-α production can be induced by fungi, viruses, parasites, and bacterial wall products, such as bacterial lipopolysaccharide of the Gram-negative bacteria. TNF-α production can be also induced by bacterial toxins as well as by some cytokines, as IL-1 and IFN-γ. TNF-α has a wide range of biological effects including cell differentiation, tissue development and death, and proliferation. Kuo et al. (2011), studying the proteomic profile of cells incubated with ergosterol, found that the expression of NF-κB p65, which is identified as the dominant transcription factor for induction of TNF-α expression, was suppressed. As a result, ergosterol was able to suppress the expression of TNF-α in LPS-stimulated RAW 264.7 macrophages. Ergosterol peroxide, which is also extracted by Ascomycetes, is able to suppress proliferation of human lymphocytes under

stimulation with PHA (Kuo et al., 2003). No studies evaluated the biological effects of 7-dehydroporiferasterol, as well as of a mixture of sterols extracted by *Dunaliella tertiolecta.* However, it has been hypothesized that the biological effects of 7-dehydroporiferasterol could be similar to those of ergosterol because the two sterols differ only for an extra methyl group (Francavilla et al., 2010). In our study ergosterol (E), the mixture of sterols from *Dunaliella tertiolecta* (AE) and the purified Algae extract of 7-dehydroporiferasterol and ergosterol (PE) were able to suppress cell proliferation in sheep PBMC stimulated with ConA. These results seemed to suggest a similarity between the mixture of 7-dehydroporiferasterol, ergosterol, and the mixture of sterols based on their effects on PBMC. The increase of suppressive effects exerted at 0.8 mg/ml on sheep cell proliferation only by the mixture of ergosterol and 7-dehydroporiferasterol extracted and purified from *Dunaliella tertiolecta,* however, led us to the hypothesis that the presence of 7-dehydroporiferasterol may strengthen the inhibitory effects of ergosterol on cell proliferation with increasing concentrations of both sterols. A possible explanation of our results may be the synergic effects exerted by 7-dehydroporiferasterol and ergosterol at increasing concentrations. Suárez et al. (2005) demonstrated that the effects of sterols on cell proliferation depend on their structural features. Results from our study suggested that 7-dehydroporiferasterol and ergosterol, when administrated together, have synergic effects in reducing sheep cell proliferation. Another possible explanation of the suppressive effects of the mixture of ergosterol and 7-dehydroporiferasterol on cell proliferation could be their cytotoxic effects on ovine PBMC, caused by massive overload of their ambient environment. Anyway, we excluded this hypothesis considering that other authors tested higher concentration of

phytosterols on cell proliferation without finding effects on cells viability (Valerio and Awad, 2011; Kobori et al., 2007). The proved suppressive effects of the mixture of ergosterol and 7-dehydroporiferasterol on the immunological responses of ovine PBMC could be of some relevance when used as a feed supplement in sheep production systems to control inflammatory diseases and reduce tissue damages derived from uncontrolled immune activation.

When the effects of different sterols from *Dunaliella tertiolecta* on cytokine production by stimulated PBMC were tested, we found that ergosterol, the mixture of sterols from *Dunaliella tertiolecta,* and the purified 7-dehydroporiferasterol and ergosterol from *Dunaliella tertiolecta* were able to suppress TNF-α production by LPS and ConA stimulated sheep cells, thus supporting previous findings on the anti-inflammatory effects of sterols (Kobori et al., 2007; Kuo et al., 2011). IL-6 is a pleiotropic cytokine and has a wide range of immunological functions including stimulation of B cells and cytotoxic T cells. Both the expression and production of IL-6 are strongly controlled and mainly occur under inflammatory conditions, with IL-6 being responsible for the increase of acute phase proteins. IL-1 is known as an inflammatory cytokine involved in lymphocyte activation and in acute-phase response. Bacterial lipopolysaccaride, cytokines, such as TNF-α, interferon-γ, and IL-2, are able to induce IL-1 production (Stylianou and Saklatvala, 1998). In our study PBMC production of IL-6 in response to stimulation with LPS and ConA was reduced in presence of both ergosterol and of the mixture of ergosterol and 7-dehydroporiferasterol extracted and purified from *Dunaliella tertiolecta,* confirming a potential anti-inflammatory effect of both sterols and of the purified extract from *Dunaliella tertiolecta*. Kobori et al. (2007) found suppressive effects of ergosterol peroxide

on LPS-stimulated cells after 6 hour of incubation by a reduction of IL-1β expression. Our results on the absence of effects of sterols on IL-1β production may be caused by a general suppressive effects exerted by IL-6 on production of IL-1β by stimulated cells. In humans, IL-6 provided a negative feed-back signal on IL-1β production after 48 hours of cells incubation with LPS (Schindler et al., 1990). IL-6 has the ability to inhibit also the expression of IL-1β and TNF-α, and to stimulate expression of IL-1-receptor antagonist and soluble TNF-receptor (Bannerman, 2008). As a consequence, discrepancy between our and previous results on suppressive effects of sterols on IL-1β expression by stimulated cells may be attributed to different times of incubation of cells and subsequent increase in IL-6, which acted on cells with a reduction of IL-1β production. Furthermore, different results can be attributed to differences in the type of cells stimulated (sheep PBMC vs mouse RAW 264.7 macrophages).

IL-10 is a regulatory cytokine with anti-inflammatory properties which refer to its ability to down-regulate the production of proinflammatory cytokines and the activity of both macrophages and dendritic cells. In our study, cells stimulated with LPS and ConA displayed an increase in IL-10 production in presence of both ergosterol and of the mixture of ergosterol and 7-dehydroporiferasterol purified from *Dunaliella tertiolecta,* thus further suggesting the anti-inflammatory role of the extract containing the two sterols by *Dunaliella tertiolecta.* A number of studies demonstrated that the presence of phytosterols is able to up-regulate the IL-10 production in different cell lines (Valerio and Awad, 2011, Nashed et al., 2005). In our study both ergosterol and the mixture of ergosterol and 7-dehydroporiferasterol extracted from *Dunaliella tertiolecta* dose-dependently increased the production of IL-10 in LPS-stimulated sheep cells. In particular, our

results suggest that the optimal concentration of the mixture of ergosterol and 7-dehydroporiferasterol from *Dunaliella tertiolecta* to obtain the highest production of IL-10 was 0.4 mg/ml; further increase in the mixture of ergosterol and 7-dehydroporiferasterol from *Dunaliella tertiolecta* did not result in a further increase in IL-10 secretion. On the contrary, ergosterol alone achieved the highest production of IL-10 at 0.8 mg/ml. Kobori et al. (2007) reported an inhibition of the C/EBPβ DNA-binding activity in the nuclear extracts of macrophages incubated with both ergosterol peroxide and ergosterol at a concentration of 30 and 60 μM. Macrophages have been demonstrated to require C/EBPβ for IL10 production, when exposed to *E. coli* (Csóka et al., 2007). Based on previous findings a tentative explanation of the reduction of IL-10 production by sheep cells at increasing concentrations of the mixture of ergosterol and 7-dehydroporiferasterol from *Dunaliella tertiolecta* could be the possible inhibition of C/EBPβ DNA-binding activity exerted at increased concentrations of the mixture. It is worth noting that the inhibitory effects of both ergosterol peroxide and ergosterol on C/EBPβ DNA-binding activity were obtained by the highest concentrations tested by Kobori et al. (2007), which were similar to the highest concentration of sterols tested in our study. As a consequence, the increased IL-10 production by sheep cells in the presence of 0.4 mg/ml of the mixture of ergosterol and 7-dehydroporiferasterol from *Dunaliella tertiolecta* could be attributed to the absence of inhibition on C/EBPβ DNA-binding activity; on the contrary the increased concentration of the mixture up to 0.8 mg/ml could have inhibited the C/EBPβ DNA-binding activity, thus leading to a reduction of IL-10 production. The increase of IL-10 production by PBMC in our study can help to explain the reduction in IL-6 production in stimulated cells both in presence of

ergosterol and of the mixture of ergosterol and 7-dehydroporiferasterol purified from *Dunaliella tertiolecta*. It has been demonstrated that IL-10 can control inflammatory processes by regulating the production of inflammatory cytokines (Wattegedera et al., 2004). Caroprese et al. (2012) in cows found a relation between IL10 production and the decrease in IL-6 secretion. In addition, IL-10 has been demonstrated to strongly regulate proliferation of sheep PBMC (Wattegedera et al., 2004) and this could help to explain the reduction in PBMC proliferation exerted by the mixture of ergosterol and 7-dehydroporiferasterol purified from *Dunaliella tertiolecta* observed in our study.

1.5 CONCLUSIONS

We studied the immunomodulatory properties of a mixture of phytosterols, and a mixture of ergosterol and 7-dehydroporiferasterol extracted from *Dunaliella tertiolecta,* in relation to the immunomodulatory properties of ergosterol, on peripheral blood mononuclear cells isolated from sheep. The results of the present study evidenced a role of phyotosterols from *Dunaliella tertiolecta* on immune modulation and anti-inflammatory activity in sheep. The immunomodulatory and anti-inflammatory activities were more evident in the purified extract represented by a mixture of ergosterol and 7-dehydroporiferasterol, and might depend on the existence of a synergic effect of the structures of the two phytosterols.

The use of a mixture of sterols, purified and extracted from *Dunaliella tertiolecta,* could have particular innovative implications on the modulation of the immune reactions in sheep production systems to control tissue damages deriving from uncontrolled inflammatory reactions, when administrated as feed additives in sheep diet.

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Second trial

Hypothalamic-pituitary-adrenal axis activation and immune regulation in heat stressed sheep after polyunsaturated fatty acids supplementation

2.1 INTRODUCTION

Stressors of different natures and intensity can induce production of extracellular and intracellular mediators to modulate cell responses. Heat stress stimulates signal transduction pathways to alter gene expression of immune cell mediators (i.e. cytokines) and can activate heat shock response. Heat shock response evokes large changes in the secretion and activity of heat shock proteins (HSPs), which are strictly connected to the endocrine and immune system (Collier et al., 2008); HSPs are divided into a set of different families according to their molecular weights; those of approximately 90, 70 and 27 kDa are referred to as HSP 90, HSP 70, and HSP 27. Cultured sheep lymphocytes have been demonstrated to produce HSP 90 and HSP 70 in response to thermal stress (Guerriero and Raynes, 1990). The engagement of extracellular HSPs also includes the promotion of cytokine activity (Martin et al., 2009). Distinct cytokine patterns are responsible for the effector functions and development of T-helper1 (Th1) and T-helper 2 (Th2) cell responses. Th1 cells activate cellular immunity and inflammatory responses, whereas Th2 cells control humoral immunity and promote anti-inflammatory responses. Animals' ability to produce selectively Th1 cytokines (interferon-γ (IFN-γ), and IL-12), and Th2 cytokines (IL-10, IL-4, IL-13), and thereby to regulate Th1/Th2 cytokine balance, is highly temperature dependent (Park et al., 2005). Hyperthermia can down regulate Th1 cytokines in favour of the secretion of Th2 cytokines, thus suppressing cell-mediated immunity (Murzenok et al., 1997; Elenkov and Chrousos, 1999; Webster et al., 2002). Ewes subjected to heat stress displayed an impairment of their cellular immune response after the intradermal injection of mitogens, and increased cortisol concentrations (Sevi et al., 2002; Caroprese et al., 2012). It has been demonstrated that

corticosteroids, whose rise can be caused by stress, bind to DNA and inhibit the expression of genes involved in T-cell activation and cytokine production (Sgorlon et al., 2012). Anti-inflammatory properties of corticosteroids lead to decreased phagocytic cell activity, which in turn alters lymphocyte function (Rosen at al., 2001). A number of ruminant diseases, including Johne's disease, require a strong cell-mediated immune response in sheep to protect against pathogenic bacteria (Begg et al., 2005). Therefore, the maintenance of the proper Th1/Th2 balance could be a critical factor to face immunological challenges, in particular if occurring during the summer season.

Few studies have focused on the effects of heat stress on both nutrient utilization and circulating hormones to cope with immune challenges in sheep. Recently, Caroprese et al. (2012) found that in heat stressed ewes the supplementation of whole flaxseed, rich in linolenic acid (C18:3, ALA), which is a n-3 PUFA, sustains humoral responses and induces an increase in cortisol secretion. The precise mechanisms underlying cellular immune and humoral functions in sheep under high temperatures, however, remain undefined, particularly with regard to cytokine profiles in vivo and to HPA activation.

To overcome heat stress in lambs, Saker et al. (2004) suggested the use of a supplementation with a brown seaweed, *Ascophyllum nodosum* (Tasco). The high antioxidant content of *Ascophyllum nodosum* was found partially responsible for the enhanced immune function and antioxidant status in heat stressed lambs. In addition, *Ascophyllum nodosum* contains n-3 PUFA, such as eicosapentaenoic acid (C20:5, EPA), which are considered health promoting molecules, and fucoidan, which has anti-inflammatory and anti microbial activities.

No studies evaluated the combined effect of flaxseed and algae as supplementation to heat stressed sheep. We hypothesized that the administration of PUFA supplementation, as whole flaxseed, or algae, or in combination, in the diet of dairy sheep under heat stress could contribute to alter HPA activation and sustain their immune functions and Th1/Th2 balance.

The objective of the present experiment was therefore to determine the effects of supplementing the diet with PUFA from flaxseed, and algae, or from a combination of flaxseed and algae, on immune regulation, in terms of cell-mediated and humoral immune responses, and HPA activation in sheep under heat stress conditions. The balance between Th1 and Th2 cells, by monitoring ILs secretion, and the production of heat shock proteins in vivo were also investigated.

2.2 MATERIALS AND METHODS

2.2.1 Animals and experimental design

The experiment lasted 30 d, and was conducted during the summer (June-July) of 2012 at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE), Apulia. Thirty-two late-lactation Comisana ewes (d 202.1±5.3 of lactation, mean ± SD) were divided into four groups of eight, balanced for milk yield, body weight, and body condition score. Groups were separately reared in external pens of 5 x 12 m bounded with mesh-fence; the trough and the crib were located in the external areas. During the trial, ambient temperature and relative humidity in indoor and outdoor areas were monitored with thermo-hygrographs (LSI, I-20090 Settala Premenugo-Milano, Italy) placed at 1.5 m from the floor. The average

temperature-humidity index (THI) was calculated using the Kelly and Bond's (1971) formula.

All groups were individually fed twice daily and received 1.8 kg/ewe/d of oat hay. The control group also received 1 kg/ewe/d of pelleted concentrate (Mangimificio Molino Gallo, Potenza, Italy), whereas ewes in the experimental groups were supplemented with whole flaxseed (Lin Tech, Tecnozoo srl, Torreselle di Piombino Dese, Italy), or *Ascophyllum nodosum* (Tasco®, Acadian Seaplants, Canada), or their combination. Namely, ewes in the FS group received 750 g/ewe/d of pelleted concentrate, and 250 g/ewe/d of whole flaxseed; ewes in the AG group received 1 kg/ewe/d of pelleted concentrate in which 5% *Ascophyllum nodosum* was incorporated; ewes in the FS+AG group were supplemented with both flaxseed (250 g/d) and pelleted concentrate (750 g/d) incorporating 5% *Ascophyllum nodosum*. DMI was determined for each experimental group by weighing the refusals at 0800, 1200, 1600, and 2000 h. Water was available ad libitum for all groups from automatic drinking troughs at any time of day.

All procedures were conducted according to the guidelines of the Eu Directive 2010/63/EU (2010) on the protection of animals used for experimental and other scientific purposes. Ewes were healthy and their conditions were carefully examined by veterinarians throughout the trial to exclude the presence of signs of diseases.

The chemical composition of diets was carried out by standard procedures (AOAC, 1990). Ingredients and chemical composition of diets are reported in Table 2.1

Item	Diet			
	Control	AG	FS	FS+AG
Ingredients, % of DM				
Oat hay	64.50	64.39	64.37	64.38
Concentrate[1]	35.50	17.72	26.57	8.86
5% *Ascophyllum nodosum* concentrate	0	17.89	0	17.70
Whole flaxseed[2]	0	0	9.06	9.06
Chemical composition				
DM%	93.48	93.64	93.67	93.65
Ether extract, % of DM	1.93	2.04	5.02	5.12
CP, % of DM	12.53	12.84	12.89	13.20
ADF, % of DM	27.75	28.03	28.48	28.76
NDF, % of DM	53.36	53.32	54.58	54.55
ADL, % of DM	3.44	3.56	3.83	3.96
NE_L, Mcal/kg[3]	1.30	1.31	1.29	1.30

[1]Contained: Corn Meal, Soybean Meal, Wheat Germ Meal, Wheat Meal, Roasted Soybean Seeds, Barley Meal, Wheat Fine Bran, Corn Cracked, Sugarcane Molasses, Partially Debarked Sunflower meal, Bentonite, Dried Pulp, Calcium Carbonate, Sodium Bicarbonate, Sodium Chloride, Magnesium Oxide, 8.3 IU/g vitamin A, 8.2 IU/g vitamin D3, 99mg/kg vitamin E, 0.07mg/kg vitamin B1, 255mg/kg vitamin PP, 488mg/kg Cl, 293mg/kg Fe, 1.26mg/kg Co, 1mg/kg Cu, 0.4% Na.
[2] Lin Tech (Tecnozoo srl, Torrreselle di Piombino Dese, Italy).
[3]Calculated according to NRC (2001).

Table 2.1 *Ingredients and chemical composition of the diets (DM basis).*

The determination of methyl esters of the diet ingredients was carried out according to O'Fallon et al. (2007). Briefly 1 g of the samples was pipetted into a screw-cap (16 x25 mm) reaction tube. Methanol (0.5 mg) of C13:0/mL, 0.7 mL of KOH and 5.3 mL of MeOH was added into each tube, during incubation at 55°C for 1h and 30', the tubes were inverted to mix for 5 s every 20 min. After cooling in a cold water bath, 3 mL of hexane were added into each tube and vortex for 5 min. The tubes were centrifugated at room temperature for 5 min at 500g; supernatant (1 mL) was taken from each tube and transferred into vials and stored at -20°C to followed gas-chromatography (GC) analysis. Fatty acid profiles were quantified using Agilent Technologies,

6890N GC equipped with a flame ionization detector (FID). Helium was the carrier gas and the gas flow rate was 175 kPa. The oven temperature (Eulitz et al., 1999) was initially held at 70°C for 4 min, and then programmed to 175°C at 13°C/min increase and held isothermally for 45 min. The column used was a capillary column (HP88; 100 m x 0,24 mm i.d., 0,20 µm film thickness, Agilent Technologies Santa Clara, USA). Concentrations of fatty acid methyl esters (FAME)s were analysed utilizing a calibration curve with a mixture of standards of 50 fatty acid (GLC Reference standard 674, Nu-Check Prep, Inc. Elysian MN 56028, USA) with added CLA standards: C18:2-8t, 10c; C18:2-9c, 11t; C18:2-11c, 13t; C18:2-9t, 11c; C19:2-8c, 10c; C18:2 9c, 11c; C18:2-10t, 12c; C18:2-8t, 10t; C18:2-9t, 11t; C18:2-10t, 12t; C18:2-11t, 13t (GLC Reference standard UC-59M, Nu-Check Prep, Inc. Elysian MN 56028, USA).

Flaxseed fatty acid composition was mainly characterized by 53.21g/100g of total fatty acids (FA) of C18:3n3 (ALA), while *Ascophyllum nodosum* was characterized by 37.03 g/100g FA of C18:1 cis9 and by 5.03 g/100g of FA of C20:5n3 (EPA).

2.2.2 Body condition score and respiration rate

At the beginning of the experiment and then weekly the body condition score (BCS) of the ewes was measured in the morning after milking and before feeding time and recorded using a six-point scale (0 = thin, 5 = fat).

Respiration Rate (RR) was measured in all animals weekly at 1430 h by counting the rate of flank movements each 30 seconds (Caroprese et al., 2012).

2.2.3 Plasma cortisol determination

At 22 d of the experiment, plasma cortisol concentrations were measured; as demonstrated in a previous paper the effects of a diet based on flaxseed

supplementation were evident after at least 20 d from the beginning of the treatment (Caroprese et al., 2011). The ewes were intravenously injected with 2 IU adrenocorticotropic hormone (ACTH)/Kg $BW^{0.75}$ (Sigma Aldrich, Milan, Italy). Blood samples (7mL) for evaluation of cortisol concentrations were collected in heparinised vacuum tubes from the jugular vein immediately before and 60, 120 and 240 min after ACTH injection. Hormone concentration was determined by a competitive enzyme immunoassay (Radim, Cortisolo EIA WELL KS18EW, Rome, Italy), according to Caroprese et al. (2012).

2.2.4 In vivo cell-mediated immunity

At the beginning of the experiment, at 15 d, and 30 d of the trial, a skin test was performed to induce non-specific delayed-type hypersensitivity by intradermic injection of 1mg/mL phytohemagglutinin (PHA) (Sigma Aldrich) dissolved in sterile saline solution. At each sampling time, the injection was administered into the centre of a 2 cm diameter circle marked on shaved skin on the upper side of each shoulder. The determination of lymphocyte proliferation, measured as skinfold thickness, was calculated from the two measurements made with calipers as the difference between 24 h post-injection thickness - pre-injection thickness.

2.2.5 Humoral immune response

To evaluate the humoral response to ovalbumin (OVA, chicken egg albumin), and to measure anti-OVA IgG concentrations, at 0 d of the experiment the ewes were subjected to a subcutaneous injection of 6 mg of ovalbumin (OVA, Sigma Aldrich) dissolved in 1 mL of sterile saline solution and 1 mL of incomplete Freund's adjuvant (Sigma Aldrich). A second injection without adjuvant was repeated 7 d later. Antibody titers were determined in blood samples collected in

heparinized vacuum tubes (Becton Dickinson, Plymouth, United Kingdom) immediately before the first antigen injection (0 d) and then at 15 d, and 30 d of the study period. An ELISA (Enzyme Linked Immunoassorbent Assays) was performed in 96-well U-bottomed microtiter plates. Wells were coated with 100 µL of antigen (10 mg of OVA/mL of phosphate buffered saline-PBS) at 4°C for 12 h, washed with PBS (pH 7.2) and 0.05% Tween 20 (PBST) and incubated with 1% skimmed milk at 37°C for 1 h to reduce nonspecific binding, and then washed. The sheep serum (1:5,000 dilution in PBS) was added and incubated at 37°C for 1 h. The extent of antibody binding was detected using a horseradish peroxidase-conjugated donkey anti-sheep IgG (Sigma Aldrich) (1:20,000 dilution in PBS). After other washing, 100 µL of substrate (1 mg of 3,3' 5,5'- tetramethylbenzidine (TMB), 1 mL of dimethylsulfoxide (DMSO), 9 mL of phosphate-citrate buffer, 2 µL of H_2O_2) were added in each well. The reaction was completed adding 50 µL of H_2SO_4 after 30 min. Optical density was measured at a wavelength of 450 nm with titer-ELISA spectrophotometer (Rosys Anthos 2020).

Anti-Ova IgG evaluation was a quantitative assay: a standard curve (y=(0.0043-0.919)/(1+(x/233)^1.06)+0.919), R^2 = 99.99%) was made up by ovine IgG dilutions (A5295, Sigma Aldrich); all plasma samples were read against the curve. Data were expressed as mg of anti-OVA IgG/mL. The assay was optimized in our laboratory for concentrations of coating antigen, serum, and antibody.

2.2.6 Determination of cytokines in plasma samples by ELISA

The determination of IL-10 and IL-12 in plasma was carried out by an ELISA according to Kwong et al. (2002), and Hope et al. (2002), respectively, on blood samples collected at 0 d, 15 d, and 30 d of the experiment. Plates were read by a titer-ELISA spectrophotometer (Power Wave XS, Biotek, UK). The intra- and

inter-assay CV were 3.2% and 9.8%, respectively, for IL-10, and 5.4% and 10.3%, respectively, for IL-12.

The level of IL-13, IL-4, and IFN-γ in plasma were assayed by an ELISA as briefly described: 96-well plates (Sterilin, Cambridge, UK), were coated overnight at 4°C with 100 µL of anti-Bovine IL-13 (Kinghfisher Biotech, Saint Paul, USA), anti-Bovine IL-4 (AbD Serotec, Kidlington, UK), anti-Bovine IFN-γ (AbD Serotec) antibodies in PBS (2µg/mL). After washing with PBST, the antibody was blocked with PBS/1% BSA for 1h. IL-13, IL-4 and IFN-γ standard and samples were added to plate for 1h at room temperature and after washing ~~for~~ 4 times. Biotinylated secondary anti-Bovine IL-13 (Kinghfisher Biotech), IL-4 (AbD Serotec) and IFN-γ (AbD Serotec) antibodies in PBS (0.5µg/mL) were added for 1 h followed by 100 µL of streptavidin-HRP (AbD Serotec). After washing, 100 µL of TMB substrate solution were added to each well for 30 min, to stop the reaction 50 µL of H_2SO_4 (4M) were added to each well. The level of IL-13, IL-4 and IFN-γ were measured colorimetrically at 450 nm (to which were substracted the absorbance at 540nm) and quantified by interpolation from a standard curve. Data were expressed as µg/mL of IL-13, as ng/mL of IL-4 and pg/mL of IFN-γ. The intra- and inter-assay CV were 8.8% and 6.1%, respectively, for IL-13, 5.4% and 9.3%, respectively for IL-4, and 3.9% and 2.3% respectively, for IFN-γ.

2.2.7 HSPs determination in white blood cells lysate

At 0 d, 15 d, and 30 d of the experiment, white cells from blood were collected after lysis of red cells: 1mL of blood was added at 5 mL of NaCl at 0.2% and incubated at 4°C for 30 min; during incubation the samples were mixed with a gentle inversion; subsequently samples were centrifuged at 1500 g for 15 min at 4°C. After complete removal of supernatant the pellet was stored at -20°C.

Pelletted white cells were lysed with a cold RIPA Buffer (Thermo Scientific, Waltham, USA), which was implemented with Halt Protease Inhibitor Cocktail, Halt™ Phosphatase Inhibitor Cocktail according to the manufacturer's instructions. The supernatants were collected for HSPs determination.

The concentration of HSP 70 and HSP 90 in white blood cells lysate were carried out with Sheep Heat Shock Protein 70 (CSB-EQ027551SH, CusabioBiotech Co., LTD, Wuhan, China) and Sheep Heat Shock Protein 90 kits (E14H0304, Blue Gene, Shanghai, China) according to the manufacturer's instructions. Data were expressed in ng/mL of HSP 70 calculated in 10^6cells/mL of white blood cells lysate and pg/mL of HSP 90 calculated in 10^6cells/mL of white blood cells lysate.

2.2.8 Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro e Wilk, 1965). All the data were analysed using ANOVA for mixed models, having the animal as a random factor nested in the treatment. Data collected during the experiment were analysed with the diet, the time of sampling and their interactions as fixed effects. Data were covariated on initial values. For DMI weights of refusals taken at four-hour intervals during the day were used as random factor nested in the treatment. When significant effects were found (at $P<0.05$, unless otherwise noted), the LSD test was used to locate significant differences between means.

2.3 RESULTS

2.3.1 Meteorological Data

On average, maximum temperature was of about 38°C throughout the study period and average minimum night temperature was of about 21°C. Maximum THI was higher 86 during the first d of the experiment, both outside and inside the experimental pens (Figure 2.1). THI levels higher than 80 were registered outside the pens during d 9-12 and d 30 of the experiment. Inside the experimental pens THI levels higher than 80 were registered during the d 12-14, 17-19, and d 20-22 of the experiment.

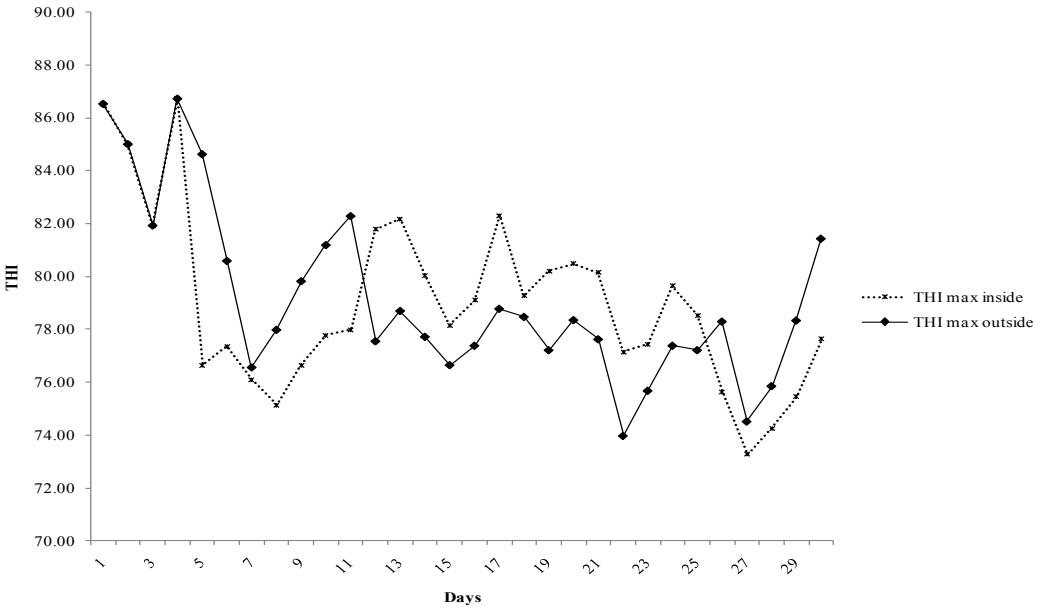

Figure 2.1 *Daily averages of maximum Temperature-Humidity Index (THI) values detected inside and outside the experimental pens during the d of the experimental period.*

2.3.2 Dry Matter Intake, Body condition score and Respiration Rate

Daily DMI are reported in Figure 2.2: ewes in AG and C groups almost entirely consumed their ration, whereas ewes in FS and FS+AG groups displayed lower DMI ($P < 0.01$).

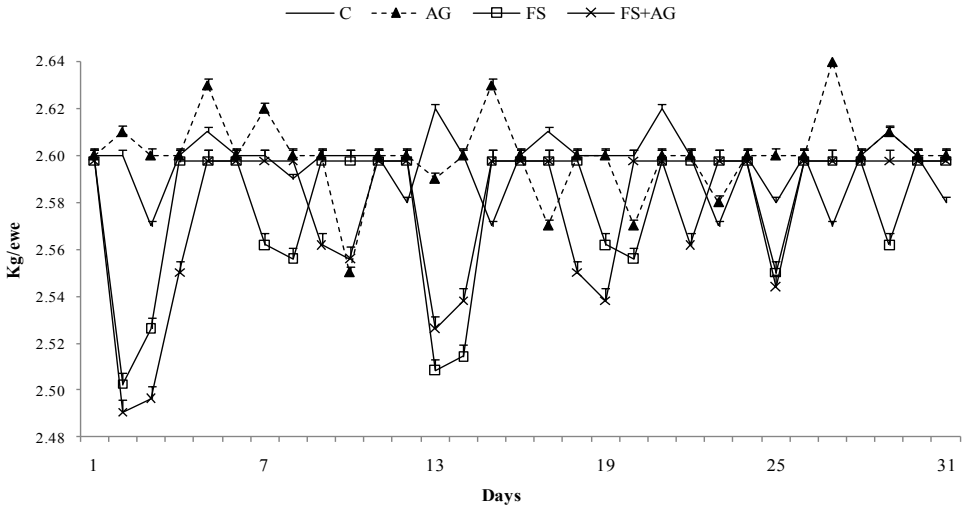

Figure 2.2 *Daily dry matter intakes (DMI±SEM) measured in sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).*

In Table 2.2 data related to sheep BCS and respiration rate at the beginning and the end of the experiments were reported; BCS was affected by time ($P < 0.001$)

		Treatment					Effects, P		
Item	Days	C	AG	FS	FS+AG	SEM	Diet	Time	Diet x Time
BCS[1]	0 d	2.54	2.53	2.52	2.53				
	30 d	2.42	2.38	2.52	2.47	0.07	NS	***	NS
Respiration rate, breath/min	0 d	120.35	130.39	128.71	129.55				
	30 d	160.35a	171.39a	128.71b	133.55b	7.64	NS	***	***

[a,b] Means followed by different letters are significantly different at P<0.05.
NS, not significant; ***, P<0.001.
[1]Body condition score.

Table 2.2 *Least Square Means ± SEM of respiration rate, and BCS of sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG*

because in all the experimental groups BCS decreased from the beginning of the experiment until wk 3 and then returned to the initial levels.

Respiration rate was affected by time ($P < 0.001$) and the interaction time x diet ($P < 0.001$); in FS and FS+AG sheep lower respiration rates than C and AG sheep were measured at 30d of the experiment.

2.3.3 Plasma cortisol

Cortisol concentrations in plasma peaked at 60 min, decreased at 120 min, and returned to basal concentrations 240 min after ACTH challenge in all groups ($P < 0.001$) (Figure 2.3). An interaction of time x diet was found: at 60 min after ACTH challenge FS ewes displayed greater cortisol concentrations in plasma than AG, FS+AG, and C ewes ($P < 0.05$).

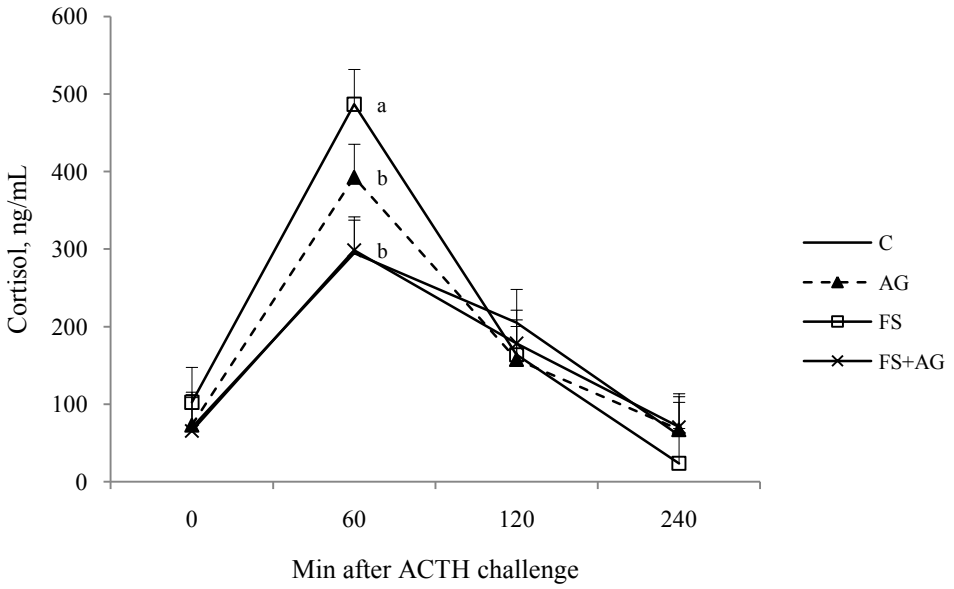

Figure 2.3 *Plasma cortisol production (Least Squares means ± SEM) measured immediately before and then at 60, 120 and 240 min after ACTH challenge at 22 d of the experiment in sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).* [a, b] *Values with different superscripts differ between feeding treatments within a sampling day ($P < 0.05$).*

2.4.4 In vivo cell-mediated immunity and humoral immune response

Cell-mediated immune response of sheep was affected by time of sampling ($P < 0.001$): from the beginning to the end of the trial a reduction of skinfold thickness was found in each group, except for the FS group, in which the skinfold thickness remained unchanged throughout the trial (Figure 2.4, a).

Anti-Ova IgG production was affected by time and the interaction of time x diet ($P < 0.001$, and $P < 0.05$, respectively). The anti-Ova IgG rose in all the groups at the end of the experiment. At 15 d of the experiment FS+AG ewes displayed less anti-OVA IgG titres than AG and C ewes (Figure 2.4, b).

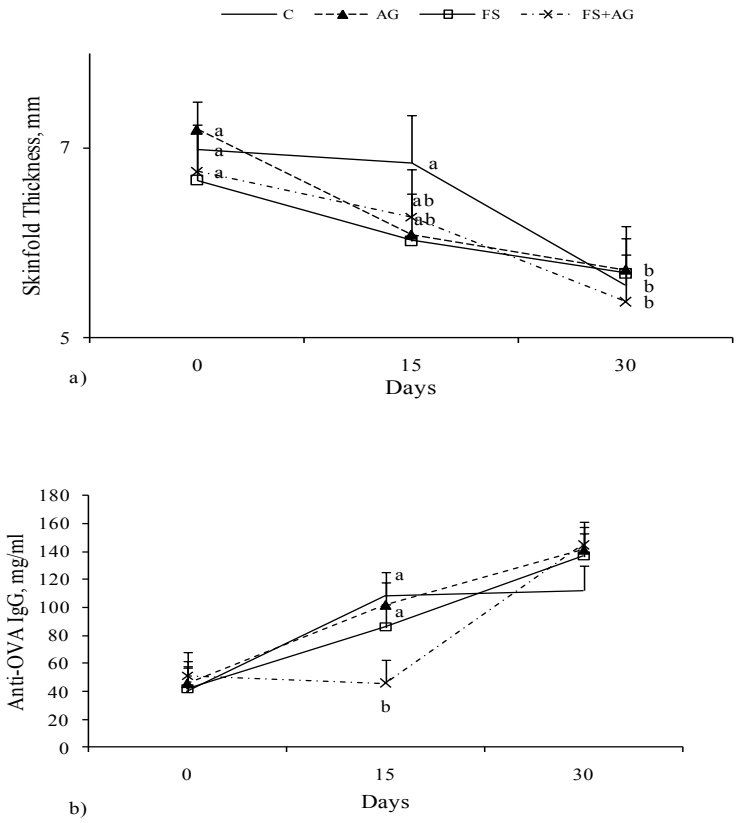

Figure 2.4 *Least Squares means ± SEM of a) Skinfold thickness measured after PHA injection at 0, 15, and 30 d of the experiment in sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG); b) Antibody titres to OVA detected at 0, 15, and 30 d of the experiment in blood of sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).* $^{a, b}$ *Values with different superscripts differ between feeding treatments within a sampling day ($P < 0.05$).*

2.4.5 Plasma cytokines

IL-10 concentrations in plasma increased from the beginning to the end of the experimental period, and then increased in all groups ($P < 0.001$) (Figure 2.5, a). At 15 d of the experiment IL-10 concentrations were greater in AG, FS and FS+AG than in the control group ($P < 0.05$).

IL-4 concentrations in plasma samples decreased throughout the experiment in all the groups ($P < 0.001$). No differences among the different groups emerged (Figure 2.5, b).

On average, FS+AG ewes showed lower IL-13 plasma mean concentrations than FS, AG and C ewes ($P < 0.05$). IL-13 plasma concentrations decreased in all groups at 30 d of the experiment compared with previous sampling times ($P < 0.001$). FS+AG ewes displayed lower IL-13 plasma concentrations than control and AG ewes at 15 d, and lower than control, AG and FS ewes at 30 d of the experiment ($P < 0.05$) (Figure 2.5, c).

IL-12 plasma concentrations were affected by time of sampling ($P < 0.001$), and displayed an increase in all groups at 30 d of the experiment. In particular, FS and FS+AG ewes displayed greater IL-12 plasma concentrations than C and AG ewes at 30 d of the experiment ($P < 0.05$) (Figure 2.5, d).

IFN-γ plasma concentrations decreased at 30 d of the experiment in all groups ($P < 0.001$). At that time control ewes displayed greater concentrations of plasma IFN-γ than FS+AG ewes (Figure 2.5, e).

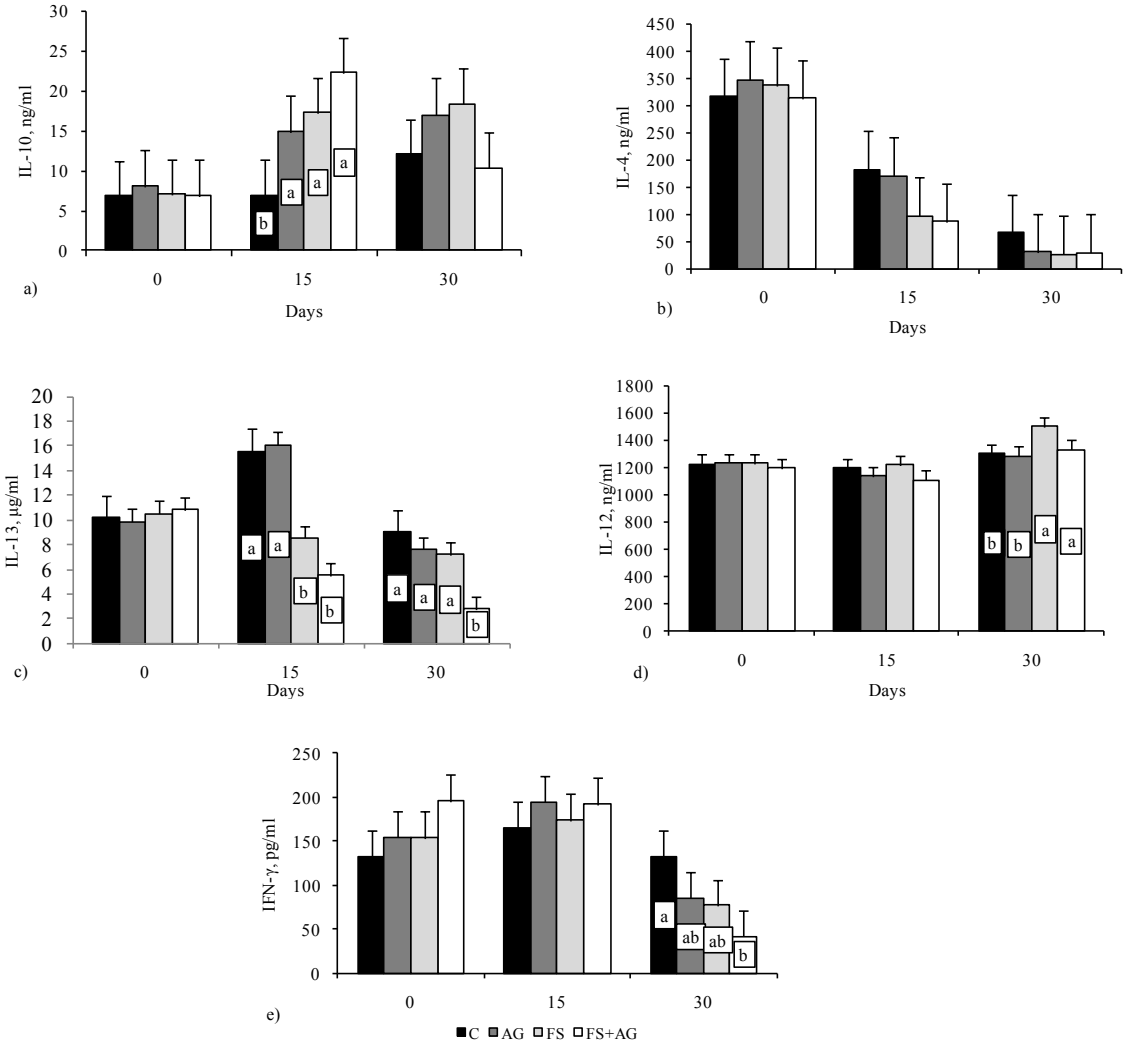

Figure 2.5 *Least Squares means ± SEM of a) IL-10 production; b) IL-4 production; c) IL-13 production; d) IL-12 production; e) IFN-γ production measured at 0, 15, and 30 d of the experiment in plasma of sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).* $^{a, b}$ *Shows a significant difference between feeding treatments within a sampling day (P < 0.05).*

2.4.6 Heat shock proteins

HSP 70 concentrations in white blood cells lysate were affected by time x diet: FS ewes displayed lower HSP 70 at 30 d of the experiment than at the beginning and at 15 d of the experiment ($P < 0.05$). On the contrary in AG ewes the concentrations of HSP 70 in white blood cells lysate were greater at 30 d than at the beginning of the experiment (Figure 2.6, a).

At 30 d of the experiment, FS and C ewes displayed greater concentrations of HSP 90 in white blood cells lysate than FS+AG ewes ($P < 0.05$) (Figure 2.6, b).

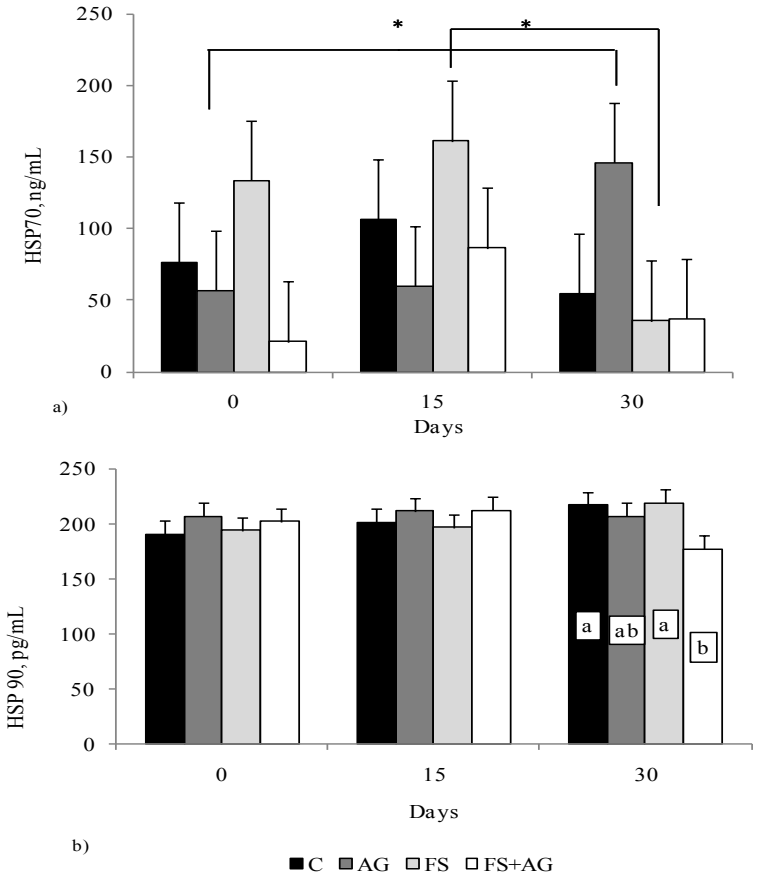

Figure 2.6 *Least Squares means ± SEM of a) HSP70 concentration measured at 0, 15, and 30 d of the experiment in white blood cells lysate of sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).* **Shows a significant difference between different sampling days within feeding treatments ($P < 0.05$); b) HSP90 concentration measured at 0, 15, and 30 d of the experiment in white blood cells lysate of sheep fed control diet (C), or supplemented with Ascophyllum nodosum (AG), flaxseed (FS), and a combination of Ascophyllum nodosum and flaxseed (FS+AG).* $^{a, b}$*Shows a significant difference between feeding treatments within a sampling day ($P < 0.05$).*

2.5 DISCUSSION

In this study sheep were used as in vivo models to evaluate the effects of PUFA supplementation from different sources on HPA activation and immune regulation under heat stress conditions. The positive influence of supplementation on HPA activation and immune regulation could have a role in sustaining altered immune response found in sheep under heat stress.

Prolonged exposure to maximum air temperature over 30°C and to THI higher than 80 prevent lactating ewes from maintaining their thermal balance, thus inducing heat stress (Sevi et al., 2001). The meteorological conditions registered both inside and outside the experimental pens may account for the high respiration rate observed. The respiration rate displayed by the ewes in the present study suggested they were under heat stress, according to Silanikove (2000), who defined the panting rate of 80-120 as indicative of high heat stress in farm animals. The lower respiration rates measured in FS and FS+AG sheep than in C and AG confirmed previous findings on the positive peripheral vasodilator effect of flaxseed, even in combination with *Ascophyllum nodosum,* in helping sheep to cope with heat stress (Caroprese et al., 2012). However, it should have to be mentioned that the administration of flaxseed in the diet slightly reduced DMI of ewes.

Acute heat stress can act on HPA axis activation by increasing plasma cortisol secretions in order to increase glucose availability to meet the enhancement of energy demand for thermoregulation (Matteri et al., 2000). In ruminants the diet can also influence the activation of the HPA axis and expression of genes involved in stress-response, as stated in a number of studies (Munksgaard et al., 2006; Stefanon et al., 2009; Sgorlon et al., 2012). The increase of cortisol

secretion in ewes under heat stress and supplemented with flaxseed, which was found in our experiment, was in agreement with a previous study (Caroprese et al., 2012), and demonstrated the ability of flaxseed supplementation to interfere with cortisol secretion. On the other hand, the reduced cortisol secretions in sheep supplemented with the combination of flaxseed and *Ascophyllum nodosum* compared with cortisol secretions of FS sheep might support the hypothesis of the existence of a negative synergy of flaxseed and algae as regards HPA activation.

Hyperthermia and the consequent increase in cortisol secretion can suppress cell-mediated immunity, down regulating Th1 cytokines in favour of the secretion of Th2 cytokines (Murzenok et al., 1997; Elenkov and Chrousos, 1999; Webster et al., 2002). Th2 cells mainly produce IL-4, IL-13, and IL-10, which are able to sustain humoral immunity; IL-4 and IL-10 stimulate the differentiation of B cells into antibody-secreting B cells, and can inhibit macrophage activation, pro-inflammatory cytokine production and T cells proliferation. IL-10 is produced by T lymphocytes, B cells, eosinophils, mast cells, and monocytes (Asadullah et al., 2003), and is considered the key antagonist of Th1 response by regulating cytokine production and acting on post-transcriptional mechanisms (Moore et al., 2001). Flaxseed supplementation in heat stressed dairy cows resulted in a reduction of IL-10 production and in an enhanced cellular immune response, supporting the hypothesis that whole flaxseed was able to modulate immune responses of cows under heat stress by opposing lymphocytes shift from Th1 to Th2 type, that usually occurs under heat stress conditions (Caroprese et al., 2009). In the present study, cell-mediated immune response decreased in all the experimental groups with the exception of the FS group, probably as a result of down regulation of Th1 responses exerted by heat stress. Diet supplementation

administrated under heat stress conditions could have different effects on the regulation of immune responses based on the extent of heat stress, and according to species differences. The increased concentrations in plasma IL-10 in the experimental groups compared with the control group confirmed a possible role of the diet in regulating immune responses, probably by altering the expression of different genes involved in stress response. Furthermore, glucocorticoids have a direct effect on IL-10 production because they are able to up-regulate the IL-10 secretion by T cells (Elenkov and Chrousos, 1999). Ewes fed flaxseed showed increased cortisol concentrations compared with all the other groups, and this could have deeply influenced their IL-10 production. The increased IL-10 secretions in FS ewes could be claimed to explain the absence of the expected direct positive effect of flaxseed supplementation on the cellular immune response found in the cows. In a previous study carried out in heat stressed ewes, the increase in cortisol secretion was responsible for the impairment of their cellular immune response after the intradermal injection of mitogens (Sevi et al., 2002). In the present experiment, FS ewes, despite the increased cortisol and IL-10 concentrations, displayed stable and durable cellular immune responses, according to previous studies on cows (Caroprese et al., 2009). The increase in IL-10 production registered in all the experimental groups at 15 d of the experiment, might have a direct suppressive effect on Th1 cell response, by reducing the secretion of INF-γ (Elenkov and Chrousos, 1999). The reduction of INF-γ concentrations observed in AG+FS ewes at 30d of the experiment could be the direct result of the high IL-10 production measured in this group. On the other hand, it is well known that IL-12 is a critical factor for cell-mediated immune response in vitro, stimulating IFN-γ production (Macatonia et al., 1993; Brown

and Estes, 1997). IL-12, which is produced by monocytes, dendritic cells and neutrophils, can alter the balance between Th1 and Th2 responses by enhancing cell-mediated immune responses and impairing the production of immunoglobulins involved in the Th2 humoral responses. Brown and Estes (1997) reported that IL-12 increased IFN-γ production in bovine peripheral blood mononuclear cells (PBMC) after mitogen or parasite antigen stimulation. In the present experiment, FS ewes had IFN-γ concentrations similar to those found in control ewes, despite the increased concentrations of both plasma IL-10 and glucorticoids observed. The increased concentrations of plasma IL-12 detected in FS ewes at the end of the experiment could be, at least partially, responsible for IFN-γ production, and cell-mediated immune responses of FS ewes at the end of the trial. In mitogen-activated ovine PBMC, the addition of recombinant ovine IL-12 enhances IFN-γ production, and has a small effect on cell proliferation. On the contrary, recombinant ovine IL-10 has a great inhibiting effect on cell proliferation and a small negative effect on IFN-γ production; no consistent relationship between IL-10 and IFN-γ production was found (Wattegedera et al., 2004). Previous findings in an in vitro experiment seemed to be consistent with the findings of our in vivo experiment. Our results confirmed the complexity of the cross-talk between IL-10 and IL-12 and the importance of the balance of cytokine profile in controlling the in vivo Th1 and Th2 responses in sheep, in terms of cell-mediated and humoral immune responses. Based on our data, it could be argued that whole flaxseed supplementation contributed to sustain Th1 response in ewes subjected to heat stress.

IL-4 and IL-13 play an important role in the regulation of Th2 cells and in the development of humoral immune responses, even if IL-13, differently from IL-4,

cannot stimulate the growth of activated T cells (Zurawski and de Vries, 1994). Based on these findings, the lowest IL-13 plasma concentrations found in FS+AG ewes can be claimed to explain the trend of anti-OVA IgG production in FS+AG ewes at 15 d of the experiment. It was found that in humans the expression and production of IL-13 was more rapid and longer lasting than the expression and production of IL-4 in T cells stimulated with ConA; in addition, a dose- and time-dependent increase in IL-13 production in PBMC stimulated with PHA was observed (de Waal Melefyt at al., 1995; Luttmann et al., 1999). Luttmann et al. (1999), based on the different kinetics of IL-4 and IL-13 production, suggested that IL-4 could be restricted to the first phases of an initiated Th2 immune response, whereas IL-13 could play a role in the ongoing immune response. In IL-4 deficient mice, IL-13 transgene expression did not reverse IgG1 deficiency, suggesting a marginal role for IL-13 in regulating production of IgG1 (McKenzie et al., 1998). Results of immunological responses regarding FS+AG sheep seemed to suggest a negative synergistic effect of n-3 PUFA from flaxseed, mainly C18:3, and from *Ascophyllum nodosum,* mainly EPA, when administrated in combination, in influencing immune activation in sheep under heat stress; a reduction of both Th1 responses, by decreasing IFN-γ production, and of Th2 responses, by decreasing anti-OVA IgG and IL-13 productions, was indeed observed.

Sheep can synthesize heat shock proteins HSP 70 and HSP 90 in vitro; however, differences in the synthesis of HSP in different livestock species has been demonstrated (Guerriero and Raynes, 1990). On the cell surface of monocyte-derived dentritic cells, which contain high concentrations of pro-inflammatory cytokines, very high concentrations of inducible-HSP 70 in

hyperthermic stress was found (Oosterveld and Rasker, 1994). Heat stress and IL-13 can amplify the production of inducible HSP 70 (Martin et al., 2009). As a consequence, the rise of IL-13 in AG ewes at 15 d of the experiment can justify the rise of in vivo HSP 70 production in AG ewes at 30 d of the experiment. Analogously, the reduction of IL-13 in FS ewes measured at 15 d of the experiment can be claimed to explain, at least partially, the reduction in the concentrations of plasma HSP 70 in FS ewes measured at 30 d of the experiment. HSP 70 can induce the production of IL-10 (Wendling et al., 2000), suggesting a possible role for HSP 70 in increasing plasma concentrations of IL-10 in FS ewes at 15 d of the experiment. Plasma concentrations of HSP 90 in FS ewes can be explained by the increased IL-10 concentrations measured in FS ewes at 15 d of the experiment. IL-10 can up-regulate gene expression of HSP 90 in PBMC and in a human epatoma cell line (Hep G2), activating the HSP 90β gene promoter, and increasing HSP 90 concentrations (Ripley et al., 1999). Nevertheless, given the greater cortisol secretion observed in FS group compared with other groups, a role of cortisol on HSP 90 plasma concentrations cannot be excluded.

2.6 CONCLUSIONS

Findings from the present experiment confirmed a role for flaxseed supplementation in helping sheep to cope with heat stress. PUFA from flaxseed elicited HPA axis activation by enhancing cortisol secretion, probably to meet increased energy demand for thermoregulation. In addition, the supplementation of flaxseed in the diet contributed to sustain Th1 responses, in terms of in vivo cell-mediated immune response to PHA, by a complex cross-talk between IL-10, IL-12, and IFN-γ production. In flaxseed supplemented sheep, the reduced concentrations of IL-13 were connected to the reduction of HSP 70, the increased concentrations of IL-10 to the rise of HSP 90.

The supplementation of sheep with *Ascophyllum nodosum* did not lead to an enforcement of cell-mediated and humoral responses or an alteration in HPA axis activation, though it resulted in increased concentrations of HSP 70.

The combined supplementation of flaxseed and *Ascophyllum nodosum* to sheep under heat stress appeared to influence immune responses with a reduction of both Th1 responses, by decreasing IFN-γ production, and Th2 responses, by decreasing anti-OVA IgG and IL-13 productions, thus suggesting a synergistic negative effect of n-3 PUFA from flaxseed, mainly C18:3, and from *Ascophyllum nodosum,* mainly EPA.

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THIRD TRIAL

Inflammatory responses of sheep peripheral blood mononuclear cells as affected by PUFA supplementation in the diet under high ambient temperature

3.1 INTRODUCTION

Stress can affect immune system by inducing activation of inflammatory processes. When the induction of inflammatory response is not connected with the presence of pathogens it is termed “sterile inflammation” (Rock et al., 2010; Fleshner, 2013). Inflammation in response to non-pathogenic challenges is activated by the release of tissue alarm signals named damage associated molecular patterns (DAMPS), which control the gene expression activation of different cytokines, cell adhesion molecules, and immunological components to trigger inflammation. Cox et al. (2014) in rats subjected to tail shock demonstrated that glucocorticoids secretion is responsible for IL-1β stress-induced increase in rat subcutaneous adipose tissues. In sheep both physiological stress connected to twin gestation and lambing, and psychological stress caused by isolation from conspecifics, can increase in vivo plasma secretions of pro-inflammatory cytokines, such as IL-6 and IL-1β (Caroprese et al., 2006 and 2010). The balance between pro-inflammatory and anti-inflammatory cytokines seems a mechanism to balance the ongoing pro-inflammatory mediators during an immunological challenge and to avoid tissue damage resulting from excessive inflammation. IL-10 is a well-known anti-inflammatory cytokine whose main role is to reduce the production of inflammatory mediators after immunological challenges (Murray, 2006).

Farmed animals are subjected to different types of stressors, i.e. those caused by management procedures or environmental conditions. During summer season in the Mediterranean basin dairy animals are exposed to climatic conditions which often result in a depression of immune system (Lacetera et al., 2005). In dairy sheep and cows under heat stress the administration of a whole flaxseed

supplementation, rich in α-linolenic acid (C18:3n3, ALA), results in an enhancement of humoral and cell-mediated responses, and in an alteration of IL-10 secretion in vivo (Caroprese et al., 2012, and 2009).

Recently, there is a grown interest on the effects of dietary fats on immune cell functions (Calder et al., 1996 a, b, 1997, and 1998). Feeding fish oil to rats results in a decrease of tyrosine phosphorylation state of phospholipase C (PLC)-γ1 in spleen lymphocytes, thus exerting a reduction of lymphocyte activity (Sanderson and Calder, 1998).

To the best of our knowledge no studies evaluated the effects of supplementation of macroalgae, such as *Aschophyllum nodosum*, rich in eicosapentaenoic acid (C20:5n3, EPA), to dairy sheep during summer, on the activation of their inflammatory responses. The knowledge of the effects of high ambient temperatures on activation of inflammatory response and its alteration after supplementation of polyunsaturated fatty acids from different sources in the diet could be crucial to understand sheep immune reactivity during summer season in order to reduce susceptibility to infectious diseases.

We hypothesized that the supplementation of PUFA from whole flaxseed or from the macroalgae *Aschophyllum nodosum* to dairy sheep during summer season might influence the ex vivo activation of inflammatory response, in terms of pro-inflammatory and anti-inflammatory cytokine secretions and lymphocyte proliferation. This study, therefore, was undertaken to evaluate the effects of PUFA supplementation, administrated as whole flaxseed, *Aschophyllum nodosum,* and a combination of flaxseed and *Aschophyllum nodosum,* in the diet of dairy sheep under high ambient temperatures on ex vivo lymphocyte proliferation and inflammatory responses.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Experimental Design

The experiment was conducted during the summer (June-July) of 2012 at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE). Sixteen late-lactation Comisana ewes (d 202.1±5.3 of lactation, mean ±SD) were divided into four groups of four each, balanced for milk yield, body weight, and BCS. Each animal received 1.8 kg/ewe/d of oat hay in two meals a day. Control group also received 1 kg/ewe/d of pelleted concentrate (Mangimificio Molino Gallo, Potenza, Italy), whereas ewes in the experimental groups were supplemented with whole flaxseed (Lin Tech, Tecnozoo srl, Torreselle di Piombino Dese, Italy) or *Ascophyllum nodosum* (Tasco®, Acadian Seaplants, Canada), or their combination. Namely, ewes in the FS group received 750 g/ewe/d of pelleted concentrate, and 250 g/ewe/d of whole flaxseed; ewes in the AG group received 1 kg/ewe/d of pelleted concentrate in which was incorporated 5% *Ascophyllum nodosum*; ewes in the FS+AG group were supplemented with both flaxseed (250 g/d) and pelleted concentrate incorporating 5% *Ascophyllum nodosum.* Water was available ad libitum for all groups from automatic drinking troughs at any time of day.

During the trial, ambient temperature and relative humidity in indoor and outdoor areas were monitored with thermo-hygrographs (LSI, I-20090 Settala Premenugo-Milano, Italy) placed at 1.5 m from the floor. Average of temperature-humidity index (THI) were calculated using the Kelly and Bond's (1971) formula.

All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU (2010) on the protection of animals used for

experimental and other scientific purposes. Ewes were healthy and their conditions were carefully examined by veterinarians throughout the trial to exclude the presence of signs of diseases.

The chemical composition of diets was carried out by standard procedures (AOAC, 1990). The control diet contained 12.53% crude protein, 1.93% ether extract, 53.36 %NDF, and 1.30 Mcal/kg NE_L (NRC, 2001); FS diet contained 12.90 % crude protein, 5.02 % ether extract, 54.58% NDF, and 1.29 Mcal/kg NE_L, AG diet contained 12.84% crude protein, 2.04% ether extract, 53.32% NDF, and 1.31 Mcal/kg NE_L; AG+FS diet contained 13.20% crude protein, 5.12% ether extract, 54.55% NDF, and 1.30 Mcal/kg NE_L (calculated on DM basis). The fatty acids composition of whole flaxseed and of *Ascophyllum nodosum* is reported in Table 3.1.

Fatty acids, g/100g of total fatty acids	**Flaxseed**	***Ascophyllum nodosum***
C14:0	0.06	10.63
C16:0	5.53	12.80
C16:1cis	0.08	1.27
C18:0	3.56	0.82
C18:1cis-9	16.29	37.03
C18:2cis-9cis-12	16.75	9.33
C18:3n3	53.21	5.82
C20:5n3	0.01	5.03
C22:5n3	0.01	0.02

Table 3.1 *Fatty acid composition of diet flaxseed and Ascophyllum nodosum supplemented in the sheep diet.*

The determination of methyl esters of the diet ingredients was carried out according to O'Fallon et al. (2007). Briefly 1 g of the samples was pipetted into a screw-cap (16 x25 mm) reaction tube. Methanol (0.5 mg) of C13:0/ml, 0.7 ml of KOH and 5.3 ml of MeOH was added into each tubes, during incubation at 55°C for 1h and 30', the tubes were inverted to mix for 5 s each

20 min. After cooling in a cold water bath, 3 ml of hexane were added into each tube and vortex for 5 min. The tubes were centrifugated at room temperature for 5 min at 500g; supernatant (1 ml) was taken from each tube and transferred into vials and stored at -20°C to followed gas-chromatography (GC) analysis. Fatty acid profiles were quantified using Agilent Technologies, 6890N GC equipped with a flame ionization detector (FID). Helium was the carrier gas and the gas flow rate was 175 kPa. The oven temperature (Eulitz et al., 1999) was initially held at 70°C for 4 min, and then programmed to 175°C at 13°C/min increase and held isothermally for 45 min. The column used was a capillary column (HP88; 100 m x 0,24 mm i.d., 0,20 µm film thickness, Agilent Technologies Santa Clara, USA). Concentrations of fatty acid methyl esters (FAME)s were analysed utilizing a calibration curve with a mixture of standards of 50 fatty acid (GLC Reference standard 674, Nu-Check Prep, Inc. Elysian MN 56028, USA). Flaxseed fatty acid composition, as reported in Table 1, was mainly characterized by 53.21g/100g of total fatty acids (FA) of ALA, while *Ascophyllum nodosum* was characterized by 37.03 g/100g FA of C18:1 cis9 and by 5.03 g/100g of FA of EPA.

3.2.2 Isolation of PBMC

At 15 d of the experiment blood samples (15 ml) were collected in vacuum tubes from the jugular vein of sheep. Isolation of PBMC was performed by density gradient centrifugation according to Wattegedera et al. (2004). Blood samples collected into heparinised vacutainer tubes were diluted 1:5 in phosphate buffer saline (PBS) (pH 7.4, at 4°C), and centrifuged at 670 g, 4°C for 20 min. The buffy coat was recovered, layered over a Ficoll gradient (1.077g/ml), and centrifuged at 1130 g, 15°C for 30 min. The mononuclear cell band was collected

and washed three times by centrifugation at 240 g, 4°C for 10 min in Hank's buffered saline solution (HBSS) supplemented with 10U/ml heparin (Sigma Aldrich, Milan, Italy), 50µg/ml gentamicin (Euroclone, Milan, Italy) and 2% FBS (Biochrom, Berlin, Germany). PBMC were finally resuspended at a final concentration of $2x10^6$ cells/ml in Iscove's Modified Dulbecco's medium (IMDM) (Euroclone) supplemented with 10% FBS and 50µg/ml gentamicin.

3.2.3 PBMC for Lymphocyte Stimulation Assay and ILs determination

Lymphocyte proliferation assays were performed adding 100 µl of cell suspension into quadruplicate wells of 96 well U-bottom plates. PBMC were activated with 50 µl of PHA (Sigma-Aldrich) at a final concentration of 5 µg/ml (Stimulated Cells, SC) or not activated with mitogen (NSC). The plates were incubated at 37°C and 5% CO_2 in a humidified incubator for 96 h. After 96 h of incubation, cell suspensions were centrifuged at 300 g, 4°C for 10 min, and cell-free supernatants from each well were collected and stored at -20 °C until ELISA to measure cytokine production. After cell-free supernatant collection, cells were incubated with Bromodeoxyuridine (BrDU) (Exalpha Biologicals, Inc., Shirley, USA), in order to test lymphocyte proliferation. After 18h of incubation, BrDU incorporation during DNA synthesis was measured by determining optical density with a titer-ELISA spectrophotometer (Power Wave XS, Biotek, UK) at 450 nm.

3.2.4 Determination of ILs in culture supernatant by ELISA-test

The levels of IL-6 in cell-free supernatants were determined by capture ELISA performed on 96-well microtiter plates, according to Caroprese et al. (2006) with some modifications. Mouse mAb specific for ovine IL-6 (Serotec Ltd, Kidlington, UK) (100 µl, at final concentration of 2 µg/ml) dissolved in PBS (pH 7.2) were used to coat wells and incubated overnight at 4 °C. After washing with PBS (pH

7.2) and 0.05% Tween 20 (PBST) the plates were incubated with 200µl/well of 10% reconstituted commercial bovine skimmed milk at 37°C for 1 h to block non-specific binding. Plates were then washed 4 times with PBST and the supernatants (100 µl per well) were added and incubated for 1 h. PBST provided negative control wells. Plates were then washed 4 times with PBST. Rabbit polyclonal anti-ovine IL-6 Ab (Serotec Ltd, UK), were used as detecting antibodies (0.005 mg/ml diluted in 1% BSA in PBST) to determine captured IL-6 and incubated at 37°C for 1 h. The presence of bindings for IL-6 was detected using sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP, Sigma Aldrich, Italy). Optical density was measured at a wavelength of 450 nm. Culture supernatants were read against a standard curve obtained using scalar dilution of recombinant ovine IL-6 (Kingfisher Biotech Inc., St Paul, USA). Data were expressed as ng/ml of IL-6.

IFN-γ and IL-10 in plasma were assayed by an ELISA test as briefly described: 96-well plates (Sterilin, Newport, UK), were coated overnight at 4°C with 100 µl of anti-bovine IFN-γ and IL-10 Ab (Serotec) in PBS (2µg/ml). After washing with PBST, the antibody was blocked with PBS/1%BSA for 1h. IFN-γ and IL-10 standard and samples were added to plate for 1h at room temperature after washing 4 times. Biotinylated secondary anti-bovine IFN-γ and IL-10 Ab (Serotec) in PBS (0.5 µg/ml) were added for 1 h followed by 100 µl of streptavidin-HRP (2 µg/ml in PBS, Serotec). After washing, 100 µl of TMB substrate solution were added to each well for 30 min; to stop the reaction 50 µl of H_2SO_4 (4M) were added to each well. The level of IFN-γ and IL-10 were measured colorimetrically at 450 nm (by subtracting absorbance at 540nm) and quantified by interpolation from standard curve. Data were expressed as pg/ml of IFN-γ and as ng/ml of IL-10.

3.2.5 Statistical Analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro e Wilk, 1965). Then, data were processed by ANOVA using the GLM procedure of SAS (1999). The model utilized was:

$$y_{ijkl} = \mu + \alpha_i + \beta_{ij} + \gamma_k + (\alpha\gamma)_{ik} + \varepsilon_{ijkl}$$

Where μ=the overall mean, α=diet, β=animal effect within feeding treatment, γ=stimulation, $\alpha\gamma$=diet x stimulation, and ε=error. When significant effects were found (at $P<0.05$), the Student *t*-test was used to locate significant differences between means.

3.3 RESULTS

3.3.1 Proliferative response to PHA

Dietary treatment had a significant effect ($P<0.05$) on proliferation of PBMC; on average proliferation of PBMC was lower in mononuclear cells collected by sheep fed *Ascophyllum nodosum* (AG) than in sheep fed a combination of flaxseed and *Ascophyllum nodosum* diet (FS+AG) and C diet (Fig.3.1).

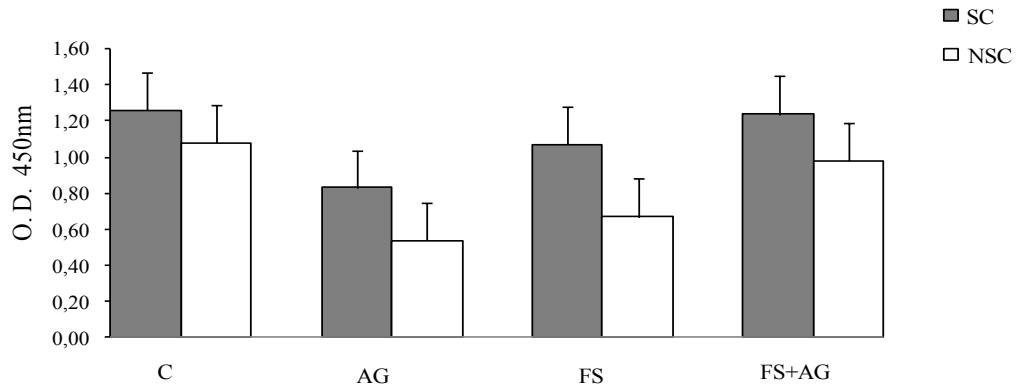

Fig. 3.1 *Mean proliferation of sheep PBMC following in vitro stimulation (Least Squares means ± SEM). PBMC were isolated from sheep fed Control diet (C) or supplemented with Ascophyllum nodosum (AG), flaxseed (FS) or a combination of Ascophyllum nodosum and flaxseed (AG+FS).*

PBMC from FS diet displayed an intermediate proliferation. An effect of stimulation was found ($P<0.05$): proliferation of PBMC in presence of PHA increased compared with proliferation of PBMC with no stimulation.

3.3.2 Cytokines production by PBMC

A significant interaction between the diet and the stimulation was found ($P<0.05$, Figure 3.2); in C, AG, FS supernatants from PBMC stimulated with PHA the levels of IL-10 were lower than in C, AG, FS supernatants from PBMC without stimulation. In supernatants from PBMC stimulated with PHA and

collected from FS+AG sheep, the levels of IL-10 were lower than in supernatants from PBMC collected from C sheep stimulated with PHA.

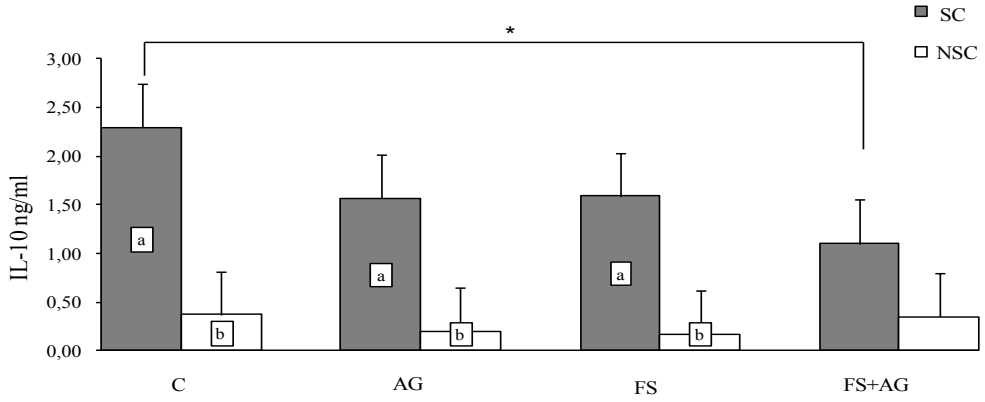

Figure 3.2 *IL-10 production (Least Squares means ± SEM), in PHA stimulated (SC) or not (NSC) sheep peripheral blood mononuclear cells (PBMC). PBMC were isolated from sheep fed Control diet (C) or supplemented with Ascophyllum nodosum (AG), flaxseed (FS) or a combination of Ascophyllum nodosum and flaxseed (AG+FS).[a, b] Values with different letters within diet treatment differ (P < 0.05). *Shows a significant difference between diet treatments (P<0.05).*

Diet did not affect IFN-γ production, whereas a significant effect of stimulation with PHA (P<0.001) emerged, and on average PBMC stimulated with PHA secreted a higher concentration of IFN-γ than unstimulated PBMC (Figure 3.3).

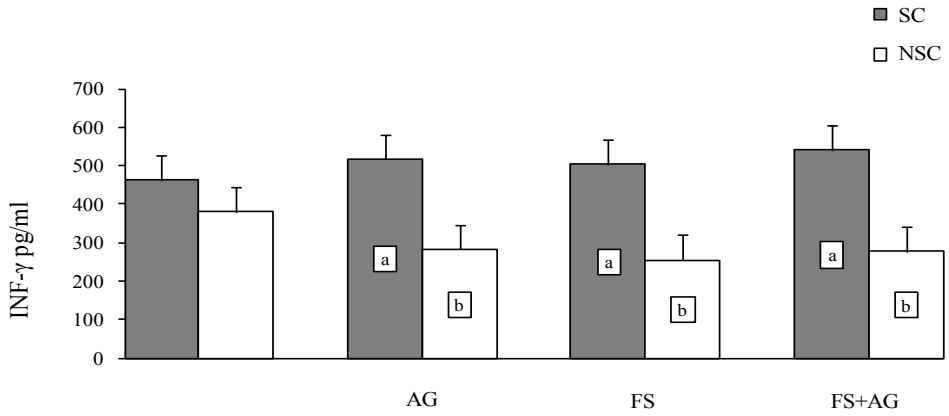

Figure 3.3 *IFN-γ production (Least Squares means ± SEM), in PHA stimulated (SC) or not (NSC) sheep peripheral blood mononuclear cells (PBMC). PBMC were isolated from sheep fed Control diet (C) or supplemented with Ascophyllum nodosum (AG), flaxseed (FS) or a combination of Ascophyllum nodosum and flaxseed (AG+FS).[a, b] Values with different letters within diet treatment differ (P < 0.05).*

Ex vivo production of IL-6 was affected by diet ($P<0.05$): data showed that on average the production of IL-6 was higher in supernatants from PBMC collected from FS+AG sheep than in supernatants from PBMC collected from AG and C sheep. Moreover, the production of IL-6 was higher when PBMC were stimulated with PHA ($P<0.01$).

The interaction of the diet x stimulation with PHA ($P<0.05$) affected production of IL-6, that in supernatants from non stimulated PBMC from FS and FS+AG sheep increased compared with IL-6 production in supernatants from PHA stimulated PBMC collected from FS and FS+AG sheep (Figure 3.4). In addition, FS+AG non stimulated cells produced higher levels of IL-6 than both C and AG non stimulated cells ($P<0.01$ and $P<0.05$, respectively).

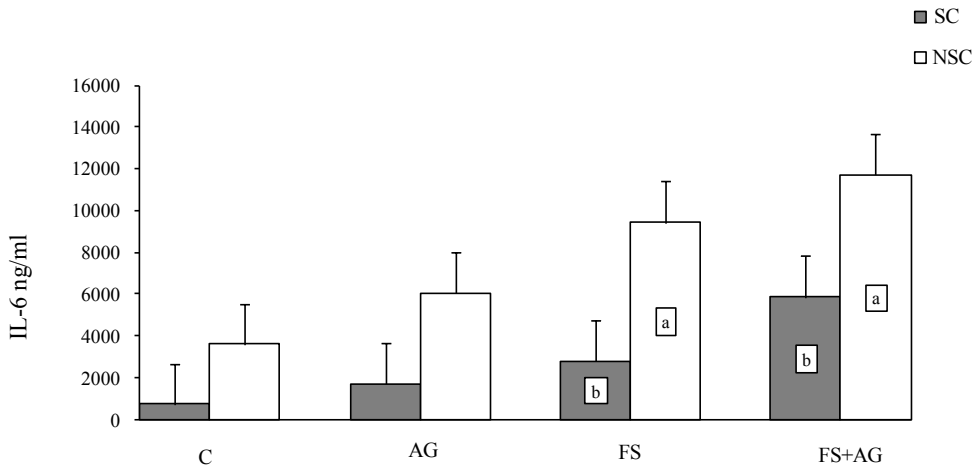

Figure 3.4 *IL-6 production (Least Squares means ± SEM), in PHA stimulated (SC) or not (NSC) sheep peripheral blood mononuclear cells (PBMC). PBMC were isolated from sheep fed Control diet (C) or supplemented with Ascophyllum nodosum (AG), flaxseed (FS) or a combination of Ascophyllum nodosum and flaxseed (AG+FS).[a, b] Values with different letters within diet treatment differ (P < 0.05). ***Show a significant difference between diet treatments (P<0.05).*

3.4 DISCUSSION

The main objective of this research was to analyze the effects of high ambient temperatures on activation of inflammatory response and its alterations as a result of supplementation of PUFA from different sources in the diet. Our hypothesis was that PUFA from different source in the diet of dairy sheep during summer season might influence the ex vivo activation of inflammatory response.

Ex vivo cell culture models are widely used in inflammation research: nutritional adjustment of the diet could have a direct effect on immune functions, and supplements provided could have physiological effects that influence immune activity (Caroprese et al., 2009 and 2012).

PHA is a one of most potent T cell mitogens and it stimulates T cells in a manner that promoted their long-term growth (Geppert, 1998). PHA stimulates T cells to proliferate with the increase of DNA synthetic (S) phase of the cell cycle (Schwarm et al., 2013). It is worth noting that interaction of T cells with mitogens initiates a cascade of gene expressions with the transcription of IL-2 and IFN-γ mRNAs that induce the T-cells to proliferation and differentiation into memory cells or effector cells (Tsai et al., 2008). Our results confirmed that PHA stimulation enhanced proliferation of sheep PBMC, regardless of the diet treatment.

Extracts from the microalgae *Dunaliella tertiolecta* showed a suppressive effects on sheep PBMC proliferation in vitro (Caroprese et al., 2012). The decreased proliferation observed in PBMC collected from sheep supplemented with *Ascophyllum nodosum* could be ascribed to the EPA content of *Ascophyllum nodosum*. Previous studies reported the effects of EPA reduction of proliferation

in culture (Calder, 2002). It was been demonstrated that rats fed fish oil showed altered functioning of T lymphocytes, by reducing production of cytokines and expression of IL-2 receptor on lymphocytes (Sanderson et al., 1998). A previous study on the influence of fish oil supplementation on cattle demonstrated a stimulation of lymphocyte blastogenic response, suggested that n-3 PUFA action could be related to the alteration of absorption of the other nutrients relevant to immune status, or to a direct effect of n-3 PUFA on the immune system (Wistuba et al., 2005). Therefore, the effects of PUFA in the animals diet on their immunological response is ascribed to species involved in the experiment, stress acting, and to types and amount of fatty acid included in the diet.

According to a previous study on cow PBMC, in which no effects of ALA were found on lymphocyte proliferation in vitro (Thanasak et al., 2005), ex vivo stimulation of PBMC proliferation was not affected by flaxseed supplementation in sheep diet. Sheep exposed to solar radiation and subjected to heat stress, showed a decrease of cell-mediated immune response (Sevi et al., 2001). The supplementation of sheep under heat stress with whole flaxseed, however, did not improve cell-mediated responses. On the contrary, during postpartum in cows fed flaxseed, a reduction of the proliferative response of PBMC was found (Lessard et. al., 2003). In vitro and in animal feeding studies it was found that ALA, in sufficient amounts, can partially inhibit lymphocyte proliferation (Calder 2002).

When sheep were fed the combination of PUFA from *Ascophyllum nodosum* and flaxseed, their lymphocytes proliferation increased compared with proliferation of PBMC from sheep fed *Ascophyllum nodosum*. This result could be caused by the simultaneous action exerted on the immune system by PUFA from algae and flaxseed, namely EPA and ALA, supplemented in the diet of sheep. It

has been stated in a number of animal and human studies that n-3 fatty acids have evident effects on immunomodulatory activities, and among them, EPA from fish oil is more biologically potent than ALA (Calder et al., 2002).

Interferon-γ (IFN-γ) has pleiotropic effects by coordinating the link between immunological challenges recognized by innate immune cells and the activation of acquired immunity mainly in terms of cell-mediated immunity (Schroder et al., 2004). IFN-γ is in vivo produced during the response to Gram-negative bacteria and amplifies cellular responses against bacteria, as demonstrated in a study on IFN-γ receptor deficient mice (Car et al., 1994). IFN-γ treatment of human monocytes causes an augmentation of NF-κB activation after Gram-negative bacteria challenge (de Wit et al., 1996). IFN-γ gene is a pivot in the growth of T lymphocytes induced by antigens (Tsai et al., 2008) and is an up-regulator of the expression of chemokines, with the TNF-α and IL-1β combined action (Bohem et al., 1997). Data from our experiment showed that the dietary supplementation of PUFA from flaxseed and *Ascophyllum nodosum* did not exert an effect on the production of IFN-γ. It has been reported that in mitogen-activated ovine PBMC there was no correlation between proliferation and IFN-γ production in response to mitogen (Wattegedera et al., 2004).

In sheep the regulation of proliferation and INF-γ production of ovine PBMC are profoundly linked to IL-10 production (Wattegedera et al., 2004). IL-10 is a cytokine produced by lymphocytes, B cells, eosinophils, mast cells, and monocytes (Asadullahet et al., 2003) and is a key cytokine for controlling inflammatory responses and sustaining immune homeostasis (Hu et al., 2014). Calder et al. (2002) reported that feeding fish oil to laboratory rodents caused a reduced production of inflammatory mediators and the expression of adhesion

molecules exerting anti-inflammatory effect. In cows fed flaxseed under heat stress, Caroprese et al. (2009) showed a decrease of IL-10 production in vivo, which supported immune responses under heat temperature with activation of cell-mediated immunity. Our present results, accordingly, showed a decrease of IL-10 production by PBMC from sheep fed FS+AG, and activated with PHA compared to IL-10 production in PBMC from C sheep. The absence of increased IL-10 production by PBMC from FS sheep compared to PBMC from C sheep could be attributed to the in vivo increase in cortisol secretion observed in FS sheep (Caroprese et al., unpublished). It is well known that glucocorticoids have a direct effect on IL-10 production because they are able to up-regulate the IL-10 secretion by T cells (Elenkov and Chrousos, 1999). Data on the decrease of the production of IL-10 in sheep fed with PUFA from flaxseed and *Ascophyllum nodosum*, demonstrated the possible synergistic positive effect of the EPA, from *Ascophyllum nodosum,* and ALA, from flaxseed, to generate ex vivo an pro-inflammatory profile.

Stress activates HPA axis to synthesize cortisol, that controls the actions and the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α (Black, 2002; Caroprese et al., 2006 a). IL-6 release and activity is strongly connected with inflammatory responses activation, and is responsible for the increase of certain acute phase proteins, and of the stimulation of B cells and cytotoxic T cells (McWaters at al., 2000; Murata et al., 2004). It has been demonstrated in previous studies both in animals and in humans, that the supplementation with PUFA from plant or fish oil can alter the functions and the production of cytokines and a wide range of immunological mediators (Yaqoob and Calder, 1995; Calder, 2002, Caroprese et al., 2009). In Maric et al. (2014) the

expression of pro-inflammatory cytokines in hypothalamic tissue of rats fed diets rich in saturated fats was upregulated by direct activation of TRL-4 receptors. In our experiment a synergistic reinforcing interaction between PUFA from flaxseed and *Ascophyllum nodosum* was observed, in the increase of IL-6 secretions by PBMC from FS+AG sheep compared to C and AG sheep. The increase of expression of pro-inflammatory cytokines after acute physiological stress could be useful to predict the ongoing of stress, both in animal and human (Black, 2002). Indeed in multiple lambing sheep high levels of IL-6 in vivo were found, as a result of activation of immune system to cope with physiological stress connected to multiple pregnancy (Caroprese et al., 2006).

The interaction between stress and inflammation is a well known biological mechanism which offer an animal engaged in, a survival advantage since an inflammatory response would help deal with any infectious organisms introduced during acting stress (Black, 2002). The increase in IL-6 production by PBMC from FS+AG sheep can be also related to the concomitant reduction of IL-10 secretion by PBMC from FS+AG.

PBMC from sheep under high ambient temperatures and supplemented with PUFA from flaxseed and flaxseed in combination of *Ascophyllum nodosum*, showed a reduction of the IL-6 levels, when activated by PHA. In human PBMC activated by mitogens, the inclusion in the diet of PUFA from flaxseed and fish oil caused a reduction of in vitro secretion of IL-1β, IL-6, and TNF-α (Kelley, 2001). Several studies (Kelley et al., 1993, Caughey et al., 1996) reported that large amounts of ALA intake inhibited in vivo and ex vivo indices of immune response.

PBMC from sheep supplemented with *Ascophyllum nodosum,* rich in EPA, exhibited an anti-inflammatory profile. Two different mechanisms for the n-3 PUFA down regulation of the inflammatory response by the control of inflammatory mediators gene expression has been described (Calder, 2002). One included a direct action on the intracellular signalling pathways, by reducing activation of one or more transcription factors, such as NF-κB, which induces a range of inflammatory genes. Xi et al., (2001) reported that feeding mice with fish oil caused a decrease of the level of the NF-κB in the nucleus of the mytogen stimulated spleen lymphocytes. A second mechanism proposed for the down regulation of inflammatory response by PUFA, and EPA in particular, is the activation of the peroxisome proliferator-activated receptors (PPAR), exerting their anti-inflammatory role.

3.5 CONCLUSION

This study demonstrated that the supplementation of PUFA from *Ascophyllum nodosum* in diet of sheep under high ambient temperatures contributed to the expression of anti-inflammatory profile ex vivo by a reduction of lymphocyte proliferation and of IL-6 production. The combination of the supplementation of PUFA from flaxseed and from *Ascophyllum nodosum*, based on the presence of both ALA and EPA, showed a pro-inflammatory response as demonstrated by the increase in IL-6 and by the decrease in IL-10 productions. Results of the experiment demonstrated that the supplementation with PUFA from different sources in sheep can influence their immunological responses under high ambient temperatures in relation to the composition of fatty acid supplementation

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FOURTH TRIAL

Effects of dietary n-3 from flaxseed on immune suppression of dairy ewes during post partum

4.1 INTRODUCTION

In dairy animals the post partum period is typically characterized by an increase in the exposition to risk of infectious disease due to an impaired immune status both before and immediately after parturition (Singh et al., 2008). In dairy cows the periparturient period is characterized by changes in the metabolic and endocrine profile, that could partially explain the immune suppression during post partum (Lacetera et al., 2005). To contrast invasion of pathogens, after parturition, the immune system reacts producing specific IgG and pro-inflammatory cytokines, such as necrosis tumor-factor(TNF)-α, Interleukin(IL)-6 and IL-8, via TLR system in placental tissue and fetal tissue (Young et., al 2002). Around lambing sheep activated both cell-mediated and humoral responses by the secretion of pro-inflammatory cytokines, such as IL-6 and IL-1β (Caroprese et al., 2006). Furthermore, cytokines can be used as biomarkers for predicting the outcome of intramammary infections, as well as the physiological stress connected to parturition (Bannerman et al., 2009; Caroprese et al., 2006), and in the treatment of intramammary infections caused by pathogens, such as *Escherichia coli* and *Staphylococcus aureus* (Sordillo and Peel, 1992; Bannerman et al., 2009).

Nutritional manipulation of the diet in animal management practices could contribute to decrease the incidence of diseases and mortality and to guarantee welfare of animals (Karcher et al., 2014). Furthermore, during post partum, dietary changes have an effect on immune responses and may influence duration of immune suppression (Lessard et al., 2003). In Calder et al. (2005) it has been demonstrated that α-Linolenic acid (ALA, n-3 series), a polyunsaturated fatty acid, has the ability of influencing inflammatory processes, by promoting both

pro- and anti-inflammatory responses (Fritsche et al., 2008). In human mononuclear cells, n-3 fatty acids have been showed to modulate cytokine gene expression (Caughey et al., 1999); additionally, in Holstein calves the supplementation with n-3 fatty acids from fish oil and flax oil in the diet affected gene expression of cytokines (Karker et al., 2014). Data from an increased intake of n-3 PUFA in the animals'diet, however, are conflicting; in dairy cows, flaxseed supplementation increased anti-OVA IgG concentration and reduced IL-10 secretion (Caroprese et al., 2009). Lessard (2003) reported that dietary n-3 PUFA affected lymphocytes and monocytes/macrophages responses in dairy cows during post partum, without interfering in the production of IgG and Interferon(IFN)-γ. Recently, flaxseed supplementation in the diet of sheep subjected to heat stress was able to modulate in vivo cell-mediated immune response, by a complex cross-talk between IL-10, IL-12, and IFN-γ production (Caroprese et al., 2014).

Few studies have investigated the immune status of ewes during post partum in terms of interleukins production, and in the absence of infections. We hypothesized that supplementing n-3 fatty acids to dairy ewes during post partum would help to overcome immune suppression conditions that normally occur during post partum. Furthermore the aim of the experiment was to characterize the immune profile of ewes during post partum fed flaxseed.

4.2 MATERIAL AND METHODS

4.2.1 Animals and experimental treatments

Twenty parturient Comisana ewes were randomly selected from an intensively managed flock of Segezia research station of the Italian Istituto Sperimentale per la Zootecnia of Segezia – Foggia (latitude: 41° 27' 6'' and

longitude: 15° 33’ 5’’). The ewes involved in the experiment, were balanced for age, body weight and body condition score, and divided in two groups of ten, respectively. All groups were individually fed twice daily and received 1.8 kg/ewe/d of oat hay. Control group (C) received 1 kg/ewe/d of pelleted concentrate (Mangimificio Molino Gallo, Potenza, Italy) individually; flaxseed group was fed with a supplementation with whole flaxseed (Lin Tech, Tecnozoo srl, Torreselle di Piombino Dese, Italy), receiving 750 g/ewe/d of pelleted concentrate, and 250 g/ewe/d of whole flaxseed, the ewes were individually fed twice daily. Diet both in C and FS ewes was administrated 1wk before lambing. The chemical composition of diets was carried out by standard procedures (AOAC, 1990). Ingredients and chemical composition of diets are reported in Table 4.1.

	Diet	
Item	Control	FS
Ingredients, % of DM		
Oat hay	64.50	64.37
Concentrate[1]	35.50	26.57
Whole flaxseed[2]	0	9.06
Chemical composition		
DM%	93.48	93.67
Ether extract, % of DM	1.93	5.02
CP, % of DM	12.53	12.89
ADF, % of DM	27.75	28.48
NDF, % of DM	53.36	54.58
ADL, % of DM	3.44	3.83
NE_L, Mcal/kg[3]	1.30	1.29

[1]Contained: Corn Meal, Soybean Meal, Wheat Germ Meal, Wheat Meal, Roasted Soybean Seeds, Barley Meal, Wheat Fine Bran, Corn Cracked, Sugarcane Molasses, Partially Debarked Sunflower meal, Bentonite, Dried Pulp, Calcium Carbonate, Sodium Bicarbonate, Sodium Chloride, Magnesium Oxide, 8.3 IU/g vitamin A, 8.2 IU/g vitamin D3, 99mg/kg vitamin E, 0.07mg/kg vitamin B1, 255mg/kg vitamin PP, 488mg/kg Cl, 293mg/kg Fe, 1.26mg/kg Co, 1mg/kg Cu, 0.4% Na.
[2] Lin Tech (Tecnozoo srl, Torrreselle di Piombino Dese, Italy).
[3]Calculated according to NRC (2001)

Table 4.1 *Ingredients and chemical composition of the diets (DM basis).*

Ewes were healthy and their condition was judged as good at the commencement of the trial. Before starting and throughout the trial the ewes were carefully examined by veterinarians to exclude the presence of signs of clinical mastitis (pain, gland swelling, fever) and a small quantity of milk was checked visually for signs of mastitis. No cases of mastitis were detected during the study period.

To determine the ewe humoral response during post partum, 1 and 7 d after lambing the animals received an i.m. of the antigen ovalbumin (OVA, Sigma Aldrich, Milan, Italy) to which the animals had not been previously exposed. At d 0 of the experiment the ewes were subjected to subcutaneous injection of 6 mg of ovalbumin (dissolved in 1 mL of sterile saline solution and 1 mL of incomplete Freund's adjuvant (Sigma Aldrich, Milan, Italy). A second injection without adjuvant was repeated 7 d later.

4.2.2 Sampling and analysis of blood

Peripheral blood samples from each ewes were collected from the jugular vein in heparinized vacuum tubes (Becton Dickinson, Plymouth, United Kingdom) immediately before the first OVA (0 d) and then at 7, 14, 21, 28, and 42 d from the day of parturition. Blood samples were centrifuged (1500 g for 20 min) to obtain plasma, then stored frozen at -20°C until time of assay. Plasma samples were used to perform ELISAs to evaluate the anti-OVA IgG titers; at 0, 14, and 42d from parturition IL-1β, IL-6, IL-10 and TNF-α concentrations were measured in the plasma samples.

4.2.3 ELISA for anti-OVA IgG detection in plasma

To evaluate the humoral response to ovalbumin (OVA, chicken egg albumin) and to measure anti-OVA IgG concentrations at d 0 of the experiment the ewes were subjected to subcutaneous injection of 6 mg of ovalbumin (OVA, Sigma

Aldrich, Milan, Italy) dissolved in 1 mL of sterile saline solution and 1 mL of incomplete Freund's adjuvant (Sigma Aldrich, Milan, Italy). A second injection without adjuvant was repeated 7 d later. Antibody titers were determined in blood samples collected in heparinized vacuum tubes (Becton Dickinson, Plymouth, United Kingdom) immediately before the first antigen injection (0d) and then at 7, 14, 21, 28, and 42 d of the study period. An ELISA (Enzyme Linked Immunoassorbent Assays) was performed in 96-well U-bottomed microtiter plates. Wells were coated with 100 µL of antigen (10 mg of OVA/mL of phosphate buffered saline-PBS) at 4°C for 12 h, washed and incubated with 1% skimmed milk at 37°C for 1 h to reduce nonspecific binding, and then washed. The sheep serum (1:5,000 dilution in PBS) was added and incubated at 37°C for 1 h. The extent of antibody binding was detected using a horseradish peroxidase-conjugated donkey anti-sheep IgG (Sigma Aldrich, Milan, Italy) (1:20,000 dilution in PBS). After other washing, 100 µL of substrate (1 mg of 3,3' 5,5'-tetramethylbenzidine (TMB, Sigma Aldrich, Italy), 1 mL of dimethylsulfoxide (DMSO Sigma Aldrich), 9 mL of phosphate-citrate buffer, 2 µL of H_2O_2 were added in each well. The reaction was completed adding 50 µL of H_2SO_4 after 30 min. Optical density was measured at a wavelength of 450 nm with titer-ELISA spectrophotometer (Rosys Anthos 2020).

Anti-Ova IgG evaluation was a quantitative assay: a standard curve (y=(0.0674-1.48)/(1+(x/27.4)^0.763)+1.48), R^2 = 99.97%) was made up by ovine IgG dilutions (A5295, Sigma Aldrich, Milan, Italy); all plasma samples were read against the curve. Data were expressed as mg of anti-OVA IgG/mL. The assay was optimized in our laboratory for concentrations of coating antigen, serum, and antibody.

4.2.4 Capture ELISA for IL-6, IL-1β, IL-10 and TNF-α detection in plasma

The levels of IL-6 and IL-1β in plasma were determined by capture ELISA performed on 96-well microtiter plates, according to Caroprese et al. (2006) with some modifications. All the incubation were at room temperature. Mouse monoclonal antibodies specific for ovine IL-6 and ovine IL-1β (Serotec Ltd, Kidlington, UK) (100 µl, at final concentration of 2 µg/mL) dissolved in PBS (pH 7.2) were added to microtiter plates (Sterilin, UK) to coating wells and incubated overnight at 4 °C. After washing with PBS (pH 7.2) and 0.05% Tween 20 (PBST) the plates were incubated with 200µl/well of Blocking solution (3% of BSA diluited in PBS) for 1 h to block non-specific binding. Plates were then washed 4 times with PBST and the plasma (100 µl per well) were added and incubated for 1 h. PBST provided negative control wells. Plates were then washed 4 times with PBST. Rabbit polyclonal antibody anti-ovine IL-6 and anti-ovine IL-1β (Serotec Ltd, UK), were used as detecting antibodies (0.5 µg/mL diluted in 1% BSA in PBST) to determine captured IL-6 and incubated at 37°C for 1 h. The presence of bindings for IL-6 was detected using sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP, Sigma Aldrich, Italy). Optical density was measured at a wavelength of 450 nm. Samples were read against a standard curve obtained using scalar dilution of recombinant ovine IL-6, and IL-1β (Kingfisher Biotech Inc., St Paul, USA). Data were expressed as ng/mL of IL-6 and as ng/mL of IL-1β.

The determination of IL-10 and TNF-α in plasma were assayed by an ELISA test according to Kwong et al (2002) and Hope et al (2003) with some modifications: 96-well plates (Sterilin, Newport, UK), were coated overnight at 4°C with 100 µl of anti-Bovine IL-10 (Serotec, 2µg/mL in PBS) and anti-Bovine

TNF-α (Serotec, 1/500 in Carbonate Buffer, pH 9.6). All the incubations were at room temperature. After washing with PBS and Tween 20 at 0.05% (PBST), the antibody was blocked with PBST/1%BSA for 1h. IL-10 and TNF-α standard and samples were added to plate for 1h at room temperature and then washed for 4 times. The biotinylated secondary anti-Bovine IL-10 antibodies (Serotec, 0.5µg/mL in PBS) and the biotinylated secondary anti-Bovine TNF-α antibodies (Serotec 1/1000 in PBS) were incubated and then 100 µl of streptavidin-HRP (2µg/mL for IL-10 and, 1/500 in PBS for TNF-α, Serotec) were added. After washing, 100 µl of TMB substrate solution were added to each well for 30 minutes, to stop the reaction 50 µl of H_2SO_4 (4M) were added to each well. The level of IL-10 were measured colorimetrically at 450 nm (to wich were substracted the absorbance at 540nm) and quantified by interpolation from standard curve. The ELISA for IL-10 and TNF-α detections were standardized using biologically active recombinant bovine IL-10 purchased form Kinfisher Biotech and recombinant bovine TNF-α purchased from Serotec. Data were expressed as ng/mL of IL-10 and pg of TNF-α/mL.

The assays were optimized in our laboratory for concentrations of monoclonal coating antibody, plasma, polyclonal detecting antibody and secondary conjugate antibody.

All procedures were conducted according to the guidelines of the Eu Directive 2010/63/EU (2010) on the protection of animals used for experimental and other scientific purposes.

4.2.5 Statistical analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro e Wilk, 1965). All the data were analysed using ANOVA for mixed models using the MIXED procedure of SAS (1999), having the animal as a random factor nested in the treatment. Data collected during the experiment were analysed with the diet, the time of sampling and their interactions as fixed effects. Data were covariated on initial values. When significant effects were found (at $P<0.05$, unless otherwise noted), the LSD test was used to locate significant differences between means.

4.3 RESULTS

4.3.1 Humoral immune responses

The anti-OVA IgG titers decreased starting from 28 days post partum (P<0.05). An interaction of treatment x time of sampling ($P < 0.05$) was observed for plasma antibody titers to OVA (Figure 4.1). At 14 d post partum (P<0.05) the anti OVA IgG titer was lower in the ewes supplemented with flaxseed than in control ewes. In the control ewes at 14 d post partum the anti OVA IgG titer showed higher concentrations than those monitored at 21 d (P<0.01), and at 28 and 42 d (P<0.05). The flaxseed supplementation resulted in anti OVA IgG titers at 0d higher than 7d, 28d and 42d post partum in addition, at 21d the anti OVA IgG titers were higher than the anti OVA IgG titers registered at 28 d and 42d post partum.

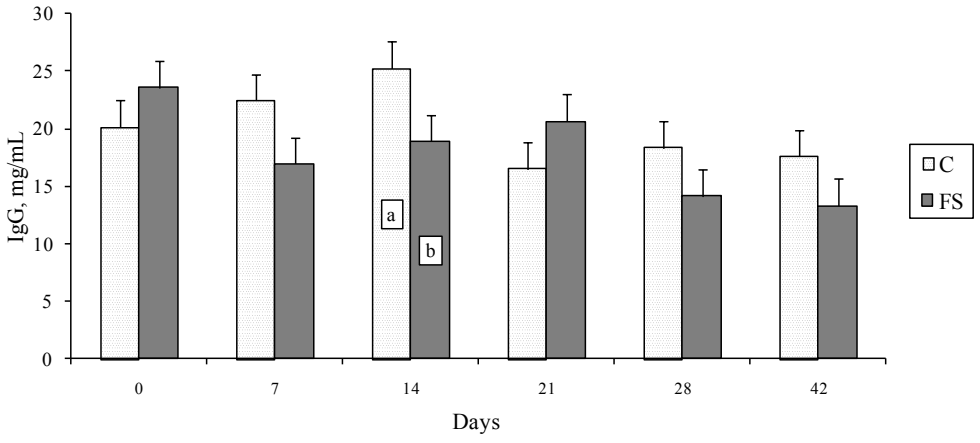

Figure 4.1 *Antibody titers to OVA (LSmeans ± SEM) detected in plasma samples of sheep fed control diet (C), or supplemented with flaxseed (FS) at d 0, 7, 14, 21, 28 and 42 after parturition.* [a, b] *Values with different superscripts differ between treatments within a sampling day (P < 0.05).*

4.3.2 Cytokines detection in plasma

IL-6 secretions (Figure 4.2) decreased from the beginning to the end of the experimental period (P<0.001), and in C ewes the IL-6 decrease from 0 d post parturition to 14d post partum (P<0.001), and from 14d to 42 d post partum (P< 0.05), whereas in FS ewes IL-6 secretions remained high until 14 d post partum and then decreased from 14 d to 42 d post partum (P<0.001). At the beginning of the experiment IL-6 secretions were higher in C ewes than in FS ewes (P<0.001). Subsequently, both at 14 d and at 42 d higher secretions of IL-6 were observed in FS than in C ewes.

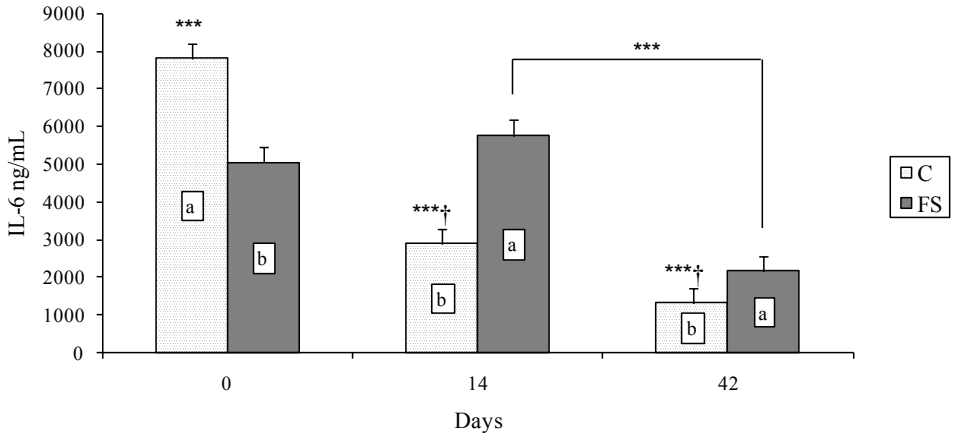

Figure 4.2 *Least Squares means ± SEM of IL-6 production at 0, 14, and 42 d of the experiment in plasma of sheep fed control diet (C), or supplemented with flaxseed (FS).* [a, b] *Shows a significant difference between feeding treatments within a sampling day (P < 0.05),* *** *shows a significant difference between different sampling days within feeding treatment (P < 0.001),* † *shows a significant difference between different sampling days within feeding treatment (P < 0.05).*

IL-10 secretion (Figure 4.3) was lower at 42 d post partum the experiment than at 0 d and 14 d post partum (P<0.001). At the beginning of the experiment IL-10 secretions were higher in the C ewes than in FS ewes, whereas at 14d IL-10 secretions were higher in the FS ewes than in C ewes ($P < 0.01$).

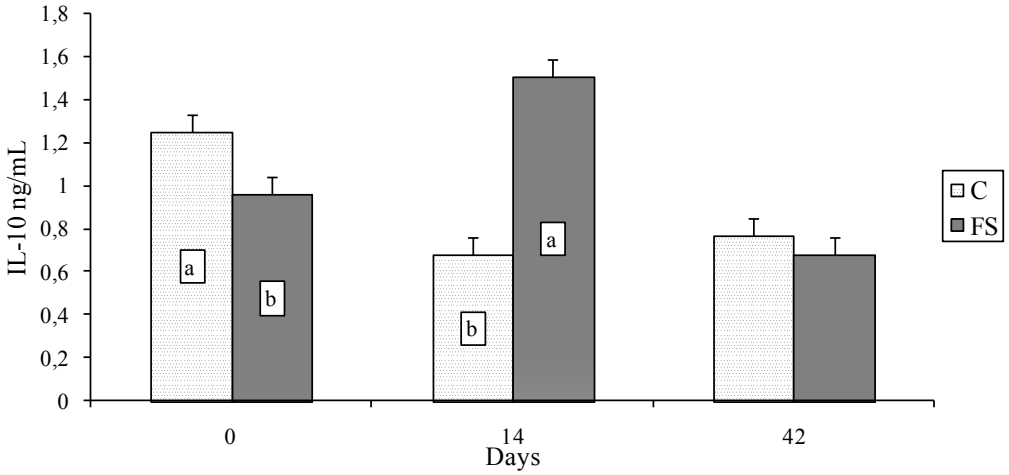

Figure 4.3 *Least Squares means ± SEM of IL-10 production at 0, 14, and 42 d of the experiment in plasma of sheep fed control diet (C), or supplemented with flaxseed (FS).* [a, b] *Shows a significant difference between feeding treatments within a sampling day (P < 0.05).*

IL-1β secretions decreased significantly after 14 d (Figure 4.4, P<0.001); in particular at the beginning of the experiment the IL-1β in FS ewes registered lower secretions than C ewes (P<0.01).

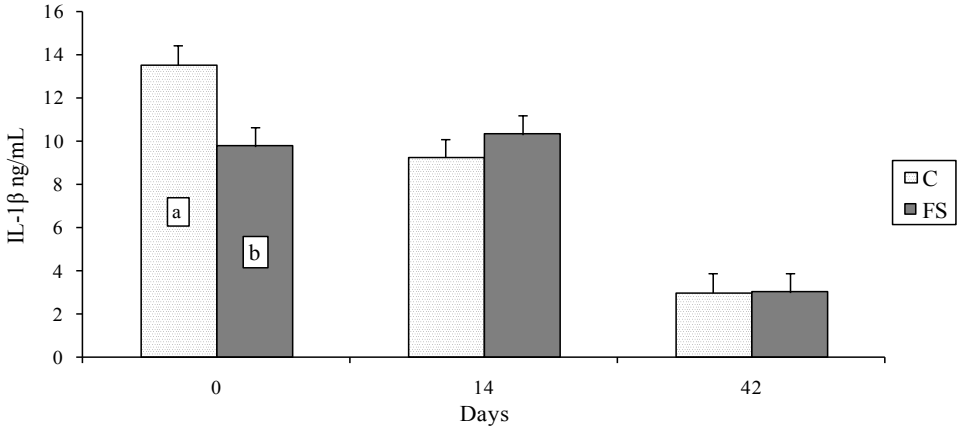

Figure 4.4 *Least Squares means ± SEM of IL-1β production at 0, 14, and 42 d of the experiment in plasma of sheep fed control diet (C), or supplemented with flaxseed (FS).* [a, b] *Shows a significant difference between feeding treatments within a sampling day (P < 0.05).*

Plasma TNF-α secretions remained unchanged until 14d from post partum, and at the end of the experiment increased (P<0.01). On average the TNF-α production was higher in FS ewes than in C ewes (P<0.05); at the end of the experiment secretions of TNF-α in C ewes showed a significant increase compared with TNF-α in FS ewes (P<0.01).

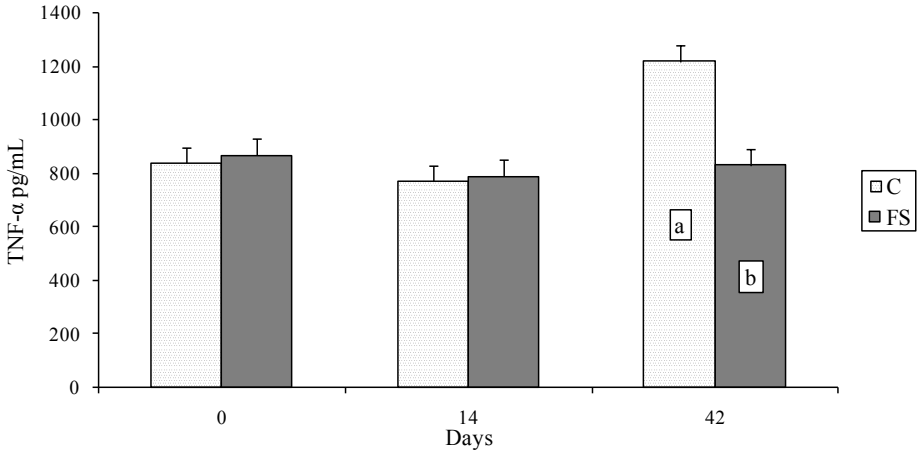

Figure 4.5 *Least Squares means ± SEM of TNF-α production at 0, 14, and 42 d of the experiment in plasma of sheep fed control diet (C), or supplemented with flaxseed (FS).* [a, b] *Shows a significant difference between feeding treatments within a sampling day (P < 0.05).*

4.4 DISCUSSION

Nutritional imbalances and neuroendocrine changes normally occurring around parturition can result in an immune depression state during post partum, which has been extensively studied in dairy cows (Lacetera et al., 2005). The present experiment focused on the effects of diet rich in PUFA on the immune responses of ewes during post partum and the consequent possible regulation of the duration and the extent of immune suppression in order to protect the animals from invading pathogens.

Dairy cows have reduced antibody secretion by B cells during the periparturient period (Kehrli et al., 1989; Detilleux et al., 1995). Mallard et al. (1998) reported that cows around parturition displayed a hyporesponsiveness if immunized with OVA following peripartum. The plasma anti-OVA IgG titres measured in this experiment displayed a reduced responsiveness to antigen stimulation both in C and in FS ewes, in accordance with Mallard et al. observations (1998). Very few experiments focused on the immune status of sheep around parturition; in a previous study an increase of antigen specific IgG was found in the week prior to parturition, even more marked in single-bearing ewes than in twin-bearing ewes (Caroprese et al., 2006). Alterations in immune responsiveness during peripartum in cows have been extensively studied, but some differences between cows and sheep have been observed, and sheep antigen-specific immune responses appeared unaltered during pregnancy (Wattegedera et al., 2008). Dairy cows, supplemented with flaxseed and other PUFA sources around parturition, both primiparous and multiparous, did not show an effect of diet on the antibody response to Ova (Lessard et al., 2003 and 2004). Similar results on the antibody responses was found also in dogs (Wander et al., 1997),

rats (Fritsche et al., 1992) and laying hens (Sijben et al., 2002) with PUFA as dietary supplementation. In this experiment, although we did not found a significant effect of diet on anti-Ova IgG titres, lower humoral responses were measured in FS ewes at 14 d postpartum. This finding was in contrast with previous studies on cows and sheep supplemented with flaxseed under heat stress, in which an increase in anti-Ova IgG titres were measured (Caroprese et al., 2009 and 2012). Hormonal and physiological changes connected with the type of stressors acting on animals can account for different dietary effects on immune responses of animals. Lymphocyte functions can be suppressed by the complex hormonal arrangements of the transition period resulting in a general immunosuppression (Shafer-Weaver et al., 1999). On the contrary, the homeostatic mechanisms activated to cope with heat stress, by maintaining a constant body temperature, can be contrasting and even stronger than those supporting homeorhetic changes connected with parturition. This can account for differences in flaxseed effects in the diet of sheep and cows, suggesting their role in enhancing humoral immune responses during heat stress but not during post partum in sheep.

The level of IL-6 rise in the bovine uterus before parturition and then return to the basal level starting from 8 days after parturition (Ishikama et al. 2004). In sheep during transition period, IL-6 plasma levels have been found to peak at parturition, highlighting the possibility of considering the level of IL-6 as indicator of the physiological stress connected with parturition (Caroprese et al., 2006). Similar trends were found in C ewes of the present experiment, in which the production of IL-6 peaked at parturition and then decreased throughout post partum. In FS ewes the levels of IL-6 registered at parturition, which remained

unchanged at 14 d post partum and higher than the levels of IL-6 of C ewes, could be due to the influence of PUFA composition of flaxseed supplemented. In Miles and Calder (1998), mice fed omega-3 FA modulated the expression of IL-1, IL-6 and TNF-α both protein and mRNA levels secreted from macrophages. Immune suppression registered during post partum in dairy cows has been reported in terms of a reduction of in vitro lymphocyte proliferation, of antibody secretion and cytokine release (Kehrli et al., 1989; Mallard et al., 1997). Results suggest that flaxseed administration may attenuate the immune suppressive effects caused by parturition, thus sustaining sheep immune functions in the days following parturition, thus exerting an important role to protect against invading pathogens during post partum period, as already reported by Lessard et al., (2004). This observation was further supported by the major levels of IL-6 of FS ewes compared with C ewes at 42d post partum.

The resolution of inflammatory conditions is orchestrated by anti-inflammatory cytokines, but the releasing of the anti-inflammatory cytokines, among which IL-10, usually occurs together with that of the pro-inflammatory cytokines (Islam et al., 2013). Cytokine profile reflects the severity and the time of infection and inflammation; high level of IL-10 were measured in cows affected from retained placenta and clinical endometritis; the levels of IL-10 found were time-dependent and peaked when clinical endometritis were in full bloom (Islam et al., 2013). This may help to explain the results of IL-10 production, which resulted two-fold higher at 14 d post partum in FS ewes than in C ewes. The concomitant enhancement of IL-6 and IL-10 in FS ewes suggested the activation of innate immune responses and the overcoming of the immune depression state around parturition in ewes supplemented with flaxseed. In a recent study, flaxseed

supplementation to sheep under high ambient temperature resulted in enhanced levels of plasma cortisol (Caroprese et al., 2012), which was responsible for up-regulation of IL-10 secretions (Caroprese et al., 2014). It could be hypothesized that flaxseed was able to stimulate a similar endocrine change in ewes during post partum, thus resulting in the enhancement of IL-10.

Dietary PUFA can stimulate the activation of immune response involving Toll-like receptor signaling (Hogenkamp et al., 2011). IL-1β have a central role in the inflammatory process in the bovine mammary gland affected from coliform mastitis (Alluwaimi et al., 2004), and is indirectly implicated in chemo-attraction of neutrophils during *Escherichia coli* infection (Shuster et al., 1997). It is demonstrated a correlation between dietary n-3 PUFA and IL-1β expression (Endres et al., 1989; Baum et al., 2012). The lower concentration of IL-1β in FS ewes around parturition than in C ewes could be related to PUFA content of flaxseed. This observation is confirmed in Caughey et al. (1996), in which high consumption of flaxseed oil in human mononuclear cells decreased the production of IL-1β by 31%. A reduction of level of the IL-1β mRNA was found also in spleen lymphocytes LPS-stimulated from mice fed PUFA from fish oil (Robinson et al., 1996). In our study, the absence of the difference in IL-1β concentration until end of the experiment may be linked to its short biological life (Persson Waller et al., 1997; Caroprese et al., 2006). Additionally, IL-6 has a general suppressive effect on IL-1β production by cells incubated with LPS (Schindler et al., 1990), and is able to inhibit IL-1β expression (Bannerman, 2009).

In infected bovine mammary glands, at the initiation of inflammation, when an increase of expression of pro-inflammatory cytokines occurs, the release of TNF-α by macrophages activates the recruitment of the neutrophils. In Sordillo et al.

(1995) mononuclear cells isolated from peripheral blood of periparturient cows displayed an increase of TNF-α levels compared with mid and late lactating dairy cows. The administration of dietary PUFA from flaxseed, however, caused a decrease of pro-inflammatory cytokines in mice (Khair-El-Din et al., 1996); moreover, a diet supplemented with n-3 fatty acids was also responsible for altering cytokines expression, with a tendency to reduction of expression of TNF-α when administrated to Holstein calves (Karcher et al., 2014). Further studies in swine and calves, exposed to LPS and fed with fish oil, supported a decrease of TNF-α serum production and of TNF-α expression (Gaines et al., 2003, Karcher et al., 2014). In contrast, some previous researches found no effects of PUFA supplementation on the production of TNF-α from monocytes activated with LPS and isolated from dairy cows (Lessard et al., 2004). The reduction of the level of TNF-α in FS ewes at the end of post partum could depend on the n-3 PUFA levels in flaxseed, as well as by the previous cited inhibition effects of IL-6 on both IL-1β and TNF-α. Furthermore, it is well known that IL-1β can induce the production of a number of pro-inflammatory cytokines, among which TNF-α. As a consequence, the low levels of IL-1β secretions measured in FS ewes could be claimed to explain the low levels of TNF-α observed in FS ewes at the end of the experiment.

4.5 CONCLUSIONS

Very few studies deal with the immune status of healthy ewes during post partum. In the present experiment dietary supplementation with n-3 PUFA from flaxseed regulated the inflammatory responses of healthy dairy ewes after parturition. Ewes supplemented with flaxseed showed an increased reactivity to inflammation by the maintenance of high levels of IL-10 and IL-6 until 2 weeks post-partum, and a decrease of TNF-α and IL-1β at 6 weeks from parturition.

Our results demonstrated that flaxseed supplementation can contribute to reduce the duration of immune depression during post partum, by altering cytokines production, and improving ewes' ability to respond to infections. Further studies are needed to verify the responses of cells from ewes supplemented with flaxseed during post partum to in vitro stimulation of inflammatory mechanisms.

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