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Utilization of grape seeds in ruminant nutrition: effects of this by-product on health conditions, milk production and quality, and ruminal metabolism in Sarda dairy sheep

dr. Fabio Correddu

Direttore della scuola	Prof.ssa Alba Pusino
Referente di indirizzo	Prof. Nicolò Pietro Paolo Macciotta
Docente guida	Prof.ssa Anna Nudda

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ABSTRACT

RESEARCH ISSUES

ABSTRACT

Grape seed is a by-product derived from winery and distillery industries, containing relevant concentrations of polyphenolic compounds. The use of this by-product in ruminant nutrition had not been evaluated yet. The present work studied the effects of dietary inclusion of grape seed, alone or in combination with linseed, as source of PUFA, on immune response, liver and kidney metabolic activity, ruminal metabolism, and milk yield, composition, fatty acid profile and oxidation stability in Sarda dairy sheep. It was demonstrated that grape seed and linseed can be included, alone or in combination, in dairy ewes diet without adverse effects on milk production traits and health status; in addition, an immunomodulatory effect of this residue was evidenced. Grape seed was not effective in reducing PUFA ruminal biohydrogenation; however, it increased the ruminal accumulation of CLA *cis*-9,*trans*-11 when used alone, and of vaccenic acid when combined with linseed, manly due to its high content in linoleic acid. Milk quality was improved, in term of FA composition, by the inclusion of grape seed and linseed, especially when used in combination. Moreover, dietary grape seed improved oxidative stability of milk, by reducing the accumulation and formation of lipid hydroperoxides and volatile secondary lipid oxidation products, i.e., hexanal and benzaldehyde, after light exposure. In conclusion, the inclusion of grape seeds in the diet of dairy ewes is an alternative use of this by-product.

RESEARCH ISSUES

The principle of sustainable development has become a very important issue in the advanced economies, but also in the developing countries, related to the increasing attention on the environmental preservation, conservation and improvement. In this field industries play a primary role because, with their activities, are involved in the environmental pollution. An important aspect related with this field concerns the production of a large amount of wastes and by-products from agro-industrial, particularly food industry, activities. Food processing wastes consist, generally, of organic material with high nutrient value, whose disposal represents a serious pollution risk. However the appropriate management and disposal of these products entails additional costs for the industries. On the other hand, due to their high nutrient content, some agro-industrial by-product could be a suitable feedstuff for animal nutrition. Grape seed represents a by-product derived from the wine making processes, containing an important amount of polyphenolic compounds, mainly proanthocyanidins, which have received great attention for their health promoting properties, principally related to their antioxidant power. Utilization of natural antioxidant in ruminant nutrition could be a suitable strategy to improve the livestock performance, by increasing animal health condition, and improving the quality of products, by modulating ruminal biohydrogenation, in order to increase beneficial FA, and by increasing oxidative stability.

The first chapter of the present work was dedicated to analyze available literature focusing the problem of agro-industrial by-products, and their potential use in ruminant nutrition; in particular a deep study was carried out on the grape seed winery by-product, in order to give an overview on its chemical properties and on the biological activities of its polyphenolic compounds. The effect of polyphenols in ruminant nutrition was also reviewed, for their effects on production, health condition, ruminal metabolism and quality of products.

The other chapters reported the experimental trial carried out to study the potential use of grape seed as dietary supplementation of dairy sheep. In particular, the effects of this by-product, alone or associated with linseed, as source of PUFA, on health conditions and milk yield and quality (chapter 2), ruminal biohydrogenation processes (chapter 3),

milk fatty acid profile (chapter 4) and milk oxidative stability (chapter 5) in Sarda dairy sheep were investigated.

CHAPTER 1
INTRODUCTION

1.1 Agro-industrial by-products

About 2.5 10^8 ton/year of waste and by-products are produced in Europe by the food processing activities (AWARNET 2004). Waste management is a crucial key in the policy of European Union as it could advantage for reducing air and water pollution, greenhouse gas emission and health problems. One of the priority objectives indicates in the "7th Environment Action Programme of EU to 2020", regarding the waste policy and managing waste, is to maximize recycling and re-use (European Union, 2014).

1.1.1 Re-use and recycling

Several strategies have been proposed for the re-use and valorization of agricultural by-products. Some products have been evaluated as alternative to fossil fuels; by-products could be chemically processed or could represent a substrate for microbial fermentation processes in order to produce bio-liquids and oils, which could be subsequently used by biodiesel industry (Giannakopoulou et al., 2010; Leiva-Candia et al., 2014). More recently, there is an increasing interest in the extraction of bioactive compounds such as carotenoids, tocopherols, and, particularly, polyphenols, which are known for their antioxidant power and health promoting properties (Schieber et al., 2001; Vauzour et al., 2010). However, transport, storage and expensive treatments of by-products are economically limiting factors for these recycling strategies. In addition, these processes result, often, in a further waste production (Federici et al., 2009). For these reasons, most frequently, agro-industrial by-products are used as feed or fertilizer (Schieber et al., 2001).

1.1.2 Utilization in animal nutrition

The use of some by-products in animal nutrition has been largely explored, and represents the easiest way to exploit these materials (Federici et al., 2009). Some agricultural residues, such as oil meals, bran, middlings, brewers' grains, beet pulp and molasses, have historically utilized as feedstuff due to their high values in fiber or protein and to their large availability. Apart from these "traditional" by-products, utilization of other "alternative" by-product residues, resulting from fruit and vegetable processes, has become interesting as feedstuff (Mirzaei-Aghsaghali and Maheri-Sis,

2008), mainly for their content in bioactive compounds (Schieber et al., 2001) and for its chemical composition (Tables 1.1 and 1.2).

Beet pulp, resulting from the sugar manufacturing process, corn gluten feed, resulting from the extraction processes of the starch, gluten and germ from corn, and soybean hulls, mainly consisting in the outer covering of the soybean, are characterized by high level of fiber (Table 1.1), and are largely employed in order to decrease the content of non-fiber carbohydrates (NFC) in diets of ruminant. Beet pulp was used in lamb diet for partly replacing barley grain in fattening (Bodas et al., 2007). Sheep diet with 340 g/kg DM of beet pulp guaranteed similar performance compared to the control diet, without significantly effects on feed intake and nutrient digestibility (Abbeddou et al., 2011). The total replacement of ground corn with soybean hulls in diet of early lactating goats improved the nutrient digestibility and did not change the milk production and composition (Zambom et al., 2012). The inclusion of corn gluten feed to replace up to 25 or 50 % of corn in diet of beef heifers gave similar results, in term of feed intake and nutrient digestibility, compared to the control diet; in addition utilizing in restriction intake could maximize its feeding value (Hussein and Berger, 1995).

Processes of oil seeds extraction results in by-product production; soybean meal, linseed meal, corn gluten meal, cottonseed meal and sunflower meal are obtained by grinding the material resulting after oil extraction. These by-products are characterized by high content in protein (Table 1.1) and are largely used as protein source in animal feed industry. Supplementation of by-products with high protein content in sheep and goat diet results in increased apparent digestibility of this nutrient (Table 1.3) (Economides, 1998; Solomon et al., 2008; Ermias et al., 2013), probably as consequence of the positive effect of proteins on the growth of rumen microbial population, which increases fermentation processes (Solomon et al., 2008).

Several by-products, resulting from the processing of olive, tomato, citrus, orange, apple, pomegranate, have been evaluated as alternative feedstuff (Table 1.2).

Use of olive by-products has been recently explored. The inclusion of 10 and 20% of destoned exhausted olive cake in the diet of lambs did not affected feed intake but reduced the final BW compared to the control diet, most likely due to a decrease in the

apparent digestibility of DM and fiber (both NDF and ADF) in lamb that received the olive cake (Tufarelli et al., 2013). The inclusion of olive cake (340 g/kg DM) in the sheep diet, reduced the digestibility of most of gross nutrients (OM, CP, ADF, NFC) probably related to serious problem of palatability of this by-product (Abbeddou et al., 2011). Residues from tomatoes processing (20.4 % on DM of diet), mainly composed by seeds and skin, fed to Comisana sheep showed a positive effect on milk quality, in term of increase of PUFA, CLA and n-3/n-6 ratio (Romano et al., 2010). The inclusion of tomato waste (125 g/kg DM) in diets of dairy goats had no effect on apparent digestibility, whereas improved the milk fatty acids profile (increased PUFA, RA, LA and LNA) and reduced ruminal NH_3 production and CH_4 emission (Romero-Huelva et al., 2012).

Chemical composition of these materials (Table 1.2) is largely variable; in addition the differences in the processing methods and the mixing of different fractions of the by-products produce wide differences within the same type of by-product (Mirzaei-Aghsaghali and Maheri-Sis, 2008). Of course, the cost of traditional feedstuff, the safety for the animal health and attractiveness of alternative uses influence the choice for by-product utilization (Mirzaei-Aghsaghali and Maheri-Sis, 2008).

Recently, the inclusion of some agro-industrial by-products in animal diet is becoming attractive, due to their content in bioactive compound, particularly polyphenols. Moderate concentrations of these compounds in the diet of animal have demonstrated positive effects on health conditions and productive performance. The transfer of these antioxidant in animal tissues can improve the quality of livestock products, related to their ability to increase oxidative stability. In ruminant they have shown ability to improve nitrogen utilization, reduce methane emission and increase beneficial FA, by affecting ruminal metabolism. The effects of the inclusion of polyphenols in ruminant diets are detailed in Chapter 3.

1.1.3 Winery by-products

Grape is the one of the most important fruit in the world, with its production exceeding 67 million tonnes (FAOSTAT, 2012). In Italy its production is more than 5.8 million (FAOSTAT, 2012) tonnes and most of the grapes is used to produce wine. Italy is one

of the most important producers of wine in the world, with more than 4 million tons produced annually (FAOSTAT, 2012). This process generates a large amount of by-product consisting in wine lee, grape stalk and grape pomace (GP). Grape pomace represents, approximately, 20% of the total weight of processed grapes and is composed by grape skin and seed. Grape pomace, has low pH (mean values ranged from 3.4 to 5.4), high level of organic matter (873-947 g/kg DM), macronutrients, especially K and Ca (mean values of 24.2 and 9.4 g/kg DM, respectively), high concentration of polyphenols (0.9-13.6 g/kg DM) and low concentration of heavy metals and micronutrients (Bustamante et al., 2008). When the disposal of this by-product is not managed properly, it can cause serious environmental problems, because of the high concentration of phenolic compounds which can exert phytotoxic and antibacterial effects. Negative effects on the soil have been associated to the immobilization of nitrogen (Bustamante et al., 2007). Moreover, the antibacterial effect of polyphenols could be a serious problem for the soil health and plant productivity, as they are closely connected with the activities of microbial communities (Chaparro et al., 2012). The problem of the winery wastes disposal is also related to their seasonal production; in fact, a large amount of wastes are generated in a short period (August-October).

In order to reduce the environmental impact of winery residues, grape pomace and wine lee must be sent to alcohol distilleries, producing exhausted grape mark and liquid waste (European Council Regulation, 1493/1999). Moreover, the recycling of these residues is heartily encouraged by EU. For this reason, several alternatives have been investigated and proposed for the valorization of winery and distillery wastes.

Due to their content in oil, grape seeds have been studied as material for potential biodiesel production. However, the high degree of unsaturation and PUFA concentration in grape seed oil, mainly related to the concentration of LA (about 70%), make this oil an inappropriate material for biodiesel production (Ramos et al., 2009). In fact, the fatty acid composition of a feedstock is the major factor influencing the possibility to use that material for fuel production. This is because the properties of biodiesel, such as oxidative stability, melting point, kinematic viscosity, ignition performance and iodine value (related to the unsaturation of the oil), are largely dependent on the FA profile of the oil. Briefly, oils with high amount of long chain

monounsaturated fatty acids (MUFA) are preferred materials for conferring good properties to the fuel, while high levels of polyunsaturated fatty acids (PUFA) negatively affect the characteristics of biodiesel (Moser and Vaughn, 2012).

It has been reported the suitable use of grape stalk in the decontamination of effluents. Levin et al. (2012) observed that grape stalk was a good lignocellulosic substrate for the grown of fungi cultures used for (effluent) decolorization purposes. Grape pomace could be used as alternative source for energy production (Cáceres et al., 2012), and for extraction and recovery of a great range of products, such as grape seed oil, ethanol, tartrate, citric acid, hydrocolloids, dietary fiber and phenolic compounds (Schieber et al., 2001). Most of the phenolic compounds of grape are contained in grape seed. Because the great antioxidant power, the extract of grape seed (GSE), mainly composed by proanthocyanidins, is actually commercially available as dietary supplement of natural antioxidant, and it is used to increase shelf-life and enhance health promoting compounds in foods (Carpenter et al., 2007; Brannan, 2009). Literature available about the use of grape seed in animal feeding is limited, probably because of the high lignin and tannin content (Spanghero et al., 2009) that reduce digestibility and energy value (Baumgärtel et al., 2007). However, the presence of moderate concentrations of dietary polyphenols was found to be able to increase productive performances in ruminant, by increasing utilization efficiency of nitrogen, reducing gas emission and improving the quality of derived products and improving health (Waghorn and McNabb, 2003). As reported by Santana-Méridas et al. (2012) there is an increasing interest in the utilization of a large number of by-products in food and feed, due to their content in polyphenols. For these reasons, recently, the utilization of grape pomace in ruminant feeding has become an attractive research field (Spanghero et al., 2009; Abarghuei et al., 2010; Moate et al., 2014; Santos et al., 2014).

1.2 Grape seed

Grape seeds are characterized by high in fiber, essential oil, and phenolic compounds (de Campos et al., 2008). In particular, the high content of lignin (Spanghero et al., 2009) is the main limitation of use of this by-product in ruminant diets. The chemical composition of grape seed (means of some red and white Italian cultivar of *Vitis vinifera* means; Table 1.4) revealed high concentration of NDF, ADF and Lignin and a high total phenol content (70 g/kg of DM).

Oil and polyphenols are the most important components of grape seed, both before and after vinification processes. The Fe and Cu are the most abundant minerals in grape seed.

1.2.1 Grape seed oil

Grape seed oil is a high quality nutritional oil, due to the high levels of unsaturated fatty acids (UFA) (Table 1.5). The PUFA ranged from 63.64 to 75.80%, MUFA from 14.19 to 22.20 % and SFA from 9.66 to 14.94 % (Lutterodt et al., 2011; Fernandes et al., 2013). Linoleic acid is the most abundant FA (about 70%), followed by oleic, palmitic and stearic acids (18, 8 and 4 %, respectively) (Dalmolin et al., 2010; Sabir et al., 2012; Fernandes et al., 2013).

1.1.2 Grape seed polyphenols

Phenolic compounds, or Polyphenols, are products of the secondary metabolism of plants. More than 8000 different structures have been identified; these compounds are characterized by an aromatic ring bearing one or more hydroxyl groups, and chemical structure ranges from simple molecules as phenolic acids to more complex structure such as tannins. Synthesis of these compounds derives manly from shikimate and the acetate pathways, during the normal development of plant, or under different stress conditions (Naczk and Shahidi, 2004). Although not completely defined, the biological role of polyphenols seems to be related to some plant defense mechanisms against pathogens, herbivorous, insects (antibiotic and anti-feeding actions) and solar radiation (Quideau et al., 2011). Polyphenols are thought to be responsible for astringency of many beverages and foods and are well known for their antioxidant power (Riceevans et al., 1995) and beneficial aspect for health, such as cardioprotective (Schroeter et al., 2006; Perez-Vizcaino et al., 2009), anti-inflammatory (Kubena and McMurray, 1996) and anticancerogenic effects (Stoner and Mukhtar, 1995; Forester et al., 2014).

Grape (*vitis vinifera*) contains large amount of phenolic compounds that can be divided in flavonoid, including flavan-3-ols (catechin), flavonols (quercetin) and anthocyanins, and non-flavonoid, such as hydroxybenzoates (gallic acid), hydroxycinnamates and stilbenes (resveratrol) (Yang et al., 2009).

The relative distribution of polyphenols content in the different tissues of grape is reported to be approximately 64%, 30%, 5% and 1%, in seeds, skins pulp and juice, respectively (Yang et al., 2009). Most representative phenolic compounds in grape are anthocyanins, resveratrol, manly located in the skin, and flavan-3-ols and gallic acid manly located in seeds (Hollecker et al., 2009; Mattivi et al., 2009).

As reported by several studies, total content, profile and tissue distribution of polyphenols in grape, vary considerably among cultivars, during ripening and with climate conditions (Rodríguez Montealegre et al., 2006; Ledda et al., 2010; Ivanova et al., 2011). In addition the different methods used for their extraction and determination largely influence the results of measurements, leading difficult to compare data from different works. For these reasons the reported total phenolic content in grape shows a

wide rage. It has been reported that phenolic compounds in different grape varieties ranged from 115 mg/kg fresh weight of grapes (Cantos et al., 2002) to 930 mg/kg fresh weight (Kanner et al., 1994).

Regarding grape seeds, the total concentration of polyphenols in fresh fruit is highly variables among different works being 9.7-41.31 mg/g FW (Liang, 2012), 108-190 mg/g FW (Ivanova et al., 2011), and 79.2-154.6 g/kg on FW (Bozan et al., 2008). Vinification process can cause a decrease in the total content of polyphenols; this reduction is more evident in red wine production, because the whole fruit is fermented and polyphenols are partly extracted by ethanol. However, by-product resulting after vinification represents a good source of polyphenols and other nutritionally beneficial health-promoting compounds, such as tocopherols, macro- and micro-elements (Lachman et al., 2013). The chemical characteristics of grape seed resulting after vinification of different varieties of red and white grape has been reported by (Lachman et al., 2013); the mean of total phenols concentration was found to be 32.2 and 58.2 g/kg on DM for grape seeds of red and white varieties respectively, with a wide variability among varieties, ranging from 15.1 to 76.7 g/kg on DM. Additionally, the distillery processes can decrease the total polyphenol content in the by-product; Bustamante et al. (2008) compared the chemical characteristics of different by-product, resulting from winery or distillery processes, found total polyphenols content of exhausted grape pomace (from distillery) were 61.5% of that observed in the grape pomace (from winery).

The most common grape seeds polyphenols (Figure 1.1) are represented by flavan-3-ols monomers, such as (+)-catechin, (-)-epicatechin, (-)-epigallocatechin, and (-)-epigallocatechin 3-*O*-gallate, and by dimers, trimers and more polymerized procyanidines (proantocyanidines); composition includes also low concentrations of resveratrol and flavonols (Liang et al., 2012).

1.2.2.1 Biological activity

The major characteristic of phenolic compounds is represented by their antioxidant activity. This capacity is linked to their ability to scavenge free radical (Sanchez-Moreno et al., 1999), but also by donating hydrogen atoms or electron, or chelate metal

cations (Bravo, 1998). However, most of the works describing antioxidant mechanism of polyphenols are in vitro studies and they did not consider bioavailability and metabolism of those compounds (Williamson and Manach, 2005). The concentration in the biological system seems to be too low to explain their activity, if compared with the concentrations of natural antioxidant like tocopherol and ascorbic acid. However, low concentration could be enough to modulate some cell functions. It has been demonstrate that polyphenols can influence enzymes expression or activity: telomerase, cyclooxygenase 1 and 2, 15-lipoxygenase, xanthine oxidase, monooxygenase, metalloproteinase, angiotensin converting enzyme (ACE), phospholipase A 2, protein kinase, cytochrome P450 (CYP) and phase II enzymes. Polyphenols are also involved in the regulation of antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and the concentration of glutathione (GSH) (Jeon et al., 2003; Molina et al., 2003; Koyama et al., 2013). It was suggested that the main important mechanism involves a direct action on the gene expression; several studies showed polyphenols ability to attenuate solar UVB light-induced oxidative stress by increasing of antioxidant-responsive element-dependent genes; the authors suggested that this finding could be due to the regulation of mitogen activated protein kinases (MAPK) signaling pathways (Vayalil et al., 2003; Mantena and Katiyar, 2006).

Phenolic compounds, in particular grape polyphenols, are reported to be health promoting, showing beneficial effects on cardiovascular system, brain functions, cancer prevention, obesity, diabetes, anti-inflammatory, cardiovascular and liver disease have been reported (Georgiev et al., 2014). However, the interesting health promoting activity of grape products seems to be related to its peculiar mixture of different compounds, which have different biological activities and could act a synergism in contrasting several diseases (Zhou and Raffoul, 2012).

Cardiovascular system

Beneficial effects of grape products on cardiovascular system are well documented and reviewed (Leifert and Abeywardena, 2008). Grape seeds polyphenols are reported to be effective in decreasing oxidation of low density lipoproteins (LDL), improving endothelial and vascular function and attenuating thrombosis (Feng et al., 2010; Bijak et

al., 2013; Yubero et al., 2013). Molecular mechanisms underlying these beneficial effects are still largely unknown; inhibition of platelet aggregation and reactive oxygen intermediates production by grape seed extract has been proposed as mechanism of antithrombotic effect of grape polyphenols (Vitseva et al., 2005).

Neurodegeneration (Brain functions)

Regular consumption of fruit and vegetables, rich in polyphenols, has been shown to delay Alzheimer's disease onset (Dai et al., 2006), decrease risk of dementia (Hughes et al., 2010) and Parkinson's disease (Okubo et al., 2012). Moderate intake of wine has been associated to a lower risk of incidence of neurological disorder (Letenneur, 2007, Pinder, 2009), most likely related to the antioxidant effect of the polyphenolic content of wine. An in vitro study on cultured hippocampal neurons showed that polyphenol-rich grape seed extract (GSE) has a significant neuroprotective effect, when glutamate toxicity was induced (Narita et al., 2011); glutamate toxicity occurs during various neurotoxic injuries, such as brain ischemia. Oral administration of grape polyphenols extract to laboratory animals (gerbils) was also found to reduce neuronal damage induced by reperfusion after ischemic event (Wang et al., 2009a), related to the releasing of reactive oxygen species (ROS) which are involved in oxidative damage of lipids, proteins and DNA (Chan, 2001). In mouse model, polyphenols extracted from grape seeds showed their ability to reduce accumulation of amyloid-beta (Aβ) (Wang et al., 2009b), which plays a key role in the Alzheimer's disease. Modulation of gene and protein expression by wine polyphenols has be proposed to be more explanatory than the direct antioxidant effect in the protection from oxidative injury (Doré, 2005).

Cancer

Several studies evidenced the cancer preventive effect of polyphenols, against skin, pancreatic, prostate, head and neck cancer. An in vitro study demonstrated that melanoma cell invasion and migration were inhibited by grape seed proanthocyanidins (Vaid et al., 2011); the authors found that the effect was related to the reduction in expression of cyclooxygenase-2 (COX-2) and production of Prostglandin-E_2 (PGE_2), which normally increase with UV radiation and have been recognized as risk factors for the skin cancers development (Black et al., 1978; Williams et al., 1999). Recently, grape seed proanthocyanidins extract was investigated for the effect on pancreatic

adenocarcinoma cells (Cedo et al., 2014); the authors found that the extract inhibits cell proliferation and increases apoptosis of MIA PaCa-2 cells, with gallic acid being the component with the highest effect. This finding agree with previews works showing inhibitory activity of grape proanthocyanidins on the growth of pancreatic cancer cells, evidenced both in vitro and in vivo studies (Prasad et al., 2012). On mice, the inclusion of grape seed extract inhibited the prostate tumor growth (Raina et al., 2007). Treatment of human head and neck squamous cell carcinoma (HNSCC), generated from the oral cavity, larynx, pharynx and tongue, with grape seed proanthocyanidins reduced the cell growth, and the results were confirmed in vivo, on mice, by the inhibitory effect on tumor xenograft growth (Prasad and Katiyar, 2012).

Obesity

Grape seed proanthocyanidins extract was tested to determine its effect on dysplipidemia in rats (Quesada et al., 2009); the work revealed the capacity of this product to prevent and treat lipid altered metabolic state, manly by a reduction in lipogenesis of very low density lipoprotein (VLDL) development in hamsters was reduced by feeding grape seed extract.

Diabetes

The administration of grape seed proanthocyanidins to rats was found to be able to protect from induction of diabetes by alloxane (El-Alfy et al., 2005); the mechanism suggested by the authors implicates a prevention and restoration of pancreatic antioxidant defense systems. The same authors supposed that the anti-diabetic effects, related to the higher release of insulin, could be exert by preserving pancreatic β-cell function. A study conducted on human revealed capacity of grape seed extract to reduce postprandial plasma glucose after consuming high carbohydrate meal (Sapwarobol et al., 2012). Diabetes is a chronic metabolic disorder, representing a worldwide health problem, characterized by a high blood glucose concentration (hyperglycemia), due to a deficiency of insulin, or an insulin resistance. Although the pathogenesis of these diseases is not completely clarified, some evidences suggested an important role of oxidative stress in the damaging of pancreatic β-cells (Baynes, 1991, Watson and Loweth, 2009). In particular, recent studies have connected the damage of β-cells with

the local production of ROS, and it has been hypotized a potential help of antioxidant plant polyphenols in reducing diabetes development (Lee et al., 2008).

1.3 Polyphenols in ruminant nutrition

Several studies have been conducted regarding the polyphenols effect on ruminant nutrition. Many studies refer negative impact of polyphenols on intake and production, manly linked to the their low palatability related to the ability to bind salivary protein and confer astringency (Jöbstl et al., 2004). Decrease in voluntary intake and organic matter digestion was observed in sheep fed high concentration of tannins (Barry and Duncan, 1984); some negative effects of tannins were associated to their interaction with digestive enzyme and epithelium lining digestive tract (Silanikove et al., 2001). However, more recent works showed beneficial effect of dietary polyphenols on health condition, ruminal metabolism, milk production and quality.

Other study showed that tanniferous tree or forage can be used in ruminant (sheep) nutrition without affect intake (Alonso-Diaz et al., 2009).

1.3.1 Effect on productions

Since the presence of polyphenols in the diet can affect the voluntary feed intake, its metabolism and digestion, consequently, the productivity of the animals could be affected. It is generally accepted that high concentrations of these compounds in the diet can negatively affect the productivity; whereas the inclusion of moderate concentration of phenolic compounds (intake under 50 g/kg DM) could improve animal productions, maybe due to a better utilization of nutrients, especially protein, with a consequent greater availability of intestinal amino acids. However contrasting results have been reported in literature, about the correlation between concentration of phenolic compounds and effects on animal performance. Montossi et al. (1997) found that 4.2 g CT from Holcus lanatus /kg DM increased the gain in liveweight in lamb. Priolo et al. (2000) and Vasta et al. (2007) observed a reduction in gain of liveweight in lamb fed carob pulp which provided, respectively, 25 and 27 g condensed tannins/kg DM of diet. Wang (1996) observed an increase in gain of liveweight in sheep fed L. *cornicolatus* (34 g condensed tannins/kg DM). This discrepancy could be explained by the fact that the concentration represents only one of the main factors influencing the effect of

phenolic compounds on animal nutrition; the chemical characteristic of polyphenols, their source, other dietary components and the specie of animal are involved in the extent of effects of phenolic compounds on animal.

Regarding milk production several works present in literature show a positive effect of the inclusion of polyphenols in the diet of ruminants at doses of < 50 g/kg DM of diet (Figure 1.2). Only few authors reported a negative effect of these compounds on milk production and, often, it is correlated with a decrease in feed intake (Grainger et al., 2009; Griffiths et al., 2013). The best dose of polyphenols leading higher milk production seems to be related to the source of these compounds and, consequently, to their chemical characteristics and to the other dietary ingredients. Similar doses of polyphenols could results in very different effects, when derived from different sources (Figure 1.2). For example, the dietary inclusion of Cassava hay (9.6 g TP/kg DM diet) increased (+ 74 %) the milk production in goats (Dung et al., 2005), while Acacia *Mearnsii* (9.0 g TP/kg DM diet) reduced (- 4 %) the milk production in cows (Grainger et al., 2009).

1.3.2 Effect on health conditions

Based on the wide range of positive effects showed in vitro and in vivo studies, beneficial role of polyphenols on ruminant health and welfare is being an important issue in ruminant livestock. Gas formation (resulting in bloat problems) and parasite infection are two of the major diseases affecting ruminant grazing. Bloat occurs when gas eructation is inhibited or reduced and it is often associated with feeding forage containing highly soluble protein. The rapid fermentation of these proteins leads to the development of a stable foam in the rumen. Due to the gas accumulation, the rumen expands and exerts pressure on the diaphragm and lungs, leading to feeding aversion and preference (Villalba et al., 2009), respiratory difficulty, and ultimately to death (Cheng et al., 1998). Bloat represents a serious problem for dairy farms; due to its irregular occurrence, farmers are reluctant, or not able, to treat it with anti-boat agents (Waghorn and McNabb, 2003). The ability of polyphenols, proanthocyanidins in particular, to reduce gas production and prevent bloat are yet recognized. This property can be explained by the proanthocyanidins capacity to precipitate proteins and reduce their fermentation. In addition, the interaction between polyphenols and protein can

enhance the flow of nitrogen leaving the rumen to the gut, improving protein utilization (Aerts et al., 1999). Gastrointestinal parasitism is an important issue related to the health condition and welfare of ruminant and represents a limitation for the economic viability of grazing ruminants (Hoste et al., 2012). Due to the large use of anthelmintic drugs, a resistance against these compounds has been developed (Waller, 1994). In order to reduce parasitism, alternative strategies have been proposed, such as utilization of natural bioactive compound with antiparasitic properties. Polyphenols have shown particular antiparasitic activity both in vitro and in vivo. The effect of polyphenols extract from *Pistacia lentiscus* L. and *Phillyrea latifolia* L. on gastrointestinal nematodes was tested, revealing its high anthelmintic potential (Azaizeh et al., 2013). Antiparasitic effect of tannin-rich foliage of *Havardia albicans* was found when this supplementation was included at the dose of 400 g/kg DM in the diet of sheep (Galicia-Aguilar et al., 2012). Inclusion of quebracho extracts (30 and 60 g on kg FW) in diet of sheep with intestinal parasitic infection, was able to reduce the development of the infection, suggesting a direct anthelmintic effect of the condensed tannins (Athanasiadou et al., 2000). Studies about toxicity of polyphenols on ruminants are limited, and most of them used compounds as tannic acid, gallic acid and ellagic acid as models for their investigations (Zhu et al., 1992). These compounds derive from degradation of hydrolysable tannins, which are known to be toxic for ruminant at high concentration (Pryor et al., 1972; Murdiati et al., 1992). The toxicity of proanthocyanidins is not directed, but it should be related to the dose-dependent anti-nutritional effect of these compounds, such as reduction in digestion of proteins and carbohydrates (Reed, 1995). Studies on sheep (Hervás et al., 2003) and goats (Silanikove et al., 1996) did not showed any toxic effect of tannins (from quebracho; and Quercus calliprinos (oak), Pistacia lentiscus (pistacia) and Ceratonia siliqua (carob), respectively), as supported by the normal values of liver and kidney function indicators.

However, specific investigation on the potential toxic effects of polyphenols, or source of polyphenols, should be done, before using these products in animal experimentation.

1.3.3 Effect on ruminal metabolism

Polyphenols, tannins in particular, affect strongly metabolism processes in ruminant. Combination of direct effect with nutrients and interaction with enzymes and ruminal

microbial population lead to intense variations in the metabolism of protein, carbohydrates and lipids. Good knowledge of these mechanisms allows to manipulate nutrients metabolism, in order to improve productive performance, the quality of meat and dairy products and to reduce the loss of nutrients.

1.3.3.1 Protein fermentation

Utilization of nitrogen (N) is one of the most important factors affecting the performance of ruminant livestock; it depends on the quantity of proteins that leave rumen and reach abomasums. Dietary proteins are exposed to proteolysis by the rumen microorganisms, which use amino acids for the microbial protein synthesis. Proteins that reach abomasums are manly represented by microbial proteins and secondary by the rate of protein escaping from the rumen. Amino acids in excess for microbial utilization are converted in NH_3 and carboxylic acids, by an oxidation process. NH_3 produced can be reutilized for the protein microbial synthesis. This reaction depends on the availability and the fermentation rate of carbohydrate. Different rumen microorganisms have been considered responsible for the proteolytic activity and for the NH_3 production; protozoa and bacteria, as clostridium and peptostreptococcus, are often cited. The preferred substrate for the protein synthesis depends on the rumen species; generally, amino acids and peptides are preferred mainly by eubacteria proteolytic species, whereas NH_3 is the favorite substrate for cellulolytic, methanogenic and amyloytic bacteria. The reduction of protein degradation results in the increase of the amount of protein digested in the small intestine, and improves N utilization. Tannins have been reported to bind dietary proteins and to decrease their rumen fermentation and NH_3 production, improving N utilization. Recently, Abarghuei et al. (2014a) observed a reduction in the fermentation rate and NH_3 production when two different levels of pomegranate extract (with 15 and 30 mg of total polyphenols DM of basal diets) were tested in an in vitro experiment in order to evaluate the fermentation patterns on rumen inoculums of sheep. Hymes-Fecht et al. (2013) found that replacing alfalfa or red clover silage with birdsfoot trefoil silage with 3 levels of tannins (low, normal and high, 8.3, 12.1 and 15.7 g/kg DM, respectively) improved the N utilization in lactating dairy cows, from about 21% for alfalfa and red clover to 26.3%, on average, for birdsfoot trefoil silages (*Lotus cornicolatus* L.); however, in the same work, the

concentration of rumen NH_3 increased for the birdsfoot trefoil silages compared with red clover. In the experiment of Bhatta et al. (2012) conducted to investigate, in vitro, the suitability of sheanut (Butryospermum Parkii) by-product in ruminant feeding, the lower production of rumen NH_3 was related to the tannin content of this by-product (about 7 % of hydrolysable and less 1 % of condensed tannins). An experiment conducted with different levels of quebracho tannins showed ability of 2% DM of QT to protect hay proteins from rumen degradation and improve growth performance of lambs (Al-Dobaib, 2009). Similarly administration of *Lotus cornicolatus*, containing 22 g/kg DM of tannins, was able to reduce rumen protein degradation, and to increase net digestion of amino acids in sheep (Waghorn et al., 1987). There is a scarcity in bibliography regarding the effect of grape or grape by-product on rumen metabolism. Abarghuei et al. (2010) investigated the effect of grape pomace (total polyphenols, 70.5 g/kg DM), on the ruminal metabolism of Ghezel sheep, when dietary alfalfa was replaced by this by-product. The ability of grape pomace polyphenols (diet contained 40.7 g/kg of TP on DM) to reduce the degradation of protein was evidenced by the decrease of NH_3 production (Table 1.6); however, the authors found that the inclusion of grape pomace in the diets of sheep had a detrimental effect on the metabolism of sheep. In the experiment of Moate et al. (2014) the inclusion of grape marc (dried or ensiled) in the diet of cows did not affect the NH_3 production (Table 1.6); however, in that experiment the mean of the levels of grape polyphenols was lower then that used in the experiment of Abarghuei (4.1 vs 40.7 g/kg DM diet).

1.3.3.2 Carbohydrate digestion

The results on the effect of polyphenols on apparent digestibility of carbohydrate reported in literature are not univocal. In the experiment carried out by Al-Doibaib (2009), increasing levels of quebracho tannins in the diet of sheep fed alfalfa hay had different effects on the fiber digestibility: dosage of 7.5 and 15 g/kg of DM had no effect, whereas 22.5 g/kg of DM decreased fiber digestibility (Table 1.6). At the contrary, in the experiment of Hymes-Fecht at al (2013) the replacing of alfalfa with birdsfoot trefoil (Lotus *cornicolatus*) showed a negative correlation between concentration of tannins and fiber digestibility (Table 1.6): birdsfoot trefoil containing low condensed tannins (8.3 g/kg DM), reduced NDF, ADF and hemicelluloses

digestibility, normal CT birdsfoot trefoil (12.1 g/kg DM) decreased only hemicelluloses digestibility and high CT birdsfoot trefoil (15.7 g/kg DM) increased digestibility of NDF (Hymes-Fecht et al., 2013). Carbohydrate digestion in sheep fed L. *pedunculatus* containing 32.9 g/kg DM of condensed tannins was reduced by tannins (Barry and Duncan, 1984); in particular, the apparent digestibility of cellulose and hemicellulose was lower in sheep fed L. *pedunculatus* than in sheep fed L. *pedunculatus* plus PEG, given in order to abolish the effects of tannins. The inclusion of grape pomace in the diet of sheep (diet contained 40.7 g/kg of TP on DM) decreased digestibility of organic matter (OMD) and of NDF (NDFD) (Abarghuei et al., 2010). Depression of OMD and NDFD was found also by Tiemann et al. (2008) in an experiment consisting in the partial replacement of *Vigna unguiculata* legume (tannin-free) with two tannin-rich shrube legume species (*Calliandra calothyrsus* and *Flemingia macrophylla,* 175 and 111 g of total condensed tannins/kg on DM, respectively) at two increasing level (150 and 300 g/kg on DM); in addition the dose-response relationships was found to be mainly linear.

1.3.3.3 Lipid metabolism

Over the last decades much attention has been directed on the content of beneficial fatty acids (FA) such as polyunsaturated fatty acids from the family of *n*-3 (PUFA *n*-3) as alpha-linolenic acid (LNA, C18:3 *n*-3) and conjugated of linoleic acid (CLA) such as rumenic acid (*cis*9, *trans*11 C18:2) in ruminant dairy products. In order to increase the amount of these beneficial fatty acids and improve food quality a number of studies on ruminant nutrition have been conducted (Nudda et al., 2006; Caroprese et al., 2010). It is well documented that diet composition and rumen microbial biohydrogenation hardly influence the fatty acid profile of dairy products (Buccioni et al., 2011). A number of studies have demonstrated that dietary supplementation of a source of PUFA, such as flax seed, can improve FA profile in milk and dairy products, by increasing concentration of nutritional FA (Mughetti et al., 2012; Nudda et al., 2013).

Dietary lipids are involved in a sequence of reactions, performed by microbial population, which include hydrolysis of esterified lipids to free fatty acids, isomerization and hydrogenation of double bonds of the free fatty acids.

Linoleic acid (LA, C18:2 cis-9,cis-12) and alpha-linolenic acid are the most abundant fatty acids in the diets of ruminants; in particular LA is the most abundant in the concentrates, whereas LNA is the most abundant in fresh forage. Therefore the pathways of biohydrogenation of these FA have been extensively investigated. LA biohydrogenation begins with an isomerization of the *cis*-12 into *trans*-11 bond, by a *cis*-12 *trans*-11 *isomerase*, with specificity for free carboxylic group and for the conjugated diene *cis*-9, *cis*-12. The resulting compound, conjugated of linoleic acid (CLA) C18:2 *cis*-9, *trans*-11 (rumenic acid, RA), is converted to C18:1 *trans*-11 (vaccenic acid, VA) by the enzymatic hydrogenation of the *cis*-9 bond, operated by a microbial reductase. The last step of biohydrogenation is the enzymatic reduction of VA to C18:0 (stearic acid, SA) and it is founded to be the rate-limiting in the process (Harfoot and Hazlewood, 1997). For this reason VA tends to accumulate in the rumen and to be more available for the absorption (Bauman et al., 1999). VA, in part, is converted by the Δ^9-desaturase of the mammary gland in CLA *cis*-9, *trans*-11, which represents about the 80 % of the total RA of milk fat (Mosley et al., 2006). The first step of biohydrogenation of LNA consists on the enzymatic isomerization of the *cis*-12 bond; the C18:3 *cis*-9,*trans*-11,*cis*-15 isomer is reported to be the most representative intermediate C18:3 isomer of this reaction (Harfoot and Hazlewood, 1997); however different pathways have been proposed including also the formation of C18:3 *cis*-9,*trans*-13,*cis*-15 (Destaillats et al., 2005) and C18:3 *trans*-10,*cis*-12,*cis*-15 isomers (Griinari and Bauman, 1999). C18:3 isomers are then converted into a number of C18:2 isomers, in which the C18:2 *trans*-11,*cis*-15 is the most abundant as reported by the study of Shingfield et al. (2010) based on several works on the biohydrogenation. The same authors indicated that the enzymatic reduction of C18:2 isomers results in the formation of a number of C18:1 isomers represented, in large part, by the C18:1 *trans*-11. It is generally accepted that the formation of CLA *cis*-9,*trans*-11 derived from the biohydrogenation of linoleic acid but is not formed from alpha-linolenic acid. However, in the recent study of Lee and Jenkins (Lee and Jenkins, 2011) on the in vitro biohydrogenation of LNA, several CLA isomers were identified, included the C18:2 *cis*-9,*trans*-11.

Several bacteria species are known to be related to the biohydrogenation process, and have been classified into two groups: group A can convert LA to VA, group B can

saturate VA to SA (Kemp and Lander, 1984). Cellulolytic bacteria, in particular Butyvibrio *fibrisolvens*, are reported to be the main responsible microorganisms of the biohydrogenation of UFA in the rumen (Kepler and Tove, 1967), in particular are the most active species of group A. Among bacteria of group B Clostridium *proteoclasticum* are the most active.

It has been demonstrated that the presence of endogenous plant factors, such as polyphenols, in diets of ruminant can influence biohydrogenation process by reducing or inhibiting the activity and growth of rumen microbes (Cabiddu et al., 2009; Vasta et al., 2009a). The extent of the effects of polyphenols on ruminal biohydrogenation depends on the chemical characteristics of these compounds, on the amount and phenolic profile, but also on the other dietary components.

In the study conducted by Vasta et al. (2010) was evaluated the effect of the dietary tannins inclusion (7.86 g of total phenols/kg of DM in the diet) on the FA profile and ruminal microbial population in sheep. In this work the tannins supplementation influenced the biohydrogenation, increasing the accumulation of VA and decreasing the SA/VA ratio; this result was explained by a shift in microbial population, evidenced by an increase in B. *fibrisolvens* and a decrease in B. *proteoclasticus* bacteria (Table 1.6), which are responsible, respectively, for conversion of LA to RA and for the reduction of VA in SA. This finding suggests as tannins have a selective activity on ruminal bacteria population and confirmed previews studies showing different effects of the same type of tannins on different strain of bacteria (Jones et al., 1994; Min et al., 2005). Moreover the work of Vasta et al. (2010) confirmed, in vivo, the ability of condensed tannins to inhibit the last step of biohydrogenation of UFA, consisting in the enzymatic reduction of VA to SA, as suggested by previous in vitro studies (Khiaosa-Ard et al., 2009; Vasta et al., 2009a).

Diet composition is another factor that can influence the effect of polyphenols on ruminal biohydrogenation. The inclusion of quebracho powder (4 % tannins of DM in the diet), was able to affect ruminal biohydrogenation in sheep, as evidenced by the increase of the VA concentration (Vasta et al., 2009b); moreover in this trial the inclusion of tannins was tested with two different dietary treatments, herbage and concentrate; the authors found that tannins exert a greater effect when are included in

the diet based on concentrate than in the forage as suggested by the finding that the first treatment leads to a greater accumulation of VA in the rumen than second one (+ 96.78 % and + 19.75 %, respectively). As supposed by the authors the greater extent of biohydrogenation could be related to the higher NDF content in the forage than in the concentrate, which could have promote the proliferation of cellulolytic bacteria as B. *fibrisolvens*, mainly responsible of biohydrogenation.

The activity of ruminal microflora leads to the formation of an important class of FA: the odd- and branched-chain fatty acids (OBCFA) (Fievez et al., 2012). It is well known that the synthesis of this class of FA is to a great extent influenced by the dietary treatment, because of its effect on the relative abundance of ruminal microorganism (Vlaeminck et al., 2006). The depressive effect of polyphenols on the growth of ruminal microorganisms is reported to decrease the synthesis of OBCFA (Vasta et al., 2009a); moreover, as, among microorganism, individual OBCFA are produced in different levels, the profile of this class of FA have been proposed as a diagnostic tool to predict shift in microbial population related to the dietary variations (Lee et al., 1999, Vlaeminck et al., 2004).

1.3.4 Effect on quality of animal products

In the last decades the consumers have increasingly paid attention to the relationship between diets and prevention of chronic diseases. The inclusion of functional and nutraceutical compounds in animal food products has become an important issue for the producers, to satisfy consumers needs. The occurrence of plant phenolic compounds in food is of particular interest thanks to their health promoting properties. In addition the antioxidant power of these compounds can be used to reduce the oxidation processes, which take place in foods. In fact, polyphenols have been proposed as substitute of typical synthetic antioxidant such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), used in different type of food (Naveena et al., 2008; Aladedunye and Matthäus, 2014; Lorenzo et al., 2014). Dietary polyphenols, or their metabolites, can be transferred in the final products and can influence the quality and the characteristics of products, mainly thanks to their antioxidant properties (Monino et al., 2008; O'Grady et al., 2008).

1.3.4.1 Milk fatty acid profile

In ruminant, the effects of the dietary inclusion of polyphenols on the milk and dairy products depend, also, on the influence of these compounds on the rumen metabolism, particularly on the lipid fermentation. The major aim of polyphenols inclusion in ruminant nutrition, linked to amelioration of food, is the reduction of biohydrogenation of PUFA, and the increase of the accumulation of VA in rumen, increase the amount of these FA reaching mammary gland and, consequently, the amount PUFA, VA and CLA (derived from the enzymatic desaturation of VA) in milk. However, literature focusing on the alteration of ruminal biohydrogenation process and manipulation milk fatty acids profile is limited (Benchaar and Chouinard, 2009) and results are not univocal. Some works reported the ability of polyphenols to decrease SFA and increase the concentration of MUFA and PUFA in milk (Table 1.7). The LNA transfer rate from feed to milk was found to be influenced by the different flowering catch crop included in the diet of lactating dairy cows, according to the polyphenols content of the diets (Kälber et al., 2011). Turner et al. (2005) showed increased levels of ω-3 FA, LNA in particular, in milk from cows fed *Lotus cornicolatus* as source of tannins. In a recent work, the inclusion of pomegranate extract, as source of secondary metabolites, in the diet of lactating dairy cows (4.56, 5.58 and 6.60 g of total phenols/kg DM diet), was effectiveness in improving milk fatty acid profile, by decreasing the concentration of SFA and the ration n6/n3, and increasing the amount of EPA and DHA (Abarghuei et al., 2014b). Although it has been demonstrated the ability of polyphenols to inhibit the enzymatic reduction of VA to SA, no works report, to our knowledge, the increase of CLA in milk as consequence of this action. In the experiment of Toral et al. (2011) the supplementation of diet of dairy ewes, receiving a diet containing sunflower as source of LA, with a mixture (1:1, w/w; 10 g of tannins/kg DM) of two extracts of quebracho condensed tannins (CT) and chestnut hydrolysable tannins (HT) was not able to increase the amount of VA and RA in milk. A similar experiment, conducted with 20 g tannins/kg on DM (from quebracho extract), failed to raise beneficial fatty acids concentration in milk (Toral et al., 2013).

As suggested by Vasta et al. (2008), the lack of effect in the increasing RA concentration in milk by the inclusion of polyphenols in ruminant diet, could be related to a reduction in the rate of conversion of VA into RA in mammary gland, as several

studies have shown the inhibitory effect of PUFA on the Δ9-desaturase enzyme expression (Sessler and Ntambi, 1998; Vinknes et al., 2013). It was also hypothesized and demonstrated that dietary tannins (quebracho, 6 % of tannin on DM diet) can increase, indirectly by changing fatty acid composition in tissues, the Δ9-desaturase expression in lamb muscle (Vasta et al., 2009c); despite this finding, the concentration of RA in *longissum dorsi* muscle of lamb did not increase as expected. The authors suggested the presence of different Δ9-desaturase isoforms, regulated by different mechanisms, as possible explanation of these contrasting results.

1.3.4.2 Oxidative stability

Another aim, related to the inclusion of polyphenols in the diet of ruminants, is the improving of the oxidation stability of meat, milk and dairy products.

In fact, one of the major problems concerning the quality of the animal derived products is linked to their oxidative deterioration. The oxidation of unsaturated fatty acids are the major responsible event of this degradation, and results in the formation of lipid hydroperoxides (primary lipid oxidation products) which can be converted in free radicals and volatile compounds (secondary lipid oxidation products) (Bradley et al., 2006). The presence of oxygen (O'Brien and O'Connor, 2002), enzyme (Steffensen et al., 2002), metal ions (Timmons et al., 2001), and exposure to heat (Pikul et al., 2013) and light (Wisiiner, 1964) could promote oxidative damage of dairy products. Three pathways have been proposed for the lipid oxidation: auto-, photo- and enzymatic-oxidation.

Auto-oxidation

The auto oxidation is a free radical chain mechanism consisting in the formation of a highly reactive lipid free radical (initiation), occurring, often, from the reaction of unsaturated fatty acids with reactive oxygen species (ROS), also called “initiators”. In fact, the direct reaction between UFA and oxygen has a very high energy of activation, due to their dissimilar spin multiplicity, and is unlikely to occur under normal biological condition. The subsequently reaction between the formed lipid free radical and triplet oxygen leads to the formation of different radical species highly unstable, including peroxyradicals, which, react with other unsaturated fatty acids to form lipid

hydroperoxides and another lipid free radical (propagation). The oxidation process terminates when the free radicals combine to form stable compounds, or when antioxidants react with free radicals to form stable radical or non-radical compounds (termination) (Frankel, 1984). The hydroperoxides (primary oxidation compounds) formation involves the stabilization of the peroxilradicals, by delocalization of electrons and rearrangement of double bounds, leading to the formation of many hydroperoxides isomers. These primary oxidation compounds decompose quickly, to produce instable radicals, which decompose leading to the formation of several types of volatile and non volatile compounds (secondary oxidation products), such as alcohols, aldehydes, ketones, esters, hydrocarbons, furans, epoxides, lactones and polymers, mainly depending on the characteristics of the FA and on the conditions of the reaction.

Potho-oxidation

The photo-oxidation process starts with the interaction between light and naturally occurring photosensitizer compounds as flavins and chlorophylls.

Upon light exposure the electrons of the outer shell of photosensitizer became excitate, reaching high energy level, called singlet state. From this state the electron decays quickly to its excited triplet state. This strongly oxidizing species can directly react with a substrate (unsaturated fatty acids), by donating or accepting a hydrogen molecule or an electron (Type I mechanism); differently, it can transfer its energy by reacting with atmospheric oxygen to produce singlet oxygen, a reactive electrophilc compound, which reacts with electron rich molecular sites (Type II mechanism).

The type I and II mechanisms lead to the formation of lipid radical and lipid hydroperoxides, respectively. It has been hypothesized that singlet oxygen could be the main responsible of lipid oxidation, due to its high reactivity and capacity to react directly with unsaturated fatty acids (Rawls and Van Santen, 1970).

Enzymatic-oxidation

The enzymatic oxidation of unsaturated fat lead to the formation of lipid hydroperoxides; the enzyme involved in this reaction is the lipoxigenase, and can be found in animal tissues and in a wide range of variety of plant. The action of this enzyme is stereospecific for polyunsaturated fatty acids containing the *cis,cis* penta-1,4-diene unit (—CH═CH—CH_2—CH═CH—) (Whitaker, 1994). Consequently, monounsaturated fatty acids (MUFA) do not represent a substrate for this enzyme.

The potential antioxidant effect of polyphenols in food depends on the transmission of these compounds from feed to the different tissues, which depends on their metabolism and bioavailability. The transfer of dietary polyphenols into milk and dairy product has been documented. The presence of polyphenols, such as catechin, quercetin, ferulic acid and chlorogenic acid, in goat's cheese, was found to be related to the consumption of these compounds during grazing (Hilario et al., 2010). Jordan et al., (2010) reported the transfer of polyphenols to the milk, when distillate rosemary leaves were included in the diet of lactating goats. However not all phenolic compounds present in the diet were found in milk, as probably consequence of their metabolism. The same authors found the presence these compounds in the plasma of suckling goat, as probably consequence of their transfer during gestation and lactation periods. An analogous experiment evidenced similar results when distillate rosemary leaves were included in the diet of lactating ewes (Moñino et al., 2008). In this last experiment the transmission of polyphenols to the ewe's milk was suggested by the presence of these compounds in lamb meat. In the works of Hilario (2010) and Monino (2008) the presence of polyphenols in the diet of ruminants, and, consequently, in cheese and meat, respectively, was found able to improve oxidative stability of these products. In the work of Santos et al. (2014) the inclusion of grape residue silage in the diet of dairy cows (100 g/kg DM) increased the reducing power in milk, although no difference was found in the milk concentration of polyphenols. The authors explained this finding by the fact that high concentrations of phenolic compounds are not required to inhibit lipid oxidation, as previously reported by Sies and Stahl (1995).

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1.5 Tables

Table 1.1. Chemical composition of some traditional agro-industrial by-products used in ruminant nutrition

By products	Chemical composition[1]									Reference
	DM	OM	NDF	ADF	NFC	CP	Lignin	EE	Ash	
Barley straw	940	916	672	353	189	43	41	11	86*	Abbeddou et al., 2011
Barley straw	689	-	814	495	-	16	82	-	-	Castrillo et al., 1995
Barley straw	-	-	626	413	-	47	-	-	104	Blanco et al., 2014
Beet pulp	870	-	514	250	-	16	38	-	-	Castrillo et al., 1995
Beet pulp	921	953	488	296	375	89	37	0.4	47*	Abbeddou et al., 2011
Cottonseed hulls	895	-	878	664	-	61	224	-	27	Beckman and Weiss, 2005
Soybean hulls	913	937	621	468	154	140	74	22	63*	Zambom et al., 2012
Soybean hulls	-	-	678	486	189	118	270	15	50	Schwab et al., 2006
Corn gluten feed	-	-	441	107	228*	235	-	39	57	Segers et al., 2013
Linseed meal	939	-	231	199	178*	248	86	270	73	Ermias et al., 2013
Linseed meal	904	-	-	-	-	354	-	56	137	El-Saidy and Gaber, 2003
Sunflower seed meal	-	-	356	268	194*	355	80	18	75	Villamide and San Juan, 1998
Sunflower seed meal	959	-	-	-	-	420	-	124	76	El-Saidy and Gaber, 2003
Sunflower seed meal	-	-	466	323	-	312	-	15	71	Irshaid et al., 2003

Table 1.1. (continued)

By products	Chemical composition[1]									Reference
	DM	OM	NDF	ADF	NFC	CP	Lignin	EE	Ash	
Soybean meal	880	935	139	87	270	502	30	23	66*	Zambom et al., 2012
Soybean meal	894	933	148	103	-	495	-	21	-	Grande et al., 2014
Soybean meal	930	-	-	-	-	480	-	48	60	El-Saidy and Gaber, 2003
Cottonseed meal	957	939	606	407	-	253	76	55	-	Solomon et al., 2008
Cottonseed meal	903	-	-	-	-	410	-	147	79	El-Saidy and Gaber, 2003
Cottonseed meal	934	935	248	200	240	431	65	16	65*	Abbeddou et al., 2011
Broken lentils	969	921	283	41	436	245	20	5.3	135*	Abbeddou et al., 2011

[1]Data expressed as g/kg DM.
*Value calculated by author, according to Weiss (1999) as follows: NFC (g/kg DM) = 100 − (NDF + CP + ash + EE).

Table 1.2. Chemical composition of some alternative agro-industrial by-products used in ruminant nutrition

By products	Chemical composition									Reference
	DM	OM	NDF	ADF	NFC	CP	Lignin	EE	Ash	
Apple	179	-	107	80	842	4	24	-	-	Rodrigues et al., 2008
Pomegranate (seeds)	951	-	680	490	135	154	-	6	24	Mirzaei-Aghsaghali et al., 2011
Pomegranate (peel)	961	-	208	151	696	36	-	6	54	Mirzaei-Aghsaghali et al., 2011
Orange residue (fresh)	219	-	227	171	657*	60	17	24	32	Villanueva et al., 2013
Citrus pulp	904	831	194	128	510*	77	-	49	168	Fegeros et al., 1995
Pistachio	900	755	259	-	-	153	-	58	-	Ghasemi et al., 2012
Wet tomato pomace	142	-	636	435	-	195	-	-	-	Denek and Can, 2006
Tomato fruit	69	-	260	217	465*	170	195	28	77	Ventura et al., 2009
Tomato pomace	952	952	552	462	109	191	259	100	48*	Abbeddou et al., 2011
Tomato whole plant	177	-	457	356	276*	74	128	12	181	Ventura et al., 2009
Ficus bengalensis	-	852	425	369	279*	109	-	39	148	Dey and De, 2014
Olive cake	947	864	584	459	109	79	237	92	136*	Abbeddou et al., 2011
Olive cake	805	901	676	544	-	73	289	54	-	Molina-Alcaide and Yáñez-Ruiz, 2008
Olive cake (exhausted)	950	-	683	531	317	102	367	12	97	Tufarelli et al., 2013

[1]Data expressed as g/kg DM.
*Value calculated by author according to Weiss (1999) as follows: NFC (g/kg DM) = 100 − (NDF + CP + ash + EE).

Table 1.3. Effect of dietary inclusion of some agroindustrial by-products on nutrients apparent digestibility in ruminants

By products	Experiment	Level in diet[1]	Apparent digestibility[2]							Reference
			DM	NDF	NFC	ADF	CP	OM	EE	
Barley straw	lamb	50 g/kg FW	0.82 =	0.40 =	-	0.31 =	0.78 =	-	-	Blanco et al., 2014
Barley straw	lamb	250 g/kg FW	0.71 ↓	0.41 =	-	0.35 =	0.75 =	-	-	Blanco et al., 2014
Beet pulp	sheep	340 g/kg DM	-	0.65 ↑	0.76 =	-	0.68 =	0.68 =	0.06 ↓	Abbeddou et al., 2011
Corn gluten feed	heifers	250, 500 g/kg DM	0.63* =	0.51* =	-	-	0.60* =	0.72* =	-	Hussein and Berger, 1995
Soybean hulls	goat	413 g/kg DM	0.63 ↑	0.57 ↑	0.71 =	-	0.60 =	0.67 ↑	0.79 ↑	Zambom et al., 2012
Cottonseed meal	goat	400 g/kg DM	0.72 ↑	0.71 =	-	0.67 =	0.73 ↑	0.75 ↑	-	Solomon et al., 2008
Linseed meal	sheep	431 g/kg DM*	1.00 ↑	0.73 ↑	-	0.82 ↑	0.94 ↑	0.98 ↑	-	Ermias et al., 2013
Sunflower meal	sheep, goat	200 g/kg DM	0.57* =	0.34* ↓	-	0.28* ↓	0.84* ↑	0.59* =	-	Economides, 1998
Tomato pomace	sheep	340 g/kg DM	-	0.55 =	0.63 ↓	-	0.63 ↓	0.59 ↓	0.75 ↑	Abbeddou et al., 2011
Tomato	goat	125 g/kg DM	0.68 =	0.55 =	-	0.46 =	0.76 =	0.71 =	0.73 =	Romero-Huelva et al., 2012
Broken lentils	sheep	340 g/kg DM	-	0.62 ↑	0.82 =	-	0.68 =	0.69 =	0.20 ↓	Abbeddou et al., 2011
Olive cake (exhausted)	lamb	111 g/kg DM*	0.58 ↓	0.57 =	-	0.44 =	0.69 =	0.73 =	0.68 =	Tufarelli et al., 2013
Olive cake (exhausted)	lamb	223 g/kg DM*	0.57 ↓	0.53 ↓	-	0.43 ↓	0.68 =	0.72 =	0.68 =	Tufarelli et al., 2013
Olive cake	sheep	340 g/kg DM	-	0.43 ↓	0.46 ↓	-	0.64 ↓	0.48 ↓	0.73 ↑	Abbeddou et al., 2011
Wet tomato pomace	*vitro*	-	56.59	50.70	-	38.59	62.08	58.63	-	Denek and Can, 2006
Orange residue (fresh)	*vitro*	-	72.3		-	-	-	-	-	Villanueva et al., 2013
Cottonseed meal	*vitro*	-	79.4	89.1	-	-	95.2[†]	-	-	Carrera et al., 2012
Sunflower meal	*vitro*	-	61.0	64.4	-	-	91.5[†]	-	-	Carrera et al., 2012

[1]Data expressed as g of ingredient/kg DM of diet
[2]Expressed as g/g
*calculated by author.
[†]expressed as ruminal degradable protein + intestinal digestibility of ruminal undegradable protein.
↑: increased, ↓: decreased, =: not changed, value compared to the control ($P < 0.05$).

Table 1.4. Chemical composition, mineral composition and total phenolic content of grape seed from red and white Italian cultivars of *Vitis vinifera*

Grape seed	Value
Chemical composition (g/kg of DM)	
Organic Matter	961
Neutral detergent fiber	530
Acid detergent fiber	504
Crude protein	125
Ether extract	128
Lignin	426
Mineral composition (g/kg of DM)	
Ca	5.4
P	3.1
K	7.5
Mg	1.2
S	1.3
Zn[a]	13
Mn[a]	21
Fe[a]	61
Cu[a]	44
Total phenols (g/kg of DM)	70

From Spanghero et al. (2009).
[a]Expressed as mg/kg DM.

Table 1.5. Fatty acid profile of grape seed oil

Fatty acid (% of total FA)*	Grape cultivars									
	Barbera[1]	Moscato[1]	Nebbiolo[1]	Aragones[2]	Tinto cao[2]	Periquita[2]	Chardonnay[3]	Concord[3]	Merlot[4]	Syrah[4]
C14:0	0.07	0.05	0.06	0.06	0.06	0.07	-	-	0.05	0.07
C16:0	6.66	8.89	6.53	7.28	7.00	6.17	7.75	7.05	7.07	7.30
C16:1	-	-	-	0.13	0.15	0.18	-	-	0.08	0.13
C17:0	0.05	0.05	0.06	0.07	0.07	0.08	-	-	-	-
C18:0	4.04	2.84	4.16	4.09	4.85	5.04	3.63	2.52	3.95	4.40
C18:1 *cis*-9	16.00	15.30	13.60	14.80	16.10	17.10	19.30	13.90	13.13	15.10
C18:2 *n*-6	71.70	71.00	74.30	72.30	70.80	70.20	68.80	75.30	73.23	70.15
C18:3 *n*-3	0.47	0.46	0.43	0.51	0.47	0.49	0.25	0.41	0.44	0.67
C20:0	0.14	0.14	0.18	0.22	0.23	0.24	-	-	0.17	0.20
C20:1	0.13	0.11	0.15	0.19	0.17	0.19	0.12	0.57	-	-
C20:2 *n*-6	0.04	0.04	0.04	0.05	0.05	0.04	-	-	-	-
SFA	12.80	10.20	11.60	11.73	12.25	11.64	11.50	9.66	11.24	11.97
MUFA	15.20	18.80	14.30	15.19	16.50	17.49	19.50	14.50	13.21	15.23
PUFA	72.00	71.00	74.10	72.99	71.44	70.81	69.00	75.80	73.67	70.82
PUFA/SFA	5.63	6.96	6.39	6.22	5.83	6.08	6.00	7.85	6.55	5.92

* SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
[1] Fiori et al., 2014: seeds from grape mark obtained by winemakers in Northern Italy, for the harvesting years of 2011 and 2012.
[2] Fernandes et al., 2013: seeds obtained from red varieties of grape harvested at their ripening time in Valpaços, north- east of Portugal.
[3] Lutterodt et al., 2011: cold-pressed grape seed oils were obtained from Botanical Oil Innovations Inc. (Spooner, Wisconsin).
[4] Beveridge et al., 2005: seeds from grape pomace obtained during the fall crush of 2001 from Calona Wines Ltd. of Kelowna, BC, Canada.

Table 1.6. Effect of dietary polyphenols on DMI, apparent digestibility and ruminal metabolism

Source of polyphenols	Level g/kg DM	Species	DMI	pH	NH_3	CH_4	Total VFA	Ac	Pr	Bu	Ac:Pr	Digestibility	Ruminal microflora	Reference
Lotus cornicolatus	4.84	cow	ns	ns	ns	-	ns	ns	ns	ns	ns	NDF, ADF, Hemicellulose ↓	-	Hymes-Fecht et al., 2013
Lotus cornicolatus	7.24	cow	ns	ns	ns	-	ns	ns	ns	ns	ns	CP, Hemicellulose ↓	-	Hymes-Fecht et al., 2013
Lotus cornicolatus	9.71	cow	ns	ns	ns	-	ns	ns	ns	ns	ns	DM, OM, NDF ↑, CP ↓	-	Hymes-Fecht et al., 2013
Lotus cornicolatus	35	lamb	ns	-	↓	-	ns	ns	ns	ns	-	CP ↓	protozoa ↓	Wang et al., 1994
Carob pulp	25	lamb	↓	ns	↓	-	↓	ns	ns	ns	-	DM, NDF, CP ↓	-	Priolo et al., 2000
Flowering Sulla	40	sheep	ns	-	-	-	-	-	-	-	-	DM, CP ↓	-	Molle et al., 2009
Quebracho extract	30	cow	↓	ns	ns	-	↓	↑	↑	↑	↓	ns	-	Dschaak et al., 2011
Quebracho extract	30	cow	↓	ns	ns	-	↓	ns	ns	ns	↑	ns	-	Dschaak et al., 2011
Quebracho extract	7.5	sheep	ns	-	-	-	-	-	-	-	-	ns	-	Al-Doibaib, 2009
Quebracho extract	15	sheep	ns	-	-	-	-	-	-	-	-	CP ↓	-	Al-Doibaib, 2009
Quebracho extract	22.5	sheep	ns	-	-	-	-	-	-	-	-	OM, NDF, ADF, CP ↓	-	Al-Doibaib, 2009
Quebracho extract	4.2	heifers	ns	ns	ns	-	↓	ns	ns	ns	ns	ns	-	Baah et al., 2007

Table 1.6. (Continued)

Source of polyphenols	Level, g/kg DM	Species	DMI	pH	NH_3	CH_4	Total VFA	Ac	Pr	Bu	Ac:Pr	Digestibility	Ruminal microflora	Reference
Quebracho extract	9, 18	cattle	ns	-	↓	ns	↓	↓	ns	ns	↓	CP ↓	-	Beauchemin et al., 2007
Quebracho extract	7.86	sheep	↓	ns	-	-	-	-	-	-	-	-	Protozoa↑, tot. bacteria ns, fibrisolvens ↑, proteoclasticus ↓	Vasta et al., 2010
Quebracho extract	4.5, 18, 28, 83	Sheep, cow	ns	ns	ns	-	ns	ns	ns	ns	ns	ns	ns	Toral et al., 2013, Gonzalo et al., 2003, Benchaar et al., 2008
Quebracho Extract + chestnut	10	sheep	ns	ns	ns	-	ns	ns	ns	ns	-	-	-	Toral et al., 2011
Rain tree pod meal	5.28	cow	ns	ns	↓	↓	ns	↓	↑	ns	↓	-	Protozoa↓, fibrisolvens ↑, methanogens ↓	Anantasook et al., 2014
Grape pomace	40.7	sheep	-	-	↓	-	-	-	-	-	-	DM, OM, NDF, CP ↓	Protozoa↓	Abarghuei et al., 2010
Dried grape marc	5.6	cow	ns	↓	ns	↓	ns	↓	ns	ns	-	-	ns	Moate et al., 2014
Ensiled grape marc	2.2	cow	ns	ns	ns	↓	ns	ns	ns	↑	-	-	ns	Moate et al., 2014

↑:increased, ↓: decreased, ns: not significant; values compared to the control ($P < 0.05$)

Table 1.7. Effect of dietary polyphenols on milk fatty acids composition

Source of polyphenols	Level (g/kg DM)	Species	SA	VA	RA	LA	LNA	DHA	SFA	UFA	MUFA	PUFA	Reference
Pomegranate extract	1.92	cow	↓	-	-	ns	ns	↑	↓	-	ns	ns	Abarghuei et al., 2014
Pomegranate extract	2.80	cow	↓	-	-	ns	↑	↑	↓	-	ns	ns	Abarghuei et al., 2014
Pomegranate extract	3.66	cow	-	-	-	ns	ns	ns	↓	-	ns	ns	Abarghuei et al., 2014
Quebracho extract	30	cow	ns	ns	ns	ns	↑	-	-	-	-	-	Dschaak et al., 2011
Dried grape marc	5.6	cow	↑	↑	↑	↑	ns	-	↓	-	↑	↑	Moate et al., 2014
Ensiled grape marc	2.2	cow	↑	↑	↑	↑	ns	-	↓	-	↑	↑	Moate et al., 2014
Flowering sulla	40	sheep	ns	↓	↓	↑	↑	↑	-	-	ns	ns	Cabiddu et al., 2009
Quebracho tannins	> 4.5	sheep, cow	ns	ns	ns	ns	ns	-	ns	ns	ns	ns	Benchaar and Chouinard., 2009; Toral et al., 2011, 2013

↑:increased, ↓: decreased, ns: not significant; values compared to the control ($P < 0.05$).

1.6 Figures

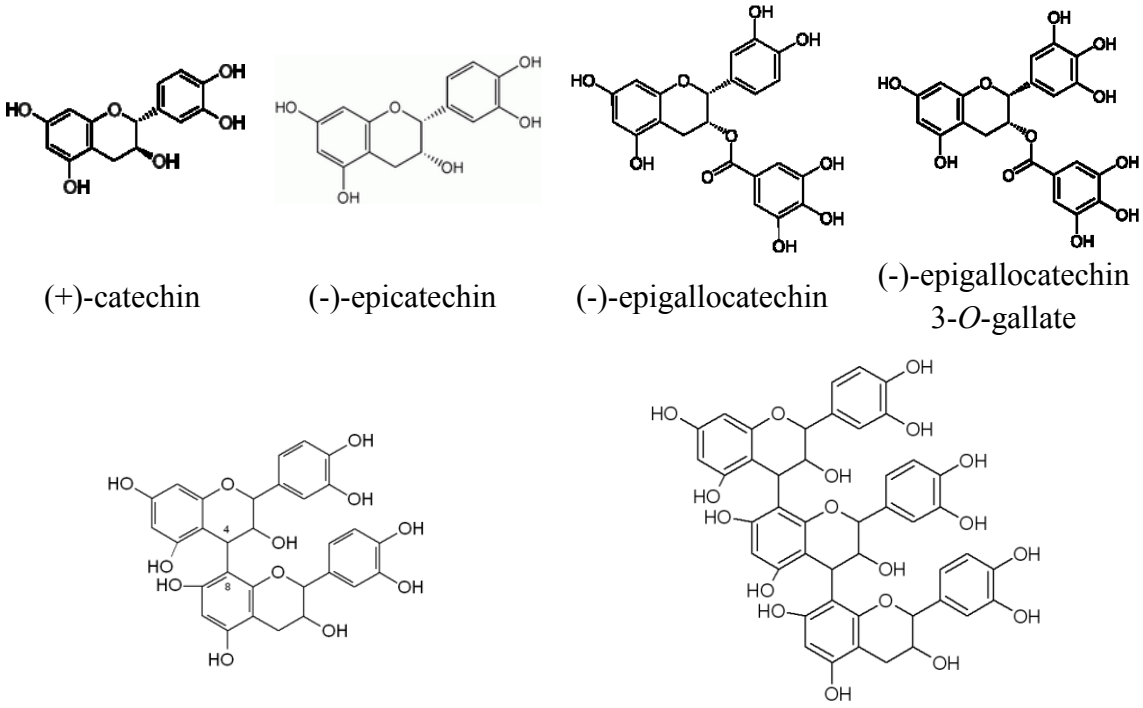

Figure 1.1. Structures of most common grape seed polyphenols

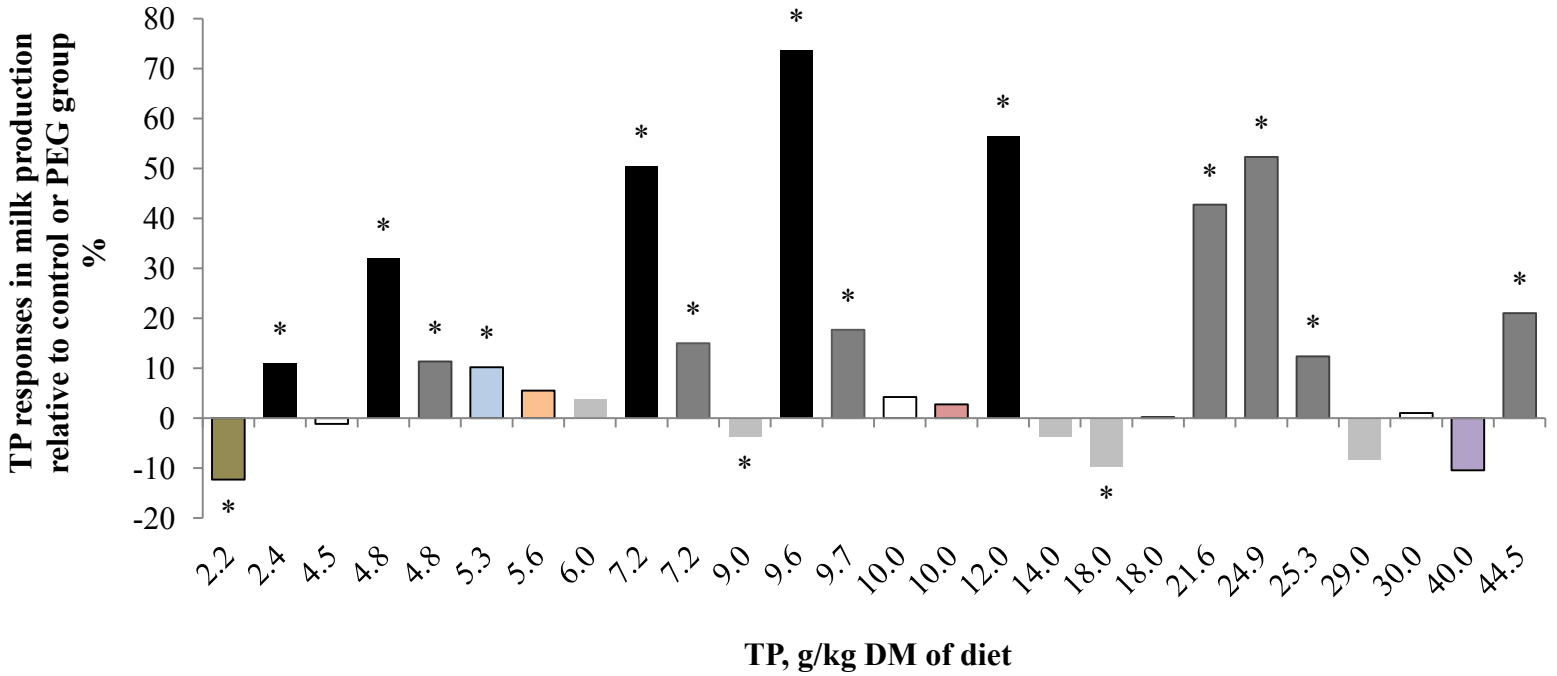

Figure 1.2. Effect of phenol concentration on milk production of ruminants, calculated as percentage compared with a control group or a group treated with PEG; * indicates a significant difference ($P < 0.05$) compared with the control group. (■), cassava hay; (■), L. *cornicolatus*; (■), acacia *mearnsii*; (■), chestnut tannins; (■), ensiled grape marc; (■), dried grape marc; (■), rain tree pod meal (Samanea saman); (■), flowering sulla; (□), quebracho extract. Sources: Wang et al., 1996b; Harris et al., 1998, 1999; Woodward et al., 2000; Dung et al., 2005; Benchaar et al., 2008; Benchaar and Chouinard, 2009; Cabiddu et al., 2009; Grainger et al., 2009; Molle et al., 2009; Dschaak et al., 2011; Griffiths et al., 2013; Hymes-Fecht et al., 2013; Toral et al., 2011, 2013; Liu et al., 2013; Anantasook et al., 2014; Moate et al., 2014.

CHAPTER 2

Effects of diets containing grape seed, linseed or both on milk production traits, liver and kidney activities, and immunity of lactating dairy ewes

A. Nudda, F. Correddu, A. Marzano, G. Battacone, P. Nicolussi, P. Bonelli, and G. Pulina

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2.1 Abstract

This study aimed to evaluate the effects of the dietary inclusion of grape seed, alone or in combination with linseed, on milk production traits, immune response and liver and kidney metabolic activity of lactating ewes. Twenty-four Sarda dairy ewes were randomly assigned to four dietary treatments consisting of a control diet (CON), a diet containing 300 g/d per head of grape seed (GS), a diet containing 220 g/d per head of extruded linseed (LIN), and a diet containing a mix of 300 g/d per head of grape seed and 220 g/d per head of extruded linseed (MIX). The study lasted 10 weeks, with two weeks of adaptation period and 8 weeks of experimental period. Milk yield was measured and samples were collected weekly and analyzed for fat, protein, casein, lactose, pH, milk urea nitrogen (MUN) and somatic cell count (SCC). Blood samples were collected every two weeks by jugular vein puncture and analyzed for hematological parameters, for albumin, alkaline phosphatase (ALP), bilirubin, creatinine, gamma glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), protein, blood urea nitrogen (BUN), and for anti-OVA IgG, IL-6 and $CD4^+$ and $CD8^+$ cells. On d 0, 45 and 60 of the trial, lymphocyte response to phytohemagglutinin (PHA) was determined in vivo on each animal by measuring skin-fold thickness (SFT) at the site of PHA injection. Humoral response to chicken egg albumin (OVA) was stimulated by a subcutaneous injection with OVA. Dietary treatments did not affect milk yield and composition. MUN and lactose were affected by diet × period. Diets did not influence hematological, kidney and liver parameters, except for BUN, which decreased in LIN and increased in MIX compared with CON and GS. Dietary treatments did not alter $CD4^+$, $CD8^+$ and $CD4^+$:$CD8^+$ ratio. The SFT was reduced in GS and MIX and increased in LIN compared with CON. The IgG and IL-6 were affected by diet × period. The reduction in IgG on d 60 and SFT in ewes fed

grape seed suggests an immunomodulatory effect of this residue. The limited variation in milk, hematological and metabolic parameters suggests that grape seed and linseed can be included, alone or in combination, in the diet of dairy ewes without adverse effects on milk production and health status.

2.2 Introduction

Grape seed is a by-product resulting from the winery and distillery industry which requires costly disposal practices. This residue contains an oil characterized by a high content of linoleic acid and by the presence of α- and β-tocotrienols and tocopherols, which exhibit strong antioxidant activity (Beveridge et al., 2005). Moreover, grape seeds are rich in polyphenolic compounds, such as proanthocyanidins mainly composed of catechin, epicatechin, gallic acid and polymeric and oligomeric procyanidins, which can be recovered by extraction (Monagas et al., 2003; González-Paramás et al., 2004). These polyphenols have antioxidant (Sharma et al., 2007), antiatherosclerotic (Leifert and Abeywardena, 2008), antibacterial, anticarcinogenic and anti-inflammatory (Vaid et al., 2011; Cheah et al., 2014; Cedó et al., 2014) actions.

The literature available on the use of grape by-products in ruminant diet is limited, probably because of their high fiber and lignin content (Spanghero et al., 2009) and the presence of secondary compounds (Makris et al., 2007; Spanghero et al., 2009), which may reduce the energy content and the digestibility of the diet (Baumgärtel et al., 2007). On the other hand, the phenolic compounds linked to the hemicellulose and the acid-soluble lignin fractions in different feedstuffs (corn bran, barley grains, and barley bran) are active antioxidants (Ohta et al., 1994; Cruz et al., 2001; Yu et al., 2001). Recently, a couple of studies have focused on the effects of the dietary use of grape residues in dairy cows. Moate et al. (2014) found that pelleted and ensiled grape marc reduced methane emissions and decreased milk fat yield, whereas only ensiled grape marc reduced milk yield in Holstein-Friesian dairy cows. Santos et al. (2014) reported that grape residue silage improved milk quality by increasing anti-oxidant activity, without influencing milk yield in Holstein dairy cows. Therefore, the release of phenolic compounds with antioxidant or anti-inflammatory activity during the fermentation of grape seed in the rumen may be expected. For this reason, the use of grape by-products in the diet of ruminants could be not only an alternative for their disposal, but also potentially beneficial to milk production and the immune system.

Linseed has been largely studied in ruminant diets to enhance the polyunsaturated fatty acids (PUFA) in milk fat (Nudda et al., 2014). This feedstuff showed positive effects in small monogastrics, such as mice (Lal Bhatia et al., 2006), broilers (Voljc et al., 2011)

and rabbits (Trebušak et al., 2014), by reducing the level of oxidative stress and improving the antioxidant defense system. However, the few studies conducted about the effects of dietary linseed on oxidative stress and immunity in ruminants have had contradictory results. For example, in dairy cows some experiments evidenced that linseed has antioxidative properties (Schogor et al., 2013), whereas others evidenced a pro-inflammatory effect (Caroprese et al., 2009). Comparing the effects of diets containing grape seed and linseed could help to elucidate their individual or combined effects on health status and production in ruminants.

The aim of this experiment was to test the effects of the dietary inclusion of grape seed, linseed, or both on milk production traits, immune response, and liver and kidney metabolic activity in lactating dairy ewes.

2.3 Materials and methods

Animals, Design, Treatments and Samples Collection

Twenty-four Sarda dairy ewes in the first part of lactation (< 50 DIM) were allocated to 4 groups (6 animals per group) balanced for milk yield, BW, DIM, and number of lactation. Each group was confined in a pen and then randomly assigned to 1 of 4 experimental diets: a control diet (CON), a diet containing 300 g/d per head of grape seed (GS), a diet containing 220 g/d per head of extruded linseed (LIN), and a diet containing a mix of 300 g/d per head of grape seed and 220 g/d per head of extruded linseed (MIX). The grape seed was obtained from different red grape varieties after winemaking and distillation processes, followed by drying (97% DM). Whole grape seeds were finely ground to obtain a grape seed meal before their use. The dose of 300 g/d per head of grape seed (total phenolic content: 333.3 ± 10.1 mg of gallic acid equivalent (GAE)/100 g of DM) was used to provide 1 g/d of total polyphenols. The dose of 220 g/d per head of extruded linseed was used to supply 70 g/d of fat.

The ingredients and chemical composition of the diets are given in Table 2.1. Sheep received a common ration consisting of beet pulps, a commercial concentrate, chopped dehydrated alfalfa and mixed hay. In addition, the ewes were offered a mixed meal composed of corn, soybean, peas, grape seed and linseed, at varying levels depending on the dietary treatment. The different levels of corn, soybean and peas in the mixed

meal used in the study aimed to obtain isoenergetic and isonitrogenous diets. Chopped dehydrated alfalfa and mixed hay were offered to each group of six ewes in the pens (group feeding). All other dietary ingredients were provided to each animal by using individual feeders and their intake was monitored and measured individually.

The daily dose of commercial concentrate was provided at the two daily milkings (0730 and 1730 h). The mixed meal was offered two hours after each milking. Subsequently, beet pulp and then dehydrated alfalfa were provided. The mixed hay was provided during the night. Clean water was always available. The study lasted 10 weeks, with two weeks of adaptation period and 8 weeks of experimental period.

Diets were formulated to meet the energy and protein requirements of the dairy ewes using the Small Ruminant Nutrition System (SRNS) model (Tedeschi et al., 2010). The nutritive value of the diets, estimated by the SRNS model, and the calculated metabolizable energy supplied are reported in Table 2.1. The fat content in the diets was 2.0, 3.2, 5.1 and 5.8% DM in the CON, GS, LIN and MIX diets, respectively (Table 2.1).

Samples of dietary ingredients were collected once before the beginning of the experiment for DM determination and chemical analysis. BW was monitored at the beginning, in the middle and at the end of the trial. On d 7, 14, 21, 28, 35, 42, 49 and 56, milk yield was recorded and milk samples were collected at the two daily milkings. On d 0 (before starting the experimental treatment), 15, 30, 45 and 60 of the experiment, a blood sample was taken from the jugular vein of each ewe and collected in a heparinized vacuum tube (Becton Dickinson, Plymouth, UK). Blood samples were centrifuged at 1,200 × *g* for 15 min at 25°C to separate plasma from the corpusculate fraction.

Feed and Milk Analysis

The dry matter (DM) content of the feed was determined by oven-drying at 105°C for 24 h. Dried feed samples were analyzed for NDF, ADF and ADL, using the filter bag equipment of Ankom (Ankom Technology Corp., Fairport, NY; Van Soest et al., 1991), for ash (AOAC, 2000; method 942.05), for CP (AOAC, 2000; method 988.05) and ether extract (EE) (AOAC, 2005; method 920.39). NFC was calculated according to Weiss (1999) as follows: NFC (g/kg DM) = 100 − (NDF + CP + ash + EE). These parameters

were expressed as percentage of DM. To analyze the phenolic content in the grape seed, an aliquot of randomly collected grape seeds was powdered by a blender. The powder (1.5 g) was then homogenized with 50 mL of a 70% acetone-water mixture using an Ultra Turrax homogenizer (Ultra Turrax T25, Janke & Kunkel KG, Staufen, Germany) at 3000 rpm for 2 min in a water-ice bath. Then the homogenate was centrifuged at 6000 × *g* for 15 min at 4°C, and the supernatant was filtered through Whatman 541 filter paper (Whatman, Maidstone, England). The concentration of phenolic compounds in the grape seed was then determined by the Folin-Ciocalteu method as decribed by Kim et al. (2003), with some modifications. Briefly, 0.5 mL of the supernatant were added to 1 mL of Folin-Ciocalteu phenol reagent (Sigma Chem. Co., St. Louis, MO) and 9.5 mL of bidistilled water in a 25 mL volumetric flask, and shaken. After 7 min, 10 mL of 7.5 % sodium carbonate (Na_2CO_3) were added, and the mixture was diluted to volume (25 mL) with bidistilled water and mixed thoroughly. The mixture was then stored in the dark for 120 min at room temperature. The absorbance was read at 750 nm against a blank solution. Total phenolic content was expressed as mg of gallic acid equivalent (GAE)/100g of DM, following a calibration curve obtained using gallic acid (Sigma Chem. Co.) as the standard.

Individual milk samples from the morning and afternoon milkings were analyzed separately for fat, protein, casein, lactose and MUN using a Milkoscan 6000 instrument (Foss Electric, Hillerød, Denmark), for SCC using a Fossomatic 360 instrument (Foss Electric) and for pH. The value of each parameter was calculated as weighted average of the morning and afternoon data.

Blood Analysis

A clinical chemistry system (Dimension RXL, Dade Behring, Ventura, CA) was used to quantify albumin, alkaline phosphatase (ALP), bilirubin, creatinine, gamma-glutamyl transferase (GGT), aspartate transferase (AST), alanine transferase (ALT), protein, urea nitrogen (BUN) in serum samples. The white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, lymphocytes (LYM), monocytes (MONO), neutrophil granulocytes (NEUT), eosinophils granulocytes (EOS) and basophiles granulocytes (BASO), were

determined in blood samples using an electronic particle counter (MS9; Melet Schloesing Laboratoires, Osny, France).

Immunological Determinations

Skin test. On d 0, 45, and 60, lymphocyte proliferation was determined in vivo in each ewe by measuring the changes in the skin-fold thickness (SFT) in response to an intradermal injection with 1 mg/mL of phytohemagglutinin (PHA; Sigma-Aldrich, Poole, Dorset, UK) dissolved in 1 mL of sterile saline solution. At each sampling, the injection was administered into the center of a 2-cm-diameter circle marked on shaved skin on each shoulder. SFT was measured with a caliper. The change in SFT (expressed in mm) was calculated as the difference between the 24-h post-injection thickness and the pre-injection thickness.

Humoral response to chicken egg albumin. At the start of the experiment (d 0), 6 mg of chicken egg albumin (OVA; Sigma-Aldrich) dissolved in 1 mL of sterile saline solution and 1 mL of incomplete Freund's adjuvant (Sigma-Aldrich, Poole, Dorset, UK) were injected subcutaneously into an inner thigh of each ewe. A subsequent injection of 6 mg of OVA in saline solution without adjuvant was administered on d 15.

ELISA test. The anti-OVA IgG titers in ewe plasma samples were determined by an ELISA test performed in 96-well U-bottomed microtiter plates according to Caroprese et al. (2009). In particular, wells were coated with 100 μL of antigen (10 mg of OVA/mL of PBS) at 4°C for 12 h, washed, and incubated with 1% skimmed milk (200 μL) at 37°C for 1 h to reduce nonspecific binding. After washing, 100 μL of plasma (1:5,000 dilution in PBS) per well were added and incubated at 37°C for 1 h. The extent of antibody binding was detected using a horseradish peroxidase-conjugated donkey anti-ovine IgG (Sigma-Aldrich; 1:20,000 dilution in PBS; 100 μL per well). Optical density was measured at 450 nm wavelength. Plasma samples were read against a standard curve obtained by scalar dilution of ovine IgG. Data were expressed as μg of anti-OVA IgG per 100 μL. Interleukin-6 (IL-6) in plasma was detected as reported by Caroprese et al. (2009). The IL-6 data were expressed as optical densities.

Determination of $CD4^+$ and $CD8^+$ cells. Plasma samples were used analyzed with flow cytometry to determine lymphocyte T-helper ($CD4^+$) and lymphocyte T-cytotoxic ($CD8^+$) cells as reported by Bonelli et al. (2013). Cells were counted in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA) and concentration was adjusted to

10^6 cells/mL. Monoclonal antibodies (mAbs) were used to perform three- and four-colour immunostaining protocols.

Statistical Analysis

Data of milk yield and composition, hematological, liver and kidney parameters were analyzed with a mixed model using PROC MIXED of SAS version 9.2 (SAS Institute Inc., Cary, NC). The model included the fixed effects of diet, period, and the diet × period interaction, plus the random effects of ewe nested within treatment. Variables obtained from blood analysis on samples collected before the beginning of the trial (i.e., on d 0) were subjected to an one-way ANOVA with the experimental group as fixed effect. The group effect was significant only for the immunological parameters ($P < 0.0001$); therefore, the value on d 0 was included as a covariable in the final model. Means were separated using Tukey test ($P < 0.05$).

2.4 Results and discussion

During the experiment the ewes were fed in pens, one per group, and received the chopped dehydrated alfalfa and the mixed hay as a group, whereas the other ingredients were provided and measured individually. This poses a limitation to the study, because it was not possible to measure the individual intake of the forages. Thus, the total dry matter and energy intake should be considered at a group level and, in this sense, individual sheep cannot be considered as experimental units and the experiment is an un-replicated demonstration. Group feeding of the forages was chosen to avoid the inevitable stress caused by individual confinement of lactating ewes in this long-term study (10 weeks in total), whose goals were to evaluate production, immunological and hematological responses to feeds with high antioxidant activity. Since individual confinement was reported to affect the immune system of sheep (Degabriele and Fell, 2001) and sheep are notoriously gregarious animals, in this experiment they were kept in groups to favor normal eating and social behaviors (Hutson, 2007).

During the trial, the feedstuffs supplied individually including those objective of the study, i.e., grape seed and linseed, were completely eaten by the animals of all groups. This result and the fact that the group intake of the dehydrated alfalfa and mixed hay

was complete, suggest that the inclusion of grape seed, linseed or both in the diet of lactating ewes did not negatively affect their DM and energy intake.

The BW did not differ ($P > 0.05$) between groups (43.2 ± 0.7 kg, mean ± SD), and increased with period ($P < 0.01$), reaching 49.1 ± 0.7 kg (mean ± SD) at the end of the trial.

Milk Yield and Composition

Milk yield and composition of the experimental groups are reported in Table 2.2 and Figure 2.1. Dietary treatments did not influence milk yield and composition ($P > 0.05$). Almost all milk parameters were influenced by period ($P < 0.001$). Lactose ($P < 0.001$) and MUN ($P < 0.05$) were affected by the diet × period interaction, but differences among diets did not reach the level of significance within each period (Figure 2.1). Grape seed in the diet of dairy ewes did not reduce yield of milk or yields of milk constituents when compared with the control diet. Similarly, Santos et al. (2014) observed no effect of the dietary inclusion of grape-residue silage on milk production and protein, fat and lactose concentrations in dairy cows. Although milk yield did not differ significantly ($P > 0.05$) among the four dietary groups, numerically greater values were observed in the two groups fed linseed (LIN and MIX) than in CON and GS. This finding is in agreement with previous observations reported in several studies on dairy goats (Nudda et al., 2013), cows (Caroprese et al., 2010; Hurtaud et al., 2010) and ewes (Gómez-Cortés et al., 2009). In our study, the differences in ME content among diets did not correspond to numerical differences in milk yield, because, as planned, ME supplied was similar among groups. In fact, the greater NFC content and the lower NDF and ADL content in the CON diet was compensated by the greater fat content of the extruded linseed in the LIN and MIX diets (Table 2.1). Therefore, the numerically higher values of milk yield in the two groups containing linseed than GS and CON may have been caused by differences in the characteristics of the nutrients, especially fat as energy source, among the diets. In all groups, milk yield decreased with period ($P < 0.05$, data not shown), following the general pattern of the lactation curve in dairy sheep (Macciotta et al., 1999).

Although MUN did not differ significantly among diets within period, the values of MUN in the animals fed LIN and, to a lesser extent, MIX showed lower values during the trial compared with the other groups (Figure 2.1). This finding agrees with other

studies in linseed-fed dairy cows (Pezzi et al., 2007) and goats (Nudda et al., 2013). We hypothesize he ewes fed linseed may have taken advantage of the low ruminal CP degradability resulting from the extrusion treatment (Solanas et al., 2005) and of the high lipid content of linseed, which can decrease protein degradability. The lack of effect of grape seed alone on MUN is in contrast to the work of Santos et al. (2014) showing a reduction in MUN content in dairy cows fed grape residue silage. Generally, the binding of polyphenols to protein reduces proteolysis in some dietary ingredients, ruminal protein breakdown and milk urea excretion. In our study, it is possible that the dose of polyphenols supplied with the grape seed (1 g/d of polyphenols) was not sufficient to interfere with rumen protein degradation compared with the CON group. In addition, the highest dose of soybean used in the GS diet, to make diets isonitrogenous with the CON group, might have counterbalanced the effects of the polyphenols present in the grape seed. In fact, soybean is characterized by a high fraction of rumen degradable protein, which is positively correlated with MUN (Pulina et al., 2006).

Blood Parameters

The haematological, liver and kidney parameters are reported in Table 2.3 and Figure 2.2. The values of most blood parameters observed in our trial were within the reference ranges of biochemical parameters in Sarda sheep (Dimauro et al., 2008). The only exception was BUN, which slightly exceeded the upper value of the reference interval (29-62 mg/dL) in the CON (63.4 mg/dL), GS (63.6 mg/dL) and MIX (68.7 mg/dL) groups.

Dietary treatments did not influence any of the haematological, kidney and liver parameters measured ($P > 0.05$), except for BUN, which decreased ($P < 0.05$) in LIN and increased ($P < 0.05$) in MIX compared with the CON and GS groups. Similar results were previously observed when dairy goats were fed a similar dose of extruded linseed as a source of PUFA (Nudda et al., 2013). In contrast to our results, the inclusion of grape marc in non-lactating dairy cattle reduced the concentration of BUN (Greenwood et al., 2012). However, it should be noted that the polyphenol content was lower in the grape seeds offered in the present study than in the grape marc used in that study (45.8 g/kg DM). In addition, grape marc, which is constituted by grape seeds, skins and stems after the winemaking process, contains other compounds which could also influence N metabolism, as hypothesized by Greenwood et al. (2012). To our

knowledge, no studies have evaluated the effects of polyphenols from grape seed extract or from grape seed by-products on liver and kidney function in ruminants. In laboratory animals some studies evidenced the hepatoprotective role of grape seed extract, when liver function was experimentally altered by adding a hepatotoxic dose of acetaminophen (Ray et al., 1999) or by ligating the bile duct (Dulundu et al., 2007).

During the trial, the level of hematological, kidney and liver parameters in blood changed with period ($P < 0.01$), except for AST, ALT and BUN (Table 2.3). In general, creatinine, albumin, and protein increased, whereas ALP decreased with period (data not shown). The variation in most blood parameters with period could be due to metabolic changes related to the progress of lactation. The decrease in ALP through period (data not shown) in all experimental groups followed the same pattern of the milk yield curve, in agreement with previous observations in dairy goats (Nudda et al., 2013). A significant diet × period interaction was found only for bilirubin ($P < 0.01$) and GGT ($P < 0.05$), although no significant differences in GGT among diets were found within period (Figure 2.2). On d 15 bilirubin was greater in LIN and GS than in CON, with MIX being intermediate. GGT increased in the MIX group from d 30 until the end of the trial, whereas it remained relatively constant in the other groups.

Immunological Determinations

The results of the immunological analyses are reported in Table 2.4 and Figure 2.3. The SFT was affected by dietary treatments ($P < 0.05$), being the lowest in the GS and MIX groups, the highest in the LIN group, and intermediate in CON. The SFT is used as an indirect measurement of lymphocyte proliferation, because lymphocytes are responsive to PHA, a mitogen that primarily stimulates T cell division and cytokine production (Morán et al., 2012). The reduced SFT in both groups fed grape seed suggests an inhibitory effect of this by-product on lymphocyte proliferation. Previous in vitro studies showed a similar inhibitory effect of polyphenols in human cells (Williamson et al., 2006) and in murine splenocytes (Yamanaka et al., 2012). In addition, Gessner et al. (2012) found anti-inflammatory effects of phenolic compounds extracted from grape seeds and grape marc in intestinal cells, under in vitro conditions. Differently, lymphocyte-derived cytokines increased in serum samples of calves fed polyphenols extracted from pomegranate, thus evidencing an immune-stimulatory effect (Oliveira et

al. 2010). The SFT was also affected by period, with a reduction at the last sampling ($P < 0.01$), probably due to a physiological adaptation of the ewes during the trial.

A significant diet × period interaction was detected for IgG ($P < 0.01$; Figure 2.3). On d 45, IgG was reduced in LIN compared with the other groups, whereas on d 60 IgG was reduced in GS and MIX compared with CON and LIN ($P < 0.05$). The available literature on immune functions of ruminants showed controversial effects of dietary antioxidants on immunoglobulin production in ruminants (Chew, 1996; Novoa-Garrido et al., 2014). Overall, our results suggest an immunomodulatory effect of grape seed, as evidenced by the reduction in IgG titers on d 60 and in SFT. Differently, the LIN group reduced IgG on d 45 but promoted lymphocyte proliferation. The increase in SFT due to dietary linseed is in agreement with the study of Caroprese et al. (2009) in dairy cows. Other studies reported that lymphocyte proliferation was attenuated in dairy cows fed linseed oil (Lacetera et al., 2007) or not affected in bovine peripheral blood mononuclear cells treated with alpha-linolenic acid in vitro (Thanasak et al., 2005).

The IL-6 concentration was influenced by diet × period interaction ($P < 0.01$; Figure 2.3), due to an increase in titers in the MIX group on d 60 compared with the other groups.

The $CD4^+$ and $CD8^+$ cells, and the $CD4^+$:$CD8^+$ ratio were not influenced by diet ($P > 0.05$), although the $CD4^+$ cells were numerically reduced in the GS group compared with the other groups. In mice, grape seed proanthocyanidins diminished regulatory $CD4^+$ cells and stimulated $CD8^+$ cells, thus inhibiting UVB-induced immunosuppresion (Vaid et al., 2011). A period effect was observed for $CD4^+$ cells ($P < 0.01$), because of an increase $CD4^+$ cells on d 45 (data not shown).

It is worth noting that most of the studies concerning the effects of polyphenols on immune function and health status have been conducted in vitro or with pathological subjects. In the review by Halliwell (2008), no evidence of pro-oxidant or antioxidant effects of flavonoids in in vivo studies was found. The author of the review suggested that those effects may be found in vitro only because of the high and non-physiological levels of polyphenols normally used. Therefore, we hypothesize that grape seed dietary treatments involve a more complex physiological system than observed under in vitro conditions and that the antioxidant effects of grape seed on immune functions may not be apparent at low doses.

2.5 Conclusions

In conclusion, this study evidenced that grape seed, alone or associated with linseed, can be included in the diet of lactating ewes without influencing production traits. The reduction in some immunological parameters, i.e., IgG on d 60 and SFT, suggests that grape seed could have an immunomodulatory effect in dairy ewes. Overall, the limited variation in milk, hematological and metabolic parameters suggests that grape seed and linseed can be included, alone or in combination, in the diet of dairy ewes without adverse effects on milk production and health status.

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2.7 Tables

Table 2.1. Ingredients and chemical composition and ME concentration of the diets supplied to lactating ewes

	Diet[1]			
	CON	GS	LIN	MIX
Ingredients (kg/d per head)				
Mixed meal				
Corn	0.15	0.17	-	-
Soybean	0.12	0.24	0.04	0.16
Peas	0.25	0.09	0.15	0.02
Grape seed	-	0.30	-	0.30
Linseed	-	-	0.22	0.22
Beet pulp	0.40	0.40	0.40	0.40
Commercial concentrate	0.50	0.50	0.50	0.50
Dehydrated alfalfa hay	0.80	0.80	0.80	0.80
Mixed hay[2]	0.20	0.20	0.20	0.20
Total DM supplied	2.20	2.47	2.11	2.39
Chemical composition				
DM (%)	90.8	91.6	91.2	92.0
NDF (% of DM)	41.8	42.8	43.7	44.5
NFC (% of DM)	33.4	28.9	28.5	24.2
ADL (% of DM)	4.6	8.9	5.0	9.4
CP (% of DM)	18.0	17.9	17.9	17.9
Ash (% of DM)	7.8	7.4	8.1	7.6
Ether extract (% of DM)	2.0	3.2	5.1	5.8
ME (Mcal/kg of DM)	2.25	2.00	2.36	2.08
ME Supplied (Mcal/d)	4.95	4.94	4.97	4.97

[1]CON: control diet, GS: diet containing 300 g of grape seed, LIN: diet containing 220 g of linseed, MIX: diet containing 300 g of grape seed and 220 g of linseed.
[2]Mixed hay was provided during the night: DM 87.3%; NDF 55%; CP 11.6; ether extract 1.08.
[3]Calculated using the Small Ruminant Nutrition Model (Tedeschi et al., 2010).

Table 2.2. Effect of dietary grape seed and linseed, alone or in combination, and period on chemical composition of milk in dairy ewes

	Diet[1]					P-value[2]	
Item	CON	GS	LIN	MIX	SEM	D	P
Yield (g/day)							
Milk	1490	1520	1620	1680	104	ns	***
Fat	87.2	93.9	96.8	100.5	5.82	ns	***
Protein	86.2	87.4	87.0	91.1	4.97	ns	***
Casein	67.3	68.7	68.6	71.6	3.90	ns	***
Lactose	73.6	76.6	81.9	84.9	5.57	ns	***
Milk composition (%)							
Fat	5.95	6.19	6.05	6.09	0.216	ns	***
Protein	5.85	5.76	5.45	5.48	0.149	ns	***
Casein	4.57	4.31	4.30	4.20	0.244	ns	***
pH	6.67	6.66	6.68	6.66	0.017	ns	***
Log SCC (x1000 cell/mL)	2.43	2.44	2.43	2.15	0.17	ns	***

[1]CON: control diet, GS: diet containing 300 g of grape seed, LIN: diet containing 220 g linseed, MIX: diet containing 300 g of grape seed and 220 g of linseed.
[2]D: effect of diet; P: effect of period; ns indicates $P > 0.05$; *$P < 0.05$; ***$P < 0.001$.

Table 2.3. Effect of dietary grape seed and linseed, alone or in combination, and period on blood hematological parameters and markers of liver and kidney activity of dairy ewes

	Diet[1]					P-value[2]	
Item	CON	GS	LIN	MIX	SEM	D	P
Hematological parameters							
WBC(x10^3cells/µL)	8.9	8.5	8.3	8.7	0.5	ns	***
RBC (x10^6 cells/µL)	9.3	9.5	9.6	9.7	0.3	ns	***
HGB (g/dL)	10.4	10.6	10.4	10.5	0.2	ns	***
HCT (%)	30.6	30.5	30.4	30.4	0.7	ns	***
MCV (fL)	33.1	32.2	31.7	31.4	0.8	ns	***
MCH (pg)	11.3	11.2	10.8	10.8	0.3	ns	***
MCHC (g/dL)	34.1	34.7	34.1	34.5	0.3	ns	***
PLT (x10^3 cells/µL)	729	643	599	557	63	ns	***
LYM (%)	53.7	52.3	51.0	52.5	2.1	ns	***
MONO (%)	3.7	4.7	4.3	3.9	0.5	ns	***
NEUT (%)	29.8	28.7	33.1	30.5	1.9	ns	***
EOS (%)	11.1	12.0	10.2	11.2	1.0	ns	***
BASO (%)	0.6	0.9	0.7	0.8	0.1	ns	***
Blood metabolites							
Albumin (g/dL)	2.6	2.5	2.5	2.5	0.1	ns	***
ALP (U/L)	108.7	164.4	128.5	226.8	36.5	ns	***
Bilirubin (mg/dL)	0.19	0.20	0.23	0.20	0.0	ns	**
Creatinine (mg/dL)	0.6	0.6	0.6	0.6	0.0	ns	***
GGT (U/L)	77.8	73.0	78.6	68.7	8.0	ns	***
AST (U/L)	142	140	119	165	15.1	ns	ns
ALT (U/L)	34.6	35.0	33.4	35.6	1.2	ns	ns
Protein (g/dL)	7.2	7.0	7.1	7.1	0.1	ns	***
BUN (mg/dL)	63.4[b]	63.6[b]	58.0[c]	68.7[a]	2.1	**	ns
Albumin/creatinine	4.2	4.2	1.2	4.1	0.4	ns	ns
Protein/creatinine	11.8	12.0	11.7	11.6	1.2	ns	ns

[a-c]means within a row with different superscripts are different (P < 0.05).

WBC: white blood cell count; RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet; NEUT: neutrophil granulocytes; LYM: lymphocytes; MONO: monocytes; EOS: eosinophils granulocytes; BASO: basophiles granulocytes; ALP: alkaline phosphatase; GGT: gamma-glutamyltransferase; AST aspartate aminotransferase; ALT: alanine aminotransferase.

[1]CON: control diet; GS: diet containing 300 g of grape seed; LIN: diet containing 220 g linseed; MIX: diet containing 300 g of grape seed and 220 g of linseed.

[2]D: effect of diet; P: effect of period; ns indicates P > 0.05; **P < 0.01; ***P < 0.001.

Table 2.4. Effect of dietary grape seed and linseed, alone or in combination, and period on immunological parameters of dairy ewes

	Diet[1]					P-value[2]	
Item[3]	CON	GS	LIN	MIX	SEM	D	P
SFT (mm)	7.25[b]	5.29[c]	9.15[a]	5.64[c]	0.680	*	**
$CD4^+$ (10^6 cells/mL)	31.0	29.7	30.6	33.4	0.64	ns	**
$CD8^+$ (10^6 cells/mL)	18.7	18.6	17.4	16.9	0.52	ns	ns
$CD4^+$:$CD8^+$	1.7	1.7	1.9	2.0	0.056	ns	ns

[a-c]means within a row with different superscripts are different ($P < 0.05$).
[1]CON: control diet, GS: diet containing 300 g of grape seed, LIN: diet containing 220 g linseed, MIX: diet containing 300 g of grape seed and 220 g of linseed.
[2]D: effect of diet; P: effect of period; ns indicates $P > 0.05$; *$P < 0.05$; **$P < 0.01$.
[3]SFT: skinfold thickness, $CD4^+$: lymphocyte T-helper cells, $CD8^+$: lymphocyte T-cytotoxic cells.

2.8 Figures

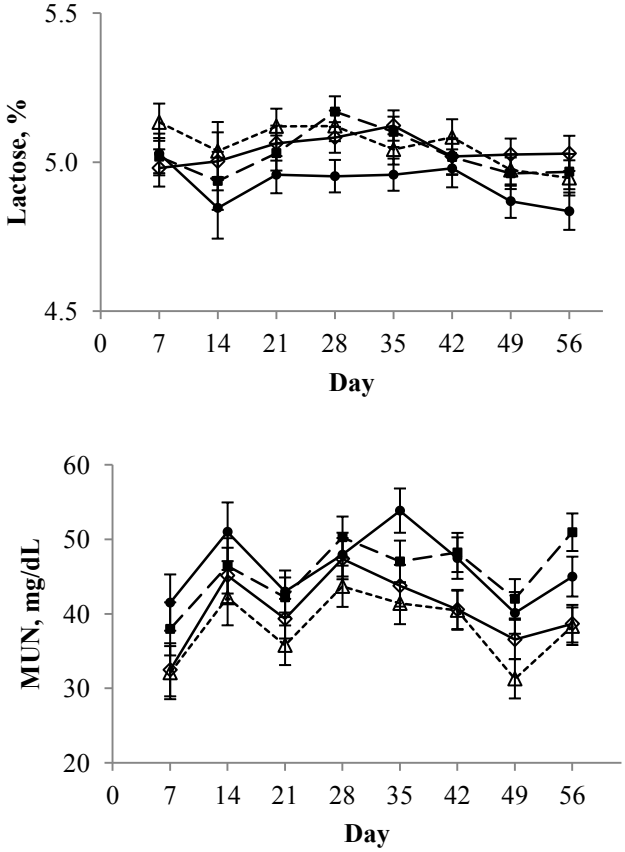

Figure 2.1. Temporal changes in milk lactose concentration and milk urea nitrogen (MUN) of dairy ewes fed control diet (solid line, ●), diet containing grape seed (long-dash line, ■), diet containing linseed (dotted line, △) and diet containing both grape seed and linseed (solid line, ◊). Differences between dietary treatments within the same day are represented by * ($P < 0.05$). Vertical bars represent the standard error of the mean.

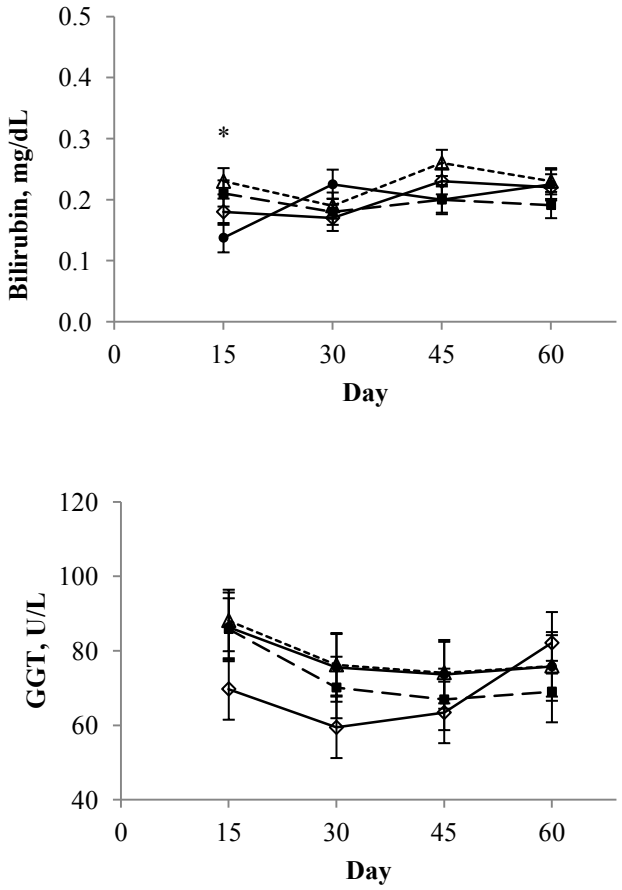

Figure 2.2. Temporal changes in bilirubin and gamma glutamyltransferase (GGT) in blood of dairy ewes fed control diet (solid line, ●), diet containing grape seed (long-dash line, ■), diet containing linseed (dotted line, △) and diet containing both grape seed and linseed (solid line, ◊). Differences between dietary treatments within the same day are represented by * ($P < 0.05$). Vertical bars represent the standard error of the mean.

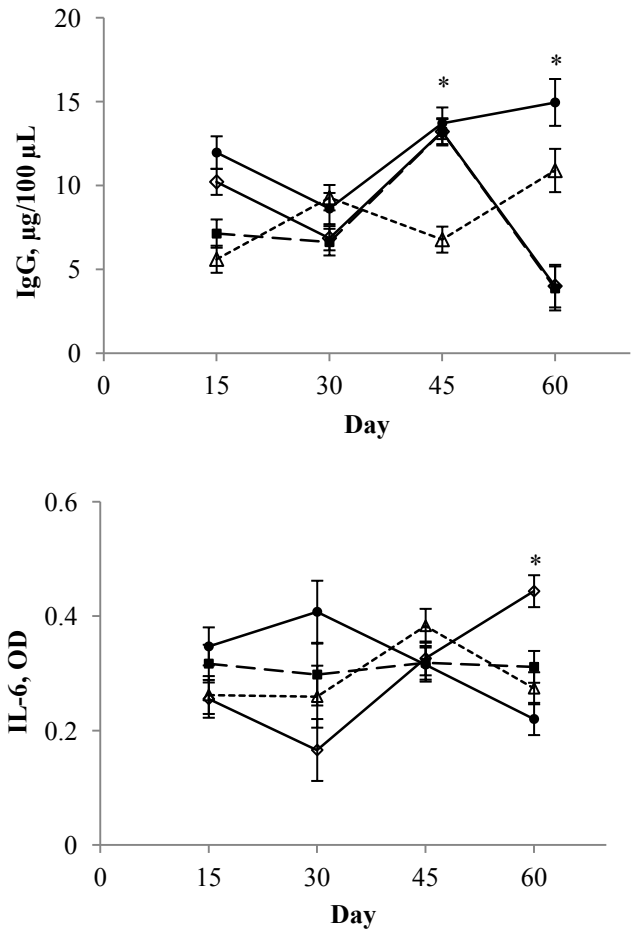

Figure 2.3. Temporal changes in antibody titers IgG to chicken egg albumin (OVA) and interleukin-6 (IL-6) in blood of dairy ewes fed control diet (solid line, ●), diet containing grape seed (long-dash line, ■), diet containing linseed (dotted line, △) and diet containing both grape seed and linseed (solid line, ◊). Differences between dietary treatments within the same day are represented by * ($P < 0.05$). Vertical bars represent the standard error of the mean.

CHAPTER 3

Effects of grape seed supplementation, alone or associated with linseed, on ruminal metabolism in Sarda dairy sheep

F. Correddu, A. Nudda, G. Battacone, R. Boe, A.H.D. Francesconi, and G. Pulina

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3.1 Abstract

Grape seed is a by-product of the winery and distillery industry which could be used in animal nutrition. To test the hypothesis that dietary supplementation with this by-product can decrease the biohydrogenation (BH) of healthy fatty acids (FA), the present study evaluated the effects of grape seed supplementation, alone or combined with linseed, on ruminal BH processes in dairy sheep. In this 60-d trial, twenty-four lactating Sarda dairy ewes were assigned to 4 homogeneous groups and fed as follows: (1) control diet (CON), (2) a diet supplemented with 300 g/d per head of grape seed (GS), (3) a diet supplemented with 220 g/d per head of extruded linseed (LIN), (4) and a diet supplemented with a mix of both grape seed and linseed (300 and 220 g/d per head, respectively) (MIX). Ammonia, pH, volatile fatty acids (VFA) and FA composition were determined in rumen liquor at 3 sampling dates (20, 40 and 60 d). Rumen pH was not influenced by diet ($P > 0.05$). The ammonia content was increased ($P < 0.05$) in GS and MIX compared with LIN and CON. The molar proportions of acetate and propionate and their ratio were not affected by the diet ($P > 0.05$), whereas the molar proportion of butyrate was the lowest in MIX. Rumenic acid (RA; CLA *cis*-9,*trans*-11) concentration increased in GS compared with CON (0.78 *vs.* 0.45 mg/100 mg FA; $P < 0.05$), whereas the percentage of vaccenic acid (VA; C18:1 *trans*-11) tended to increase ($P < 0.10$) in GS compared with CON. The concentration of VA was higher in MIX than in CON (8.18 *vs.* 3.77 mg/100 mg FA; $P < 0.05$), whereas RA did not differ between the same groups. The concentration of linoleic acid (LA; C18:2 *n*-6) decreased and stearic acid (SA; C18:0) increased in all supplemented groups, whereas linolenic acid (LNA; C18:3 *n*-3) decreased in the two groups receiving grape seed compared with CON and LIN. The concentration of total odd- and branched-chain fatty acids (OBCFA) decreased in all supplemented groups compared with CON ($P < 0.05$), evidencing that grape seed and linseed supplementation influenced the ruminal BH processes. Grape

seed was able to increase the accumulation of RA when supplemented alone, and of VA when combined with linseed; however, the rumen accumulation of SA in both groups supplemented with grape seed evidenced that this by-product was not effective in decreasing the BH of dietary polyunsaturated fatty acids (PUFA).

3.2 Introduction

Grape seed is a by-product derived from the winery and distillery industries. In countries where the wine industry is an important activity, the large production of by-products and wastes is a serious problem, because of the high cost of their management and disposal. As many other agro-industrial by-products, grape seeds are rich in polyphenols (Schieber et al., 2001), especially mono-, oligo- and polymeric proanthocyanidins (Shrikhande, 2000), which are well known for their antioxidant properties (Riceevans et al., 1995; Bagchi et al., 1997).

In the last few decades, a lot of attention has been directed to the content of healthy fatty acids (FA), especially polyunsaturated fatty acids belonging to the family of *n*-3 (PUFA *n*-3), such as alpha-linolenic acid (C18:3 *n*-3, LNA), and conjugated linoleic acid (CLA), such as rumenic acid (CLA *cis*-9, *trans*-11, RA), in ruminant milk and dairy products. A lot of studies have demonstrated that diet composition and rumen microbial biohydrogenation (BH) strongly influence the fatty acid profile of milk and dairy products (Lourenço et al., 2010; Buccioni et al., 2012). Supplementation with linseed, which is rich in LNA, is often used to improve the FA profile in sheep, goat and cow milk and cheese, by increasing the concentration of healthy FA (Caroprese et al., 2010; Mughetti et al., 2012; Nudda et al., 2013). Lipids from the diet are involved in a sequence of reactions, performed by the rumen microbial population, including hydrolysis of esterified lipids to free FA, whose double bonds can be partly isomerized and hydrogenated. These reactions can be explained by a detoxification mechanism to defend microorganisms from the toxicity of unsaturated fatty acids (UFA) as reported by Dehority (2003) and Maia et al. (2010). Because C18:2 *cis*-9, *cis*-12 (linoleic acid, LA, *n*-6 series) and LNA provided with the diet are greatly reduced (by 80% and 92%, respectively) in the rumen (Doreau and Ferlay, 1994), several strategies have been tested to protect dietary FA from rumen BH. The encapsulation of lipids in a protein

matrix is one of the techniques proposed to protect FA (Tymchuk et al., 1998; Hawkins et al., 2013). However, the occasional lack of efficiency of this method (Petit, 2003), its high costs and the need for formaldehyde utilization limit its use. Another technique that has been studied is the decrease in BH by chemical modifications of UFA, such as the transformation of UFA to fatty acyl amides (Jenkins, 1998) or to calcium salts (Lundy Iii et al., 2004). The presence of some plant compounds, such as polyphenols, in the diet of ruminants can also influence the BH process, by inhibiting the activity of rumen microbes (Cabiddu et al., 2009; Vasta et al., 2009). The use of grape seed as a supplement in ruminant nutrition could be an alternative for the expensive management and disposal of this winery by-product. However, the literature available on the use of grape by-products in ruminant nutrition is limited (Moate et al., 2014; Santos et al., 2014) and, to our knowledge, the effects of the dietary supplementation with grape seed on the BH of PUFA in the rumen has not been explored yet.

To test the hypothesis that grape seed supplementation decreases the BH activity of rumen microbes, thus boosting the effect of linseed supplementation, this work investigated the effect of grape seed supplementation, alone or associated with linseed, on rumen BH processes in dairy sheep.

3.3 Material and methods

Animals, experimental design and treatments

Twenty four Sarda dairy ewes in the first part of lactation (<50 days in milk, DIM) were assigned to 4 groups of 6 animals each, homogeneous for milk production, body weight, DIM, and lactation order. Groups were confined in four boxes and randomly assigned to one of the 4 experimental diets (Table 3.1): control (CON) diet, a diet supplemented with 300 g/d per head of grape seed (GS), a diet supplemented with 220 g/d per head of extruded linseed (LIN), and a diet supplemented with a mix of 300 g/d per head of grape seed and 220 g/d per head of linseed (MIX). The extruded linseed dose of 220 g/d was used to supply 70 g/d of fat per head. Considering that the total phenolic content of grape seed was 333.3 ± 10.1 mg gallic acid equivalent (GAE)/100 g of dry matter (DM; mean ± S.E.), the grape seed dose of 300 g/d per head was used to provide approximately 1 g/d per head of total polyphenols (approximately 0.4 g polyphenols/kg DM of diet). The grape seed used was obtained from different red grape varieties after distillation in the winemaking process. Grape seeds were ground before administration. The chemical composition and FA profile of the grape seed and linseed are reported in Table 3.2.

All animals were offered the same basal ration consisting of beet pulp, a commercial concentrate, dehydrated alfalfa hay and mixed hay. In addition, they received a mixed meal composed of corn, soybean, pea, grape seed and linseed, in different proportions depending on the dietary treatments, to obtain isoenergetic and isonitrogenous diets. Dehydrated alfalfa hay and mixed hay were offered to each group of six ewes, whereas all other dietary ingredients were provided to each animal by using individual feeders. The commercial concentrate (500 g/d per head) was provided at the two daily milkings (7:30 and 17:30). The mixed meals were provided two hours after each milking, and subsequently beet pulp (400 g/d per head) and dehydrated alfalfa hay (on average 800 g/d per head) were provided. The mixed hay (on average 200 g/d per head) was offered during the night. Both hays were offered at a fixed amount to avoid selection by the animals. Clean water was always available. Diets were formulated to meet the sheep energy and protein requirements using the Small Ruminant Nutrition Model (Tedeschi et al., 2010). Diets were offered in a fixed amount to ensure constant daily intakes of

dietary ingredients and to maintain energy balance. The experiment lasted 10 weeks, with two weeks of adaptation period and 8 weeks of data collection.

Rumen sample collection

Samples of rumen liquor were collected from all animals on days 20, 40 and 60 of the trial (Samplings 1, 2 and 3), 2 hours after the morning feeding, using a stomach tube and an evacuation pump. The collection of rumen liquor samples was performed by two teams of qualified experience and required approximately 30 min in total. In order to reduce saliva contamination, the first portion of the liquor collected (about 30 mL) was discarded. After sampling and filtering the rumen liquor, the pH value was immediately measured by a pH meter (Orion 250A, Orion Research Inc., Boston, MA, USA), equipped with a glass electrode with Polysolve reference electrolyte (model 238405, Hamilton Company, Reno, NV, USA), and a thermometer. The sample of rumen liquor of each animal was then divided into 3 subsamples, which were immediately stored at –80°C until analysis for ammonia, volatile fatty acids (VFA) and FA.

Chemical analysis

Dry matter content of feed ingredients was determined by oven-drying at 105°C for 24 h. Neutral detergent fiber (NDF) and acid detergent lignin (ADL) analyses were performed following the method of Van Soest et al. (1991), using an Ankom 220 fiber analyzer (Ankom™ technology, Fairport, NY, USA); NDF was measured using heat stable amylase and expressed exclusive of residual ash (aNDFom) and ADL was determined by solubilization of cellulose with sulphuric acid. Crude protein (CP) content was measured according to the Kjeldahl method (proc. 988.05; AOAC, 2000), extract ether (EE) by the Soxhlet method (proc. 920.39; AOAC, 2005) and ash by using a muffle at 550°C (proc. 942.05; AOAC, 2000). Non-fiber carbohydrates (NFC) were calculated according to Weiss (1999) as follows: NFC (g/kg DM) = 100 − (NDF + CP + ash + EE).

To determine the phenolic content of grape seed, the seeds were powdered by a blender and an aliquot of 1.5 g was homogenized with 50 mL of a mixture of acetone/water (70/30, v/v) using an Ultra Turrax homogenizer (Ultra Turrax T25, Janke&Kunkel KG, Germany) at 3000 rpm, for 2 min, in a water/ice bath. The homogenate was then centrifuged (6,000 x *g*, 15 min, 4°C) and the supernatant was filtered through Whatman

541 filter paper (Whatman, Maidstone, England) for determination of phenolic compounds, using the Folin-Ciocalteu method described by Kim et al. (2003) with some modifications. Briefly, 0.5 mL of the extract were added to 1 mL of Folin-Ciocalteu phenol reagent (Sigma Chem. Co., St. Louis, MO, USA) and 9.5 mL of distilled water in a 25 mL volumetric flask and shaken. After 7 min, 10 mL of 0.71 M sodium carbonate (Na_2CO_3) were added, and then the mixture was diluted to a volume of 25 mL with distilled water and mixed thoroughly. The mixture was then stored in the dark for 120 min at room temperature. The absorbance was read at 750 nm wavelength versus a blank solution. Total phenolic content, expressed as mg of GAE/100 g DM, was determined following a calibration curve obtained using gallic acid (Sigma Chem. Co., St. Louis, MO, USA) as standard.

The FA profiles of the whole diets, grape seed, linseed and rumen liquor were determined using the method of Kramer et al. (1997) with some modifications. Samples kept at −80°C were lyophilized and powdered. After adding 2 mL of sodium methoxide 0.5 M in methanol (Sigma and Aldrich, Spain) to the power, it was placed in a water bath at 50°C for 10 min. After cooling to room temperature, the samples were placed in a water/ice bath; then 3 mL of HCl/methanol (3M), prepared with acetyl chloride and methanol, were added. Subsequently, the samples were heated again in a water bath at 50°C for 10 min and cooled to room temperature; then 1 mL of a solution containing methyl nonadecanoate (C19:0) as internal standard (Sigma Chemical Co., St. Louis, MO, USA) and, subsequently, 7.5 mL of a 0.43 M solution of K_2CO_3 were added. After quick agitation the samples were centrifuged (1,500 x *g*, room temperature, 5 min) and each supernatant was kept in a vial for GC analysis. Fatty acid methyl esters (FAME) were determined using a Turbo 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and an automatic injector 8200 (CX Varian Inc., Palo Alto, CA, USA). The column (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 μm film thickness, Varian Inc., Palo Alto, CA, USA) was operated with the following program: 75°C for 1 min, increased at 5°C/min to 148°C and at 8°C/min to 165°C, held for 35 min; then increased at 5.5°C/min to 210 and, finally, at 3°C to 230°C, held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure of 37 psi. Split ratio was 1:100. The injector and detector temperatures were held at 225 and 285°C, respectively. Varian Star 3.4.1 software was

used to compute the retention time and area of each individual FAME, identified by comparing their retention times with those of known standards and with published studies as detailed by Nudda et al. (2008).

Ammonia content in rumen liquor was determined by colorimetric method, according to Chaney and Marbach (1962) with one modification (the use of salicylate instead of phenol), using a UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

The VFA analysis was performed by a high-performance liquid chromatography (HPLC) method. Briefly, a sample of approximately 2 mL was defrozen and centrifuged (15,000 x *g*, 10 min, 4°C); the supernatant was then withdrawn by syringe and injected into a HPLC system (Varian Inc., Palo Alto, California, USA) after filtration (PTFE 0.45 µm, 13 mm). The HPLC was equipped with an auto sampler (Varian 9300), a degasser (Varian 9012 Q), a UV detector (Varian 906P Polychrom) and an Aminex HPX 87H column (Biorad Laboratories, Hercules, CA, USA). The column was operated at 55°C with 0.008 N H_2SO_4 at 0.6 mL/min as eluent. Concentrations of VFA were estimated by comparison with a calibration curve obtained by injecting 5 µl of 5 standard solutions (5.6, 11.25, 22.5, 45 and 90 mmol/L of acetic acid, and 5, 10, 20, 40 and 80 mmol/L of propionic and butyric acid) obtained by appropriate dilutions of a standard mixture of VFA containing 5.40, 5.76 and 7.02 mg/mL of acetic, propionic and butyric acids, respectively, in H_2SO_4 0.1 N. The concentrations of total and single VFA were expressed as mmol/L and mol/100 mol of total VFA, respectively.

Statistical analysis

Data of pH, ammonia, VFA and FA profile were analyzed with the PROC MIXED procedure of SAS (2002). The model included the diet treatment (CON, GS, LIN and MIX), sampling (3 samplings on days 20, 40 and 60 of the trial) and their interaction as fixed effects, and the animal nested within the treatment as random effect. Means were separated using Tukey test ($P < 0.05$).

3.4 Results

The predominant FA of the lipid fraction in grape seed was LA (74 g/100 g FA), followed by oleic acid (OA; C18:1 *cis*-9) and palmitic acid (C16:0) (9.6 and 8.5 g/100 g

FA, respectively). The main FA in linseed was LNA, which accounted for 56 g/100 g FA (Table 3.2).

All dairy ewes consumed the whole daily amount of feeds supplied, as a consequence of the fixed amount of diets provided (Table 3.3). The intake of most FA varied with diets (CON<GS<LIN<MIX; Table 3.3) and reflected the EE concentration of the diets (Table 3.1).

pH and ammonia in rumen liquor

Rumen pH was not influenced by the diets, ranging from 6.68 in LIN to 6.77 in MIX (Table 3.4). Sampling affected pH, which was lower on sampling 1 (6.58) than on sampling 2 (6.85), and intermediate on sampling 3 (6.75). A diet × sampling interaction ($P < 0.01$) occurred, but no significant differences between diets within sampling were detected. During the trial, none of the animals recorded pH values below 6.0 and no significant differences were found in pH among animals within group ($P > 0.05$) (data not shown).

Rumen ammonia was affected by diet and sampling date ($P < 0.05$, Table 3.4), being higher in the GS and MIX groups than in the CON and LIN groups. On average, rumen ammonia increased ($P < 0.05$) from sampling 2 (14.71 mg/dL) to sampling 3 (17.71 mg/dL), with sampling 1 (15.67 mg/dL) not differing from them.

Rumen VFA

Concentration of total VFA, molar proportions of individual VFA and acetate:propionate ratio in the rumen are shown in Table 3.4. The concentration of total VFA and the molar proportions of acetate and propionate were not affected by diet, whereas the concentration of butyrate was reduced ($P < 0.05$) by the MIX containing both grape seed and linseed. A time effect was observed ($P < 0.01$) for total VFA and individual VFA, with the highest concentrations of propionate, acetate and butyrate being on samplings 1, 2, and 3, respectively ($P < 0.05$; Figure. 3.1). The acetate:propionate ratio was not affected by diet, but differed ($P < 0.05$) among sampling 1 (3.14), sampling 2 (4.04) and sampling 3 (3.54). This ratio was influenced by group × sampling interaction ($P < 0.05$), but no significant differences between diets within sampling were detected.

FA composition in rumen liquor

The fatty acid profile in the rumen liquor collected from the ewes of the different experimental treatments is given in Tables 3.5 and 3.6. The total concentration of short-chain fatty acids (SCFA) was lower ($P < 0.05$) in all supplemented groups than in CON, mainly due to a decrease in C4:0 and C5:0 ($P < 0.05$). The total concentration of medium-chain fatty acids (MCFA) was affected by diet ($P < 0.01$), with a significant reduction in both groups supplemented with linseed (LIN and MIX) compared with CON, mainly due to the lower concentration ($P < 0.05$) of C16:0 and some C16:1 isomers in the LIN and MIX groups. The concentration of MCFA and C16:0 in the GS group was intermediate, not differing significantly from CON and LIN.

The concentration of total odd- and branched-chain fatty acids (OBCFA) decreased ($P < 0.05$) in all supplemented groups compared with CON. The MIX group had the lowest concentration of OBCFA, even if not significantly different from the LIN group. The reduction in OBCFA caused by grape seed was mainly due to the reduction ($P < 0.05$) in *anteiso* branched-chain fatty acids (BCFA) and odd-chain fatty acids (OCFA), especially *anteiso* C15:0 and C5:0. The reduction in OBCFA caused by linseed was related not only to the reduction in these FA, but also to the decrease ($P < 0.05$) in *iso* BCFA, especially isomers of heptadecanoid acid (C17:0, *iso* C17:0 and *anteiso* C17:0). The total concentration of long-chain fatty acids (LCFA) increased with the grape seed and linseed supplementation, alone and in combination, being the highest in the MIX group ($P < 0.05$).

The concentration of C18:0 (stearic acid, SA) was higher ($P < 0.05$) in all the supplemented groups than in CON and, on average, increased ($P < 0.05$) in the last sampling. The diet × sampling interaction was also significant ($P < 0.05$). This FA was higher in MIX than in CON throughout the study, whereas it was higher in GS and LIN than in CON on sampling 2 ($P < 0.05$).

The concentration of LA decreased in all supplemented groups, being the lowest in LIN and MIX ($P < 0.05$). The concentration of LNA was higher in the LIN and CON groups than in the GS and MIX groups ($P < 0.05$). The concentrations of the geometrical isomers of LA, C18:2 *trans*-9,*trans*-12 and C18:2 *trans*-9,*cis*-12, were higher in LIN and MIX than in GS and CON ($P < 0.05$).

The concentration of RA was the highest ($P < 0.05$) in the GS group. A significant effect of sampling ($P < 0.01$) occurred, with increasing levels of this FA over time (0.11, 0.55 and 0.97 g/100 g FA, respectively, on samplings 1, 2 and 3). A significant diet × sampling interaction occurred, with GS having a higher concentration of this FA than the other supplemented groups on sampling 3 (Figure. 3.2).

The sum of total *trans* fatty acids (TFA) was influenced ($P < 0.01$) by diet, being higher in both groups receiving linseed supplementation (LIN and MIX) than in the CON, with GS not differing from CON and LIN. In particular, the concentration of C18:1 *trans*-11 (vaccenic acid, VA) was the highest in MIX and the lowest in GS and CON, with LIN being intermediate ($P < 0.05$).

Many of the FA measured during the trial were influenced by sampling, but the pattern varied among them. In general, most of the SCFA (C4:0, C5:0 and C6:0) and isomers of C18:1 showed a significant decrease ($P < 0.05$) in the last sampling, whereas most of the MCFA increased ($P < 0.01$) over time. The individual LCFA showed a variable pattern, without a significant sampling effect on total LCFA ($P > 0.05$). The PUFA decreased over time, mainly due to a decrease in PUFA n-6 (LA) ($P < 0.05$). Among OBCFA, *iso* BCFA and *anteiso* BCFA increased after sampling 1, whereas OCFA decreased over time on the last sampling ($P < 0.05$).

3.5 Discussion

The complete consumption of the feeds supplied individually and the fact that the group intake of the dehydrated alfalfa and mixed hay was complete suggest that the inclusion of grape seed, linseed or both in the diet of lactating ewes did not depress DM intake.
The pH values measured in all experimental groups throughout the trial were between 6.5 and 7.0, which is a normal range for rumen liquor pH in sheep (5.5-7.0; Dziuk, 1984). The fact that pH did not differ among the experimental groups and among animals within group could be considered an indicator that the individual forage intake was similar within group and large enough to avoid cases of low pH. Our results are in accordance with previous studies showing that rumen pH was not markedly affected when sheep were supplemented with grape pomace by-products, as a source of polyphenols (Yidiz et al., 2005; Abarghuei et al., 2010), or with extruded linseed, as a

source of PUFA (Mughetti et al., 2007). The increase in rumen ammonia observed in the groups supplemented with grape seed, alone or in combination with linseed, is in contrast with studies showing that the inclusion of polyphenols in the diet was usually associated with a decrease in protein degradation (Abarghuei et al., 2010; Dschaak et al., 2011), because of their ability to bind proteins and reduce the activity of microbial enzymes by decreasing the growth of proteolytic bacteria (Molan et al., 2001). This discrepancy could be explained by two hypotheses. The first is that the level of polyphenols in the grape seed used in the present work was too low to influence the activity of some strains of proteolytic bacteria, compared with the doses of polyphenols (values higher than 5 g/kg DM) which influenced rumen bacteria population in other studies (Hervás et al., 2003; Vasta et al., 2010; Anantasook et al., 2014). In addition, in some studies low concentrations of tannins increased the enzymatic activity and growth of some bacteria *in vitro* (Jones et al., 1994) and *in vivo* (Vasta et al., 2010), likely because the interaction between proteins and tannins can cause conformational changes in the protein structure, giving more accessible sites for some proteolytic bacteria (Mole and Waterman, 1985; Molan et al., 2001). The second hypothesis is that the high concentration of ADL in the grape seed used (410 g/kg DM) reduced the growth of cellulolytic bacteria, considering that high amounts of lignin in ruminant diets can reduce fiber digestion (Hartley, 1972; Jung and Fahey, 1984) by decreasing the growth of this type of bacteria (Akin, 1982), whose favorite substrate for protein synthesis is ammonia (Brayant, 1973; Van Soest, 1994), with a consequent accumulation of ammonia in the rumen.

The lack of effect of grape seed or extruded linseed, alone or in combination, on the concentration of total VFA and on the proportion of acetate and propionate and their ratio in dairy ewes observed in our study is in accordance with previous studies on dairy sheep fed diets rich in LA and supplemented with 10 or 20 g tannins/kg DM from quebracho (Toral et al., 2011, 2013), on cattle supplemented with quebracho polyphenols (Beauchemin et al., 2007) and on dairy cows supplemented with flax seed (Neveu et al., 2013). Differently, Ivan et al. (2013) found a reduction in acetate in dairy cows fed diets supplemented with oilseeds rich in LA or LNA and an increase in propionate in cows fed the latter supplement.

The reduction in the proportion of butyrate observed only in the rumen liquor of the ewes fed the MIX could be explained by the synergistic effect of the two sources of LA (grape seed) and LNA (linseed) and by the higher value of fat in the MIX diet than in the GS and LIN diets. Our finding is in accordance with the meta-analysis of Patra (2014) showing a negative effect of increasing levels of fat in the diets of sheep on the proportion of butyrate, likely due to the inhibition of microorganisms (protozoa and *Butyvibrio fibrisolvens*) involved in its production (Hristov et al., 2009).

Overall, the observed variations in rumen FA profile between diets suggest shifts in rumen microbial population. Considering that OBCFA derive largely from rumen microflora (Fievez et al., 2012), the observed decrease in total OBCFA in all supplemented groups indicates that the diets affected the activity and the growth of ruminal microorganisms. It is well documented that variations in dietary treatments influence the synthesis of OBCFA, by affecting the relative abundance of specific ruminal bacterial population (Vlaeminck et al., 2006). The reduction in total OBCFA by linseed supplementation is in accordance with previous findings on lactating sheep supplemented with sunflower oil (Toral et al., 2012), likely because these two supplements are a source of PUFA, which have detrimental effects on the ruminal microflora (Maia et al., 2007, 2010). The decrease in OBCFA caused by grape seed supplementation could be partly explained by the depressive effects of tannins on the microorganism growth (Baah et al., 2007; Vasta et al., 2010), and mainly by the high concentration of LA in this by-product. The lowest value of OBCFA and the highest value of LCFA in the MIX group suggest a combined effect of the two supplements, likely because of the high LA (74% of total FA) in grape seed and high LNA (56% of total FA) in linseed.

The OBCFA profile has been proposed as diagnostic tool to predict shifts in microbial population associated with the diet variation (Lee et al., 1999; Vlaeminck et al., 2004). In the present work the reduction in the *iso* C17:0 and *anteiso* C17:0 in the rumen liquor of sheep receiving linseed supplementation could be related to a negative effect of this supplement on the protozoa population, which produces a greater proportion of these FA than the bacteria population in the rumen (Or-Rashid et al., 2007). In fact, several authors found a negative effect of lipid addition on the growth of the protozoa

population (Broudiscou et al., 1994; Doreau and Ferlay, 1995; Ivan et al., 2013). The decrease in *anteiso* C15:0 in GS compared with CON suggests a negative effect of grape seed supplementation on the rumen bacterial population, which is the main producer of this FA in the rumen (Or-Rashid et al., 2007). The decrease in both *anteiso* C17:0 and *anteiso* C15:0 in ewes fed the MIX suggests that the combination of grape seed and linseed influenced both protozoa and bacteria in the rumen, as confirmed by the lowest OBCFA level of this treatment.

The greater proportion of TFA in the groups fed linseed, alone or in the MIX, than in CON was expected and was likely a consequence of the higher concentration of most C18:1 and C18:2 isomers, which derive in part from the BH of LNA. This finding is in agreement with previous studies showing that the concentration of C18:1 and C18:2 isomers increased in the rumen of steers fed linseed oil (Shingfield et al., 2011). The greater accumulation of most *trans* and *cis* isomers of C18:1 in the MIX group could be mainly a consequence of the BH of OA, before its reduction to SA. The increase in C18:2 *trans*-9,*trans*-12 and C18:2 *trans*-9,*cis*-12 observed only in the two diets containing linseed is in agreement with an *in vitro* study showing that the concentrations of these geometrical isomers of LA were increased by LNA supplementation, but not by LA supplementation (Jouany et al., 2007).

The significantly higher values of VA in MIX, and numerically higher in LIN, compared with GS and CON reflected the pattern of intake of its precursor LNA. The increase in RA with time, although with a different pattern among diets, reflected the observed reduction in its precursor LA during the trial. The highest concentration of RA in GS was likely associated mainly with the intake of its precursor (LA) and, to a lesser extent, with the presence of polyphenols in grape seed by-product. Some authors found that the accumulation of RA and VA in the rumen was increased by tannins (Vasta et al., 2010; Buccioni et al., 2011), which can inhibit the last step of the BH process of VA to SA (Khiaosa-Ard et al., 2009; Vasta et al., 2009; Rana et al., 2012). The low content of polyphenols in the grape seeds used in this trial was probably not high enough to markedly influence this BH process, as supported by the high rumen accumulation of SA and by the decrease in LA and LNA in both groups supplemented with grape seed. The higher content of SA in rumen liquor of all supplemented groups compared with

CON could be also a consequence of the high extent of OA biohydrogenation, as evidenced by the low accumulation of OA in the rumen liquor, despite its high intake. As expected, the linseed supplementation did not influence the accumulation of RA, because this FA is not an intermediate of the rumen BH of LNA (Wilde and Dawson, 1966).

3.6 Conclusions

The rumen metabolism of lactating dairy ewes was markedly influenced by dietary supplementation with grape seed alone or mixed with linseed. Although grape seed caused a decrease in LA and an increase in SA, this by-product determined an accumulation of RA in the rumen. When grape seed was mixed with linseed, it enhanced the BH of LNA, thus promoting a high accumulation of VA in the rumen. Therefore, the use of grape seed obtained after distillation in the winemaking process seems not to be effective in enhancing the effect of linseed supplementation.

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3.8 Tables

Table 3.1. Ingredients, chemical composition and fatty acid profile of diets

	Diets[a]			
	CON	GS	LIN	MIX
Ingredients (kg/day per head, as fed)				
Pea	0.25	0.09	0.15	0.02
Soybean	0.12	0.24	0.04	0.16
Corn	0.15	0.17	-	-
Grape seed	-	0.30	-	0.30
Linseed	-	-	0.22	0.22
Beet pulp	0.40	0.40	0.40	0.40
Commercial concentrate[b]	0.50	0.50	0.50	0.50
Dehydrated alfalfa hay[c]	0.80	0.80	0.80	0.80
Mixed hay[d]	0.20	0.20	0.20	0.20
Chemical composition[e]				
Dry matter (DM, g/kg)	908	916	912	920
NDF (g/kg DM)	418	428	437	445
NFC (g/kg DM)	334	289	285	242
ADL (g/kg DM)	46	89	50	94
CP (g/kg DM)	180	179	179	179
Ash (g/kg DM)	78	74	81	76
EE (g/kg DM)	20	32	51	58

Table 3.1. (continued)

Major fatty acids (g/100 g of total FAME)[f]	Diets[a]			
	CON	GS	LIN	MIX
C16:0	18.98	14.88	11.99	11.50
C16:1 *cis*-7	0.69	0.35	0.36	0.27
C16:1 *cis*-9	0.31	0.00	0.13	0.09
C16:1 *cis*-10	0.21	0.09	0.14	0.15
C17:0	0.00	0.00	0.15	0.12
C18:0 (SA)	3.33	4.47	4.39	4.68
C18:1 *cis*-9 (OA)	22.79	23.52	21.78	21.91
C18:1 *cis*-11	0.74	0.81	0.65	0.61
C18:2 *n*-6 (LA)	41.53	47.50	23.84	33.46
C18:3 *n*-3 (LNA)	8.25	5.04	34.45	24.93
C20:0	0.67	0.50	0.39	0.38
C20:1 *n*-9	0.38	0.36	0.24	0.27
C20:3 *n*-6	0.00	0.12	0.06	0.07
C24:0	0.53	0.45	0.37	0.30
SFA	24.24	20.88	17.75	17.42
MUFA	25.84	26.02	23.70	23.94
PUFA	49.92	53.11	58.55	58.64

[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.

[b] Commercial concentrate containing the following ingredients: sunflower seed flour, wheat bran, dehydrated alfalfa meal, corn gluten, rice husk, corn flour, soybean hulls, sugar beet molasses, calcium carbonate from powdered calcium rocks, distilled wheat, sodium chloride, plant oil (palm), mineral supplement (ferrous sulfate monohydrate at 106 mg/kg, calcium diiodate at 1.7 mg/kg, manganese oxide at 90 mg/kg, sodium selenite at 0.46 mg/kg, zinc oxide at 87 mg/kg, and sodium molybdate at 2.5 mg/kg), antioxidant (E310 propyl gallate at 4.3 mg/kg) and vitamin supplement (vitamin A at 17,971 IU/kg, vitamin D3 at 3,494 IU/kg, and vitamin E at 60 mg/kg).

[c] Dehydrated alfalfa hay composition: DM 936 g/kg, NDF 387 g/kg DM, CP 196 g/kg DM, EE 44 g/kg DM.

[d] Mixed hay composition: DM 873 g/kg, NDF 551 g/kg DM, CP 116 g/kg DM, EE 11 g/kg DM.

[e] NDF: neutral detergent fiber measured using heat stable amylase and expressed exclusive of residual ash, NFC: non-fiber carbohydrates, ADL: acid detergent lignin determined by solubilization of cellulose with sulphuric acid, CP: crude protein, EE: ether extract.

[f] FAME: fatty acid methyl esters; SA: stearic acid; OA: oleic acid; LA: linoleic acid; LNA: linolenic acid; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids.

Table 3.2. Chemical composition and FA profile of grape seed and linseed

Chemical composition[a]	Linseed	Grape seed
Dry matter (DM, g/kg)	911	974
NDF (g/kg DM)	240	539
NFC (g/kg DM)	105	231
ADL (g/kg DM)	31	410
CP (g/kg DM)	264	93
Ash (g/kg DM)	44	27
EE (g/kg DM)	347	109
Fatty acid (g/100 g of FAME)		
C16:0	5.5	8.5
C18:0 (SA)	4.5	4.9
C18:1 *cis*-9 (OA)	18.0	9.6
C18:2 *n*-6 (LA)	15.0	74.0
C18:3 *n*-3 (LNA)	56.3	0.3

[a] NDF: neutral detergent fiber measured using heat stable amylase and expressed exclusive of residual ash, NFC: non-fiber carbohydrates, ADL: acid detergent lignin determined by solubilization of cellulose with sulphuric acid, CP: crude protein, EE: ether extract, FAME: fatty acid methyl esters, SA: stearic acid, OA: oleic acid, LA: linoleic acid, LNA: linolenic acid.

Table 3.3. Dry matter and fatty acids intake of Sarda dairy ewes

	Diets[a]			
	CON	GS	LIN	MIX
Dry matter intake (kg/d)	2.2	2.5	2.1	2.4
Fatty acid intake (g/d)[b]				
C16:0	8.34	11.79	12.88	15.96
C16:1 *cis*-7	0.30	0.27	0.38	0.37
C16:1 *cis*-9	0.14	0.00	0.14	0.12
C16:1 *cis*-10	0.09	0.07	0.15	0.21
C17:0	0.00	0.00	0.16	0.17
C18:0 (SA)	1.46	3.54	4.72	6.49
C18:1 *cis*-9 (OA)	10.02	18.63	23.40	30.42
C18:1 *cis*-11	0.32	0.64	0.70	0.84
C18:2 *n*-6 (LA)	18.26	37.62	25.62	46.45
C18:3 *n*-3 (LNA)	3.63	3.99	37.02	34.60
C20:0	0.30	0.40	0.42	0.53
C20:1 *n*-9	0.17	0.28	0.26	0.37
C20:3 *n*-6	0.00	0.09	0.06	0.10
C24:0	0.23	0.36	0.40	0.41
SFA	10.66	16.53	19.08	24.17
MUFA	11.36	20.61	25.47	33.23
PUFA	21.94	42.06	62.91	81.39

[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.
[b] SA: stearic acid; OA: oleic acid; LA: linoleic acid; LNA: linolenic acid; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids.

Table 3.4. Effect of experimental diets with grape seed and linseed, alone or in combination, sampling and their interaction on rumen fermentation parameters in Sarda dairy ewes

Item	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
Rumen pH	6.75	6.71	6.68	6.77	0.06	ns	***
Ammonia (mg/dL)	13.66[b]	18.40[a]	13.88[b]	18.18[a]	0.62	*	*
Total VFA[d] (mmol/L)	57.52	59.18	58.67	54.10	2.30	ns	***
VFA[d] (mol/100 mol)							
Acetate	67.45	69.22	67.44	71.04	0.54	ns	***
Propionate	20.84	18.61	21.03	19.60	0.43	ns	***
Butyrate	11.71[a]	12.16[a]	11.54[a]	9.36[b]	0.37	**	***
Acetate:Propionate	3.44	3.79	3.31	3.74	0.09	ns	***

Means within a row with different superscripts (a, b) are different (P < 0.05).
[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.
[b] SEM: standard error of the mean.
[c] D: effect of experimental diet; S: effect of sampling.
[d] VFA: volatile fatty acids.
ns indicates P > 0.05;* P < 0.05; **P < 0.01; ***P < 0.001.

Table 3.5. Fatty acid composition of rumen liquor in Sarda dairy ewes

Fatty acid (mg/100 mg of FAME)[d]	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
C4:0	15.15[a]	9.59[b]	10.25[b]	8.79[b]	0.475	***	***
C5:0	3.01[a]	1.77[b]	1.86[b]	1.73[b]	0.113	**	***
C6:0	0.66	0.58	0.41	0.54	0.036	ns	***
C8:0	0.01	0.01	0.01	0.01	0.0005	ns	ns
C10:0	0.02	0.02	0.02	0.01	0.001	ns	*
C11:0	0.02	0.01	0.01	0.01	0.001	ns	**
C12:0	0.34	0.28	0.32	0.25	0.016	ns	***
iso C13:0	0.02	0.02	0.02	0.02	0.001	ns	**
anteiso C13:0	0.04	0.04	0.03	0.04	0.002	ns	**
C13:0	0.06	0.08	0.09	0.09	0.007	ns	ns
iso C14:0	0.19	0.19	0.18	0.17	0.008	ns	**
C14:0	0.69	0.77	0.78	0.64	0.032	ns	***
C14:1 *cis*-9	0.00	0.01	0.01	0.03	0.005	ns	ns
iso C15:0	0.33	0.30	0.27	0.32	0.013	ns	***
anteiso C15:0	1.40[a]	1.12[b]	1.19[ab]	0.98[b]	0.031	**	ns
C15:0	1.21	1.16	1.10	1.05	0.029	ns	***
iso C16:0	2.18[a]	2.00[a]	1.67[ab]	1.28[b]	0.060	***	***
C16:0	18.34[a]	16.84[ab]	15.68[bc]	14.21[c]	0.310	***	***
C16:1 *trans*-8	0.05[a]	0.02[b]	0.02[b]	0.02[b]	0.003	***	*
C16:1 *trans*-9	0.21[a]	0.12[b]	0.11[b]	0.01[b]	0.010	***	*
C16:1 *cis*-7	0.62	0.58	0.73	0.56	0.043	ns	***
C16:1 *cis*-9	0.06[a]	0.05[ab]	0.05[ab]	0.03[b]	0.002	**	ns
iso C17:0	0.31[a]	0.29[ab]	0.21[b]	0.23[ab]	0.010	**	***
anteiso C17:0	0.71[a]	0.58[ab]	0.51[b]	0.47[b]	0.023	**	***
C17:0	0.53[a]	0.44[ab]	0.39[b]	0.41[b]	0.014	**	*
C18:0 (SA)	19.81[b]	31.11[a]	28.02[a]	32.81[a]	0.797	***	**
C18:1 *trans*-4	0.03[c]	0.04[bc]	0.05[a]	0.05[ab]	0.002	***	***
C18:1 *trans*-6 + *trans*-8	0.40[b]	0.58[ab]	0.78[a]	0.83[a]	0.030	***	***
C18:1 *trans*-9	0.23[c]	0.36[bc]	0.45[ab]	0.50[a]	0.018	***	***
C18:1 *trans*-10	0.78	0.89	0.91	1.21	0.072	ns	**
C18:1 *trans*-11 (VA)	3.77[b]	4.76[b]	6.44[ab]	8.18[a]	0.302	**	ns
C18:1 *trans*-12	0.25[c]	0.73[b]	0.67[b]	0.91[a]	0.036	***	ns
C18:1 *cis*-9 (OA)	7.23[a]	5.97[a]	7.39[a]	5.89[b]	0.157	**	ns
C18:1 *trans*-15 + *cis*-10	0.46[b]	0.59[b]	0.85[a]	0.96[a]	0.035	***	***
C18:1 *cis*-11	0.77[c]	0.97[bc]	1.21[ab]	1.22[a]	0.031	***	ns
C18:1 *cis*-12	0.33[c]	0.61[ab]	0.44[bc]	0.71[a]	0.028	***	***

Table 3.5. (continued)

Fatty acid (mg/100 mg of FAME)[d]	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
C18:1 *cis*-13	0.03	0.03	0.05	0.05	0.003	ns	ns
C18:1 *cis*-14 + *trans*-16	0.08^{b}	0.16^{a}	0.19^{a}	0.20^{a}	0.010	***	**
C18:2 *trans*-9,*trans*-12	0.39^{b}	0.50^{b}	0.78^{a}	0.76^{a}	0.024	***	**
C18:1 *cis*-15	0.09^{c}	0.21^{bc}	0.26^{ab}	0.35^{a}	0.016	***	*
C18:2 *trans*-8,*cis*-13	0.23^{a}	0.20^{ab}	0.16^{bc}	0.14^{c}	0.007	***	ns
C18:2 *trans*-11,*cis*-15	0.03	0.03	0.01	0.02	0.003	ns	ns
C18:2 *trans*-9,*cis*-12	0.04^{b}	0.04^{b}	0.20^{a}	0.21^{a}	0.013	***	ns
C18:2 *n*-6 (LA)	5.95^{a}	4.66^{b}	3.77^{c}	3.26^{c}	0.161	***	***
C18:2 *n*-4	0.16	0.14	0.17	0.11	0.012	ns	***
C18:3 *n*-6	0.29	0.26	0.25	0.24	0.016	ns	***
C18:3 *n*-3 (LNA)	1.60^{a}	0.81^{b}	1.29^{a}	0.84^{b}	0.050	***	ns
CLA *cis*-9,*trans*-11 (RA)	0.45^{b}	0.78^{a}	0.49^{b}	0.47^{b}	0.057	**	***
C18:4 *n*-3	0.20^{a}	0.11^{b}	0.09^{b}	0.08^{b}	0.008	***	ns
CLA *trans*-9,*cis*-11 + C20	0.60	0.61	0.47	0.43	0.024	*	ns
CLA *trans*-10,*cis*-12	0.12^{ab}	0.10^{b}	0.17^{a}	0.15^{ab}	0.007	**	***
CLA *trans*-11,*cis*-13	0.03^{b}	0.08^{ab}	0.14^{a}	0.11^{ab}	0.011	*	***
CLA *trans*-11,*trans*-13	0.06^{b}	0.07^{b}	0.12^{a}	0.12^{a}	0.005	***	ns
CLA *trans*-9,11+ C20:1 *n*-9	0.13	0.14	0.14	0.13	0.004	ns	ns
C20:2 *n*-6	0.02	0.02	0.02	0.02	0.001	ns	***
C20:3 *n*-9	0.03^{ab}	0.03^{a}	0.03^{b}	0.03^{ab}	0.001	*	ns
C22:0	0.36^{a}	0.30^{ab}	0.33^{ab}	0.28^{b}	0.009	*	ns
C22:1 *n*-11	0.06^{a}	0.04^{b}	0.04^{b}	0.03^{b}	0.002	**	***
C20:5 *n*-3 (EPA)	0.20^{a}	0.15^{ab}	0.17^{ab}	0.13^{b}	0.007	*	***
C22:2 *n*-6	0.17^{a}	0.13^{b}	0.13^{b}	0.11^{b}	0.005	***	***
C24:0	0.42^{a}	0.34^{b}	0.37^{ab}	0.31^{b}	0.009	**	ns
C24:1 *cis*-15	0.53^{a}	0.41^{a}	0.38^{a}	0.33^{b}	0.020	*	ns
C22:5 *n*-3 (DPA)	0.01	0.01	0.01	0.01	0.002	ns	ns
C22:6 *n*-3 (DHA)	0.03	0.02	0.01	0.01	0.002	ns	ns

Means within a row with different superscripts (a, b, c) are different ($P < 0.05$).

[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.

[b] SEM: standard error of the mean.

[c] D: effect of experimental diet; S: effect of sampling.

[d] FAME: fatty acid methyl esters; SA: stearic acid; VA: vaccenic acid; OA: oleic acid; LA: linoleic acid; LNA: linolenic acid; RA: rumenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.

ns indicates $P > 0.05$;* $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3.6. Groups of fatty acids of rumen liquor in Sarda dairy ewes

Groups of fatty acids (mg/100 mg of FAME)[d]	Diet[a]				SEM[b]	P-value[c]	
	CON	GS	LIN	MIX		D	S
SCFA	18.86[a]	11.97[b]	12.56[b]	11.10[b]	0.583	***	***
MCFA	27.65[a]	25.16[ab]	23.72[bc]	21.31[c]	0.463	***	***
LCFA	46.37[c]	57.01[b]	57.52[b]	62.22[a]	0.799	***	ns
OBCFA	10.01[a]	8.00[b]	7.54[bc]	6.81[c]	0.164	***	*
iso BCFA	3.03[a]	2.80[ab]	2.36[bc]	2.03[c]	0.074	***	***
anteiso BCFA	2.15[a]	1.73[b]	1.73[b]	1.49[b]	0.043	***	***
OCFA	4.84[a]	3.47[b]	3.46[b]	3.30[b]	0.106	***	***
TFA	6.90[c]	8.86[bc]	11.53[ab]	13.87[a]	0.447	***	*
SFA	65.82	67.83	63.73	64.77	0.450	ns	***
MUFA	16.11[c]	17.12[bc]	21.18[ab]	22.30[a]	0.497	**	*
PUFA	10.73[a]	8.94[b]	8.64[b]	7.39[c]	0.171	***	*
n-6	6.44[a]	5.09[b]	4.17[b]	3.64[c]	0.160	***	***
n-3	2.03[a]	1.10[c]	1.57[b]	1.08[c]	0.059	***	*
CLA	1.38[b]	1.79[a]	1.52[ab]	1.42[ab]	0.067	*	***

Means within a row with different superscripts (a, b, c) are different ($P < 0.05$).

[a] CON: control diet, GS: diet supplemented with grape seed, LIN: diet supplemented with linseed, MIX: diet supplemented with both grape seed and linseed.

[b] SEM: standard error of the mean.

[c] D: effect of experimental diet; S: effect of sampling.

[d] FAME: fatty acid methyl esters; SCFA: short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA: medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA: long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; OBCFA: odd- and branched-chain fatty acids, sum of *iso* BCFA, *anteiso* BCFA, OCFA; *iso* BCFA: *iso* branched-chain fatty acids, sum of *iso* C13:0, *iso* C14:0, *iso* C15:0, *iso* C16:0, *iso* C17:0; *anteiso* BCFA: sum of *anteiso* C13:0, *anteiso* C15:0, *anteiso* C17:0; OCFA: odd-chain fatty acids, sum of C5:0, C9:0, C11:0, C13:0, C15:0, C17:0; TFA: *trans* fatty acids, sum of the individual trans fatty acids; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids; *n*-6: sum of individual *n*-6 fatty acids; *n*-3:sum of individual *n*-3 fatty acids; CLA: sum of individual conjugated of linoleic acids.

ns indicates $P > 0.05$;* $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.9 Figures

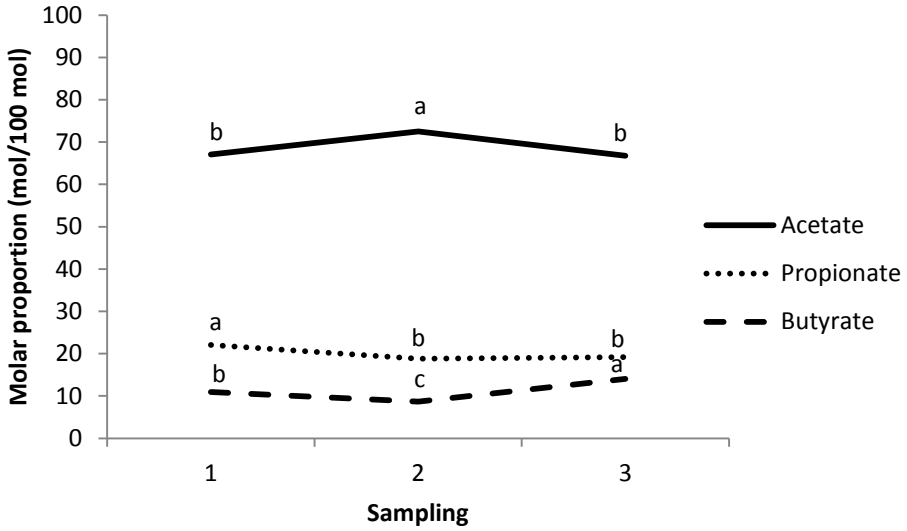

Figure. 3.1. Temporal evolution of the molar proportion of acetate, propionate and butyrate in rumen liquor on 3 samplings (days 20, 40 and 60) during the trial. Different letters (a, b, c) within the same volatile fatty acid show statistical differences ($P < 0.05$) between the samplings.

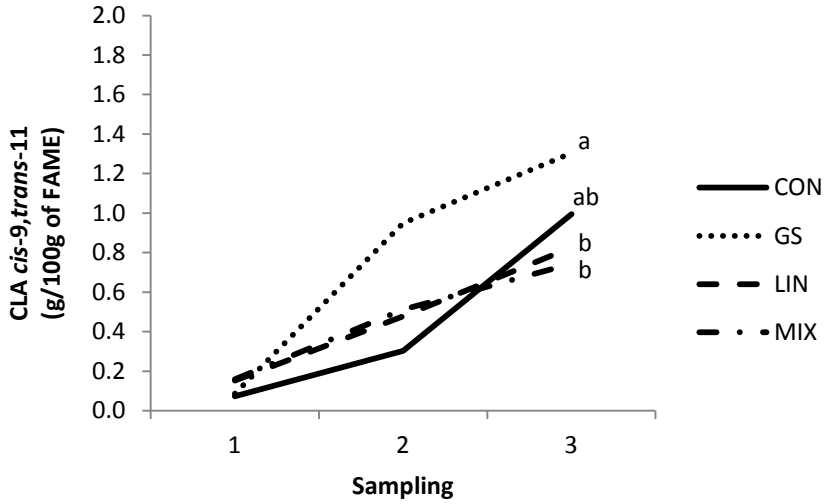

Figure. 3.2. Temporal evolution of CLA *cis*-9,*trans*-11 (RA; rumenic acid) in rumen liquor on 3 samplings (days 20, 40 and 60) during the trial in dairy ewes fed a control diet (CON), a diet supplemented with grape seed (GS), a diet supplemented with linseed (LIN) and a diet supplemented with both grape seed and linseed (MIX). Different letters (a, b) within the same sampling show statistical differences ($P < 0.05$) between the diets.

CHAPTER 4

Effect of grape seed and linseed, alone and in combination, on milk fatty acid composition in Sarda dairy sheep

4.1 Introduction

Growing interest in nutraceutical properties of food have directed the attention of researchers to increase the amount of healthy fatty acids (FA) in dairy products. Polyunsaturated fatty acids (PUFA), such as PUFA *n*-3, are recognized to be potential benefit to human health, by reducing serum triglycerides and low-density lipoprotein cholesterol (Simopoulus, 1991). Ovine milk is major source of conjugated linoleic acid (CLA), such as cis-9,trans-11 CLA (rumenic acid, RA), which have demonstrated several healthy effects, as antiatherosclerotic, anticancer, antidiabetic and anti-inflammatory activity (Bhattacharya et al., 2006).

The diet is the most important factor influencing the milk FA composition in dairy ewes. In order to increase the concentration of nutritional FA in milk, sources of unsaturated plant lipid such as linseed, soybeans, safflower and sunflower could be successful included in the diet (Nudda et al., 2014). In particular linseed supplementation gave high concentration of α-linolenic acid, CLA and VA, in sheep, cows and goats (Zhang et al 2006; Caroprese et al 2010; Nudda et al., 2013). Manipulation of ruminal biohydrogenation processes also influence the milk FA composition. It has been demonstrated, in vitro and in vivo, that the inhibition of some strain of rumen microbes, by use of dietary polyphenols, could reduce the extent of biohydrogenation of PUFA (Vasta et al., 2009, 2010), thus promoting the escape from the rumen and, consequently, increasing their concentration in milk.

Grape seed is a by-product derived from the winery and distillery industries which contain high amount of polyphenols, mainly proanthocyanidins (Schieber et al., 2001). The use of grape seed in ruminant nutrition could be useful to modulate ruminal biohydrogenation of PUFA and could represent an alternative for its high costly management and disposal. The inclusion of grape residue in ruminant nutrition was able to reduce methane emission and improve milk antioxidant activity in dairy cows (Moate et al., 2014; Santos et al., 2014).

The aim of this work was to evaluate the effect of the inclusion of grape seed and linseed, alone and in combination, on milk fatty acid composition in Sarda dairy sheep.

4.2 Material and methods

The experimental trial was conducted in a farm located in the north-west of Sardinia, during 2013. The dietary composition, chemical analysis and sheep management details have been previously described in the previous chapter. Briefly, 24 Sarda dairy sheep were allocated to the follow dietary treatments: control diet (CON), a diet containing 300 g/d per head of grape seed (GS), a diet containing 220 g/d per head of extruded linseed (LIN) and a diet containing both 300 g/d of grape seed and 220 g/d of linseed per head (MIX).

Milk samples

Individual morning milk samples were collected weekly and stored at -20 °C until analysis of FA. Milk samples collected in the last four weeks of the trial were used to analyze the milk fatty acid composition.

Fatty acid composition of milk

Milk fat extraction and fatty acid methyl esters (FAME) preparation were performed as described by Nudda et al. (2005). A Turbo 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and an automatic injector 8200 (CX Varian Inc., Palo Alto, CA) was used for determination of FAME. The FAME were separated on a capillary column (CP-select CB for FAME; 100 m x 0.32 mm i.d., 0.25 μm film thickness, Varian Inc., Palo Alto, CA) and quantified using pentanoic (C5:0) and nonadecanoic acid (C19:0) methyl esters (Sigma Chemical Co., St. Louis, MO) as an internal standards. The programmed temperature was: 75°C for 1 min, increased at 5°C/min to 148°C and at 8°C/min to 165°C, held for 35 min; then increased at 5.5°C/min to 210 and, finally, at 3°C to 230°C, held for 14 min. Helium (1 mL/min flow rate) was used as carrier gas with a pressure of 37.000 psi. Split ratio was 1:100. The injector and detector temperatures were held at 225 and 285°C, respectively. Varian Star 3.4.1 software was used to compute the retention time and area of each individual FAME, identified by comparing their retention times with those of known standards and with published studies as detailed in Nudda et al. (2005).

FA were reported as g/100 g of total FAME and groups of FA were calculated as follow: saturated fatty acids (SFA), sum of the individual saturated fatty acids; monounsaturated fatty acids (MUFA), sum of the individual monounsaturated fatty

acids; polyunsaturated fatty acids (PUFA), sum of the individual polyunsaturated fatty acids; branched-chain fatty acids (BCFA), sum of individual branched-chain fatty acids; odd- and branched-chain fatty acids (OBCFA), sum of individual odd- and branched-chain fatty acids; short-chain fatty acids (SCFA), sum of the individual fatty acids from C4:0 to C10:0; medium-chain fatty acids (MCFA), sum of the individual fatty acids from C11:0 to C17:0; long-chain fatty acids (LCFA), sum of the individual fatty acids from C18:0 to DHA; PUFA *n*-3, sum of individual *n*-3 fatty acids; PUFA *n*-6, sum of individual *n*-6 fatty acids; CLA, sum of individual conjugated of linoleic acids.

Atherogenic index (AI) and trombogenic index (TI) were calculated according to Ulbricht and Southgate (1991) except for the substitution of C18:0 with C12:0 : AI = [12:0 + (4×14:0) + 16:0]/[(PUFA) + (MUFA)], and TI = (14:0 + 16:0)/[(0.5×MUFA) + (0.5×*n*-6) + (3×*n*-3) + (*n*-3/*n*-6)]. The hypocholesterolemic/hypercholesterolemic ratio (h/H) were calculated according to Fernandez et al. (2007), as follow: h/H = [(sum of 18:1*cis*-9, 18:1*cis*-11, 18:2 *n*-6, 18:3 *n*-6,18:3 *n*-3, 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:4 *n*-6, 22:5 *n*-3 and 22:6 *n*-3)/(14:0 + 16:0)].

Statistical analysis

Data of milk FA were analyzed by with the PROC MIXED procedure of SAS (2002). The model included fixed effect of diet, sampling date (4 levels each) and their interaction; moreover to account for individual variability, the random effect of animal was nested within each treatment. The significance of group mean differences was assessed using HSD Tukey test ($P < 0.05$). In addition a multivariate approach was used to evaluate the effect of diets in milk FA pattern. In particular a total of 22 variables were analyzed (18 milk fatty acid class and 4 healthy indexes, see section *Fatty acid composition of milk* for further details) using hierarchical cluster analysis (HCA) and principal component (PC) analysis. CA was performed on the milk FA profile using as distance metrics the Euclidean distances and average linkage methods. The visualization of experimental units clustering was provided through a dendogram. Furthermore, the correlation matrix of milk fatty profiles was decomposed by PC analysis following:

$$PC_j = \alpha_{1j}y_1 + \ldots + \alpha_{nj}y_n,$$

where PC represent the generic *j*-th linear combination of the observed variables (scores) and α_{ij} coefficients of the eigenvector (loading) of correlation matrix,

corresponding to the generic *j*-th eigenvalue (i.e. the variance explained by the j-th PC). The process of extraction was stopped when the variance explained by eigenvalues summed up to 80% of the total variance. Individual PC scores were used in a one-way anova including the fixed effect of treatments.

4.3 Results and discussion

The milk FA composition was significantly altered by the experimental diets (Table 4.1 and 4.2.). The inclusion of grape seed, linseed or both in the diets reduced the concentration of FA from C6:0 to C10:1 ($P < 0.05$) and tended to reduce those of C4:0 ($P = 0.07$). The content in most of the FA from C11:0 to C17:1 *cis*-9 decreased in the GS, LIN and MIX groups compared with CON ($P < 0.05$), except for the C16:1 *trans*-6 + *trans*- 7 and C16:1 *cis*-7 which did not differ ($P > 0.05$), and except for C16:1 *trans*-8, which increased in MIX, C16:1 *trans*-9, which increased in LIN and MIX, and C16:1 *cis*-10, which increased in GS, LIN and MIX, compared with CON ($P < 0.05$). These changes resulted in reduced total concentration of short chain fatty acids (SFA) and middle chain fatty acids (MCFA) in the milk of ewes receiving grape seed, linseed or both compared with the control group ($P < 0.05$). The total concentration of long chain fatty acids (LCFA) was lower in the CON than the other groups ($P < 0.05$), following the increasing order: CON, GS, LIN and MIX. The content of C18:0 (stearic acid, SA) increased in GS, LIN and MIX compared with CON ($P < 0.05$). The concentration of most of the C18:1, C18:2 and CLA isomers increased in the milk of sheep fed linseed compared with that of CON ($P < 0.05$). These results were expected as linseed is a reach source of C18:1, C18:2 and C18:3 fatty acids, and were consistent with other works showing increased milk content of these FA in cows (Caroprese et al., 2010; Ferlay et al., 2013), goats (Nudda et al., 2006; 2013) and ewes (Mughetti et al., 2012) fed linseed. The presence of high concentrations of C18:1 isomers in LIN and MIX groups are likely the results of the ruminal biohydrogenation of C18:2 and C18:3 FA, and of the desaturation of SA in the mammary gland (Kennelly 1996). The concentration of C18:1 *trans*-11 (vaccenic acid, VA) increased ($P < 0.05$) in LIN and MIX, and tended to increase ($P < 0.10$) in GS compared with CON. This is consistent with the high amount of linolenic acid (C18:3 *n*-3, LNA) supplied by dietary linseed, as this FA is a precursor of VA produced by the ruminal metabolism, and is in accordance with the works of

Nudda et al. (2006, 2013) and Mughetti et al. (2012) which found increased levels of VA in milk of dairy goats and sheep fed linseed. VA is the precursor of CLA *cis*-9,*trans*-11, formed by the Δ^9-desaturase in mammary gland (Griinari et al., 1999). According with this fact, in our study, the concentration of rumenic acid in the milk of groups fed linseed was higher ($P < 0.05$) than in those of CON group. The inclusion of linseed in the diet resulted, also, in the increase ($P < 0.05$) of concentration of LNA, both in milk of LIN and MIX, compared with CON and GS groups. This finding agrees with other studies showing increased level of LNA, when linseed is included in the diet of cows, as reported in the meta-analysis of Glasser et al. (2008). However, in that study, the mean of the milk concentrations of LNA in linseed supplemented diets, did not exceed 1.10 % of total FA; in our work the concentration of LNA in milk from LIN group was 1.89 % of total FA, increased by 155 % compared to the CON.

The presence of moderate concentration of polyphenols in the diet of ruminant increased the milk level of beneficial FA, mainly LNA, in sheep (Cabiddu et al., 2009) and cows (Dschaak et al., 2011). This effect has been explained by the capacity of polyphenols to inhibit the activity of same strain of ruminal bacteria, involved in the biohydrogenation of unsaturated FA (Cabiddu et al., 2009; Vasta et al., 2009). Contrary to our hypothesis, the inclusion of grape seed in the ewe's diet was not able to reduce the extent the biohydrogenation of PUFA. The level of C18:3 *n*-3 in milk of GS group did not differ from those of CON group ($P > 0.05$) and decreased in milk of MIX compared with LIN group ($P < 0.05$). The low level of polyphenols in the grape seed used in the present work, compared with those of other studies, could explain the lack of effect of this ingredient to increase the concentration of LNA in milk of GS compared with CON. However, it does not explain the reduction of LNA in milk of MIX compared with those of LIN group. It must been considered that grape seed contain other compounds that could have affected the biohydrogenation of UFA. According with our result, when grape marc was included in the diet of lactating cows, the milk concentration of LNA did not increase (Moate et al., 2014). The concentration of PUFA *n*-3 mirrored the result of LNA: CON and GS showed lowest values than LIN, with MIX being intermediate ($P < 0.05$). As expected, the concentration of LA and, consequently, of PUFA *n*-6 were greater in the milk of both groups fed grape seed, than in CON and LIN ($P < 0.05$). This is likely due to the high amount of LA in grape seeds,

in agreement with the findings of Moate et al. (2014) and Santos et al. (2014), showing increased level of this FA in milk of dairy cows fed grape residue.

The total concentration of OBCFA decreased in the milk of GS, LIN and MIX groups, compared with CON, being the lowest in MIX and intermediate in GS and LIN ($P < 0.05$). OBCFA are reported to be mainly derived from the ruminal microflora (Fievez et al., 2012). The decrease in OBCFA in milk of LIN and GS groups could be explained by the high amount of LNA in linseed, and LA in grape seed. PUFA, in fact, are reported to be toxic for the grown of ruminal microorganisms (Maia et al., 2007, 2010). High amount of PUFA, mainly composed by LNA and LA, in MIX diet could give an explanation for the lowest concentration of OBCFA found in the milk of sheep of MIX group.

The concentrations of C12:0, C14:0 and C16:0 were lower in milk of groups receiving grape seed, linseed, alone or in combination, than in those of control group ($P < 0.05$). In milk of MIX the levels of those FA were also lower than GS, being intermediate in LIN ($P < 0.05$). Among the SFA, these three FA are the mainly involved in atherogenic and thrombogenic processes. In order to better understand the different effects that single milk FA might have on human health, in term of probability of increasing the incidence of atherogenesis and thrombosis, three different indices were calculated: atherogenic index (AI), thrombogenic index (TI) (Ubricht and Southgate, 1991) and hypocholesterolemic/hypercholesterolemic ratio (h/H) (Fernandez et al., 2007). As shown in Figure 4.1 the inclusion of grape seed and linseed in the diet decreased both atherogenic and thrombogenic indices and increased h/H ratio compared with CON ($P < 0.05$). Our result is consistent with the fact that dietary sources of PUFA lead amelioration of cardiac risk factors (Duda et al., 2009, Katare and Saxena, 2013), and agrees with the finding of Nudda et al. (2013) which found decreased values of AI and TI and increased ratio h/H when dairy goats were fed extruded linseed. Similar results were obtained by feeding Lacaune ewes with extruded linseed (Casamassima et al., 2014). The effect of dietary inclusion of grape seed on these indices was likely related to the high rate of decrease in C12:0, C14:0 and C16:0 and increase in MUFA. The values of AI and TI were lower and h/H was higher in LIN than GS ($P < 0.05$), suggesting that the inclusion of linseed appears to be more effectively in ameliorating

these indices than grape seed. The administration of grape seed and linseed in combination (MIX) determined lower values of AI and TI indices, and higher value of h/H than single administration (GS and LIN) ($P < 0.05$).

The HCA and PCA multivariate methods were used to better clarify the effect of the different dietary treatments on the milk FA composition. Dataset used for those analysis was obtained from the average values of four sampling date for each animal.

The results of PCA are shown in Table 4.3 and Figure 4.2. Two PC were retained for further analysis based on the proportion of variance explained by PC. The first and second principal components accounted for about 90 % of the total variability (78.3 % and 12%, PC1 and PC2, respectively). Table 4.3 shows the eigenvalues and eigenvectors of correlation matrix derived from groups of FA of milk. The PC1 was positively associated with the groups of FA characterized by long and unsaturated chains, whereas groups characterized by short-, middle-chain FA and saturated FA showed negatively association with PC1. The first component is also positively correlated with the C18:1 and C18:2 isomers and, among C18:1, the *trans* showed greater correlation than *cis* isomers. According to other studies involving other ruminants, when diet of dairy cows and goats was supplemented with vegetable oils as source of PUFA, milk samples showed increased concentration of long chain PUFA *n*-3 (Ferlay et al., 2013) or PUFA *n*-6 (Almeida et al., 2013), and decreased concentration of short- and medium-chain FA. Among PUFA, PC2 loadings were positively correlated with *n*-6 and *n*6/*n*3 ratio, and negatively with *n*-3. Moreover, PC2 negatively discriminated the OBCFA and OCFA. PC1 presented high positive loadings for h/H ratio and high negative loadings for AI and TI indices. PC2, to a lesser extent, positively correlated the AI and TI and negatively the h/H ratio.

The plot of the first two PC scores allows to describe the relationship among animals, based exclusively based on the milk FA profile (Figure 4.2). Four clusters were identified, according to the four dietary treatments, with the CON being the more isolated group, mainly discriminated by PC1 (negative scores). PC2 scores discriminated LIN (positive scores) from GS (negative scores). It could be supposed that PC1 was positively associated with the dietary inclusion of ingredients rich in PUFA, in particular with the PUFA intake (CON<GS<LIN<MIX, reported in cap 3.),

and PC1 could be named as ”PUFA intake”. The PC2 could be related with the different source of PUFA (grape seed or linseed) and consequently, with the ratio PUFA n6/n3 in the diets, with *n*-6/*n*-3 ratio in the positive range. The PC2 could be identified with the n6/n3 ratio.

The results of HCA analysis are shown in Figure 4.3. The dendogram allow to group all animals in four clusters, with the 72.80 % of similarity level. The animals of CON resulted in a unique cluster, indicating that chemical composition of milk from this group was different from those of the other groups. Another unique cluster grouped together the animals of GS, indicating that the chemical composition of milk from sheep receiving grape seed was different both from those of the CON and from those of the sheep fed linseed (LIN and MIX). The animals of these latter groups were grouped in two clusters, with few cases of incorrect assignation: two animal of MIX were assigned to the LIN, and one animal of LIN was grouped in the cluster grouping animal of MIX. This may suggest that chemical composition of milk from sheep fed linseed (LIN and MIX) was different from those of CON and GS groups, and that, within sheep fed linseed, the inclusion of grape seed caused lesser variation in milk FA composition.

The results of the statistical analysis of the relationship between groups and principal components (Table 4.4) confirmed the clustering of animals in the four dietary treatments evidenced by the plot of principal components (Figure 4.2) and by the dendogram (Figure 4.3).

4.4 Conclusions

Dietary inclusion of grape seed and linseed altered the milk FA composition in Sarda dairy ewes. When included alone 300 g/d per head of grape seed decreased the SFA content and increased those of UFA and PUFA, mainly due to a high increase of LA; the concentration of RA and VA tended to be higher than in the control group. Inclusion of 200 g/d per head of linseed alone in the diet of lactating ewes resulted in increased concentration potentially beneficial FA such as oleic acid, linolenic acid, and CLA *cis*-9,*trans*-11. The inclusion of grape seed and linseed in combination resulted in a better FA profile than the singles, except for LNA. The atherogenic index, thrombogenic

index and hypocholesterolemic/hypercholesterolemic ratio suggest that this combination could be useful to improve milk quality, in term of healthy FA.

4.5 References

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4.6 Tables

Table 4.1. Fatty acid profile of milk from sheep from different experimental diets.

Fatty acid (g/100 g of FAME)[4]	Group[1]				S.E.M.[2]	P *values*
	CON	GS	LIN	MIX		G[3]
C4:0	2.03	2.25	2.19	2.30	0.038	ns
C6:0	1.94^{a}	1.81^{ab}	1.51^{bc}	1.25^{c}	0.073	***
C8:0	2.21^{a}	1.87^{ab}	1.44^{bc}	1.04^{c}	0.110	***
C9:0	0.05^{a}	0.04^{b}	0.02^{c}	0.02^{c}	0.003	***
C10:0	9.05^{a}	6.38^{b}	4.67^{bc}	3.28^{c}	0.494	***
C10:1	0.35^{a}	0.25^{b}	0.18^{bc}	0.11^{c}	0.021	***
C11:0	0.10^{a}	0.05^{b}	0.03^{bc}	0.02^{c}	0.007	***
C12:0	6.23^{a}	3.88^{b}	2.99^{bc}	2.21^{c}	0.332	***
iso C13:0	0.06^{a}	0.03^{b}	0.02^{b}	0.02^{b}	0.004	***
anteiso C13:0	0.01	0.01	0.01	0.01	0.001	ns
C13:0	0.10^{a}	0.07^{b}	0.06^{bc}	0.04^{c}	0.005	***
iso C14:0	0.10	0.11	0.10	0.09	0.006	ns
C14:0	13.45^{a}	10.89^{b}	9.77^{bc}	8.53^{c}	0.422	***
C14:1 *cis*-9	0.33^{a}	0.23^{b}	0.20^{b}	0.16^{b}	0.018	**
iso C15:0	0.21^{ab}	0.19^{ab}	0.22^{a}	0.16^{b}	0.007	*
anteiso C15:0	0.49^{a}	0.45^{ab}	0.46^{ab}	0.39^{b}	0.013	*
C15:0	1.27^{a}	1.03^{b}	1.05^{b}	0.90^{b}	0.034	***
iso C16:0	0.29^{a}	0.24^{ab}	0.25^{ab}	0.21^{b}	0.008	**
C16:0	30.19^{a}	24.02^{b}	22.64^{bc}	21.13^{c}	0.767	***
C16:1 t6 + t7	0.06	0.06	0.07	0.07	0.002	ns
C16:1 *trans*-8	0.02^{b}	0.04^{ab}	0.03^{ab}	0.07^{a}	0.007	*
C16:1 *trans*-9	0.08^{c}	0.25^{bc}	0.34^{ab}	0.57^{a}	0.046	***
C16:1 *trans*-10	0.01^{c}	0.01^{b}	0.01^{b}	0.02^{a}	0.001	***
C16:1 *cis*-7	0.28	0.28	0.32	0.29	0.006	ns
C16:1 *cis*-9	1.19^{a}	0.78^{b}	0.74^{b}	0.64^{b}	0.061	*
C16:1 *cis*-10	0.01^{c}	0.03^{b}	0.02^{bc}	0.05^{a}	0.003	***
iso C17:0	0.36^{a}	0.32^{b}	0.36^{a}	0.28^{c}	0.009	***
anteiso C17:0	0.50^{a}	0.39^{bc}	0.44^{ab}	0.32^{c}	0.016	***
C17:0	0.66^{a}	0.53^{bc}	0.61^{b}	0.48^{c}	0.017	***
C17:1 *cis*-9	0.25^{a}	0.15^{bc}	0.17^{b}	0.11^{c}	0.012	***
C18:0	5.47^{b}	8.73^{a}	9.89^{a}	10.03^{a}	0.461	***
C18:1 *trans*-4	0.03^{b}	0.04^{ab}	0.05^{a}	0.04^{ab}	0.001	*
C18:1 *trans*-6+*trans*-8	0.20^{c}	0.47^{b}	0.59^{ab}	0.73^{a}	0.046	***

Table 4.1. (Continued)

Fatty acid (g/100 g of FAME)[4]	Group[1]				S.E.M.[2]	P *values*
	CON	GS	LIN	MIX		G[3]
C18:1 *trans*-9	0.22[c]	0.48[b]	0.56[b]	0.71[a]	0.041	***
C18:1 *trans*-10	0.52[b]	1.00[ab]	0.86[ab]	1.81[a]	0.163	*
C18:1 *trans*-11	1.04[c]	3.02[bc]	4.09[ab]	6.24[a]	0.471	***
C18:1 *cis*-9+t13+t14	13.39[b]	17.90[a]	19.66[a]	19.34[a]	0.636	***
C18:1 *cis*-10+t15	0.40[b]	0.45[ab]	0.68[a]	0.70[a]	0.037	**
C18:1 *cis*-11	0.43[b]	0.60[b]	0.82[a]	0.79[a]	0.039	***
C18:1 *cis*-12	0.28[d]	0.86[c]	0.61[b]	1.27[a]	0.079	***
C18:1 *cis*-13	0.02[b]	0.04[b]	0.08[a]	0.09[a]	0.006	***
C18:1 *cis*-14 + t16	0.16[c]	0.21[c]	0.42[a]	0.34[b]	0.024	***
C18:2 *trans*-9,12	0.42[c]	0.77[b]	1.25[a]	1.25[a]	0.076	***
C18:1 *cis*-15	0.06[c]	0.08[c]	0.24[a]	0.19[b]	0.016	***
C18:2 *trans*-8,*cis*13	0.02[b]	0.03[b]	0.07[a]	0.07[a]	0.005	***
C18:2 *cis*-9,*trans*-12	0.08[c]	0.17[b]	0.27[a]	0.25[a]	0.017	***
C18:2 *trans*-9,*cis*-12	0.15[c]	0.22[ab]	0.19[b]	0.24[a]	0.008	***
C18:2 *n*-6	2.68[b]	4.65[a]	2.97[b]	4.86[a]	0.233	***
C18:3 *n*-6	0.10[a]	0.06[b]	0.02[c]	0.03[c]	0.007	***
C18:3 *n*-3	0.74[c]	0.58[c]	1.89[a]	1.43[b]	0.115	***
CLA *cis*-9,*trans*-11+t7c9	0.70[c]	1.75[bc]	2.18[ab]	3.01[a]	0.217	***
C18:4 *n*-3	0.06[a]	0.04[b]	0.05[ab]	0.06[ab]	0.003	*
CLA *trans*-9,*cis*-11+C20:0	0.18[b]	0.19[ab]	0.23[a]	0.21[ab]	0.006	*
CLA *cis*-10,*cis*-12	0.01[b]	0.02[b]	0.11[a]	0.09[a]	0.011	***
CLA *trans*-11,*cis*-13	0.01[c]	0.02[c]	0.16[a]	0.13[b]	0.014	***
CLA *trans*-11,*trans*-13	0.04[b]	0.04[b]	0.10[a]	0.09[a]	0.006	***
CLA *cis*-11,*cis*-13	0.08[c]	0.10[bc]	0.12[ab]	0.14[a]	0.006	***
CLA t9,t11+C20:1 *n*-9	0.01	0.01	0.01	0.01	0.001	ns
C20:2 *n*-6	0.02	0.02	0.02	0.02	0.001	ns
C20:3 *n*-9	0.06[a]	0.04[b]	0.06[a]	0.04[b]	0.002	***
C20:3 *n*-6	0.03[ab]	0.03[a]	0.02[c]	0.02[bc]	0.001	***
C20:4 *n*-6	0.15[a]	0.15[a]	0.07[b]	0.08[b]	0.009	***
C20:3 *n*-3	0.01[bc]	0.01[c]	0.02[a]	0.01[b]	0.001	***
C22:0	0.09[ab]	0.07[c]	0.11[a]	0.08[bc]	0.004	***
C20:4 *n*-3	0.02[ab]	0.01[b]	0.02[a]	0.02[ab]	0.001	**
C22:1 *n*-11	0.00[b]	0.00[b]	0.01[a]	0.01[ab]	0.001	*
EPA	0.07[a]	0.03[c]	0.08[a]	0.05[b]	0.004	***

Table 4.1. (Continued)

Fatty acid (g/100 g of FAME)[4]	Group[1]				S.E.M.[2]	P *values*
	CON	GS	LIN	MIX		G[3]
C22:2 *n*-6	0.04[a]	0.03[b]	0.05[a]	0.03[b]	0.002	***
C22:4 *n*-6	0.01[ab]	0.01[a]	0.00[b]	0.00[b]	0.001	**
C24:0	0.02[a]	0.01[b]	0.02[a]	0.02[ab]	0.001	**
C24:1 *cis*-15	0.01	0.00	0.01	0.00	0.000	ns
DPA	0.07[a]	0.04[b]	0.08[a]	0.05[b]	0.003	***
DHA	0.01	0.01	0.02	0.02	0.001	ns

Means within a row with different superscripts (a, b, c) are different ($P < 0.05$).
[1] CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.
[2] SEM: standard error of the mean.
[3] D: effect of experimental diet.
[4] FAME: fatty acid methyl esters; SA: stearic acid; VA: vaccenic acid; LA: linoleic acid; LNA: linolenic acid; RA: rumenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid;
ns: $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$

Table 4.2. Groups of FA and FA healty indices of milk from sheep from different experimental diets

Item (g/100 g of FAME)[4]	Group[1]				S.E.M.[2]	P *values*
	CON	GS	LIN	MIX		G[3]
SFA	74.89[a]	63.37[b]	58.86[b]	52.80[c]	1.812	***
UFA	25.11[c]	36.63[b]	41.14[b]	47.23[a]	1.812	***
MUFA	19.34[c]	27.58[b]	31.09[b]	34.99[a]	1.283	***
PUFA	5.77[c]	9.05[b]	10.06[ab]	12.21[a]	0.553	***
BCFA	2.02[a]	1.74[bc]	1.85[ab]	1.48[c]	0.052	***
OBCFA	3.91[a]	3.21[b]	3.37[b]	2.72[c]	0.103	***
SCFA	15.64[a]	12.60[b]	10.02[bc]	8.00[c]	0.678	***
MCFA	56.25[a]	44.04[b]	40.89[b]	36.77[c]	1.572	***
LCFA	28.11[d]	43.36[c]	49.09[b]	55.24[a]	2.191	***
PUFA *n*-3	0.99[c]	0.73[c]	2.16[a]	1.64[b]	0.121	***
PUFA *n*-6	3.02[b]	4.96[a]	3.16[b]	5.03[a]	0.229	***
n6/n3	3.12[b]	7.01[a]	1.47[c]	3.09[b]	0.445	***
Total CLA	1.03[c]	2.13[bc]	2.90[ab]	3.69[a]	0.248	***
AI	3.6[a]	2.0[b]	1.6[c]	1.2[d]	0.100	***
TI	2.5[a]	1.5[b]	1.1[c]	0.9[c]	0.067	***
h/H	0.4[d]	0.7[c]	0.8[b]	0.9[a]	0.022	***

Means within a row with different superscripts (a, b, c) are different ($P < 0.05$).
[1] CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.
[2] SEM: standard error of the mean.
[3] D: effect of experimental diet.
[4] FAME: fatty acid methyl esters; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids; BCFA: branched-chain fatty acids; OBCFA: odd- and branched-chain fatty acids; SCFA: short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA: medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA: long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; PUFA *n*-3: sum of individual *n*-3 fatty acids; PUFA *n*-6: sum of individual *n*-6 fatty acids; CLA: sum of individual conjugated of linoleic acids. AI: Atherogenic index; TI: trombogenic index; h/H: hypocholesterolemic/hypercholesterolemic ratio.
*** $P < 0.001$.

Table 4.3. Eigenvalues and Eigenvector of correlation matrix based on group of milk fatty acid

Item[1]	PC1	PC2
UFA	0.246	-0.027
MUFA	0.244	-0.058
Toal C18:1	0.243	-0.058
LCFA	0.243	-0.055
PUFA	0.241	0.044
Toal C18:2	0.235	0.154
TFA	0.233	0.055
total CLA	0.231	-0.004
h/H	0.230	-0.099
Tot C18:1-*trans*	0.227	0.085
Tot C18:1-*cis*	0.195	-0.188
PUFA *n*-6	0.158	0.455
PUFA *n*-3	0.139	-0.470
n-6/*n*-3	-0.021	0.559
BCFA	-0.174	-0.288
OBCFA	-0.202	-0.249
SCFA	-0.231	0.108
TI	-0.236	0.093
MCFA	-0.238	0.030
AI	-0.238	0.021
SFA	-0.246	0.027
Eigenvalues	16.45	2.51
% var explained	78.3	11.9

[1]Class of FA and FA healthy indices calculated on individual FA reported in Table 4.1; SFA: saturated fatty acids, sum of the individual saturated fatty acids; MUFA: monounsaturated fatty acids, sum of the individual monounsaturated fatty acids; PUFA: polyunsaturated fatty acids, sum of the individual polyunsaturated fatty acids; BCFA: branched-chain fatty acids; OBCFA: odd- and branched-chain fatty acids; SCFA: short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0; MCFA: medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0; LCFA: long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA; PUFA *n*-3: sum of individual *n*-3 fatty acids; PUFA *n*-6: sum of individual *n*-6 fatty acids; CLA: sum of individual conjugated of linoleic acids. AI: Atherogenic index; TI: trombogenic index; h/H: hypocholesterolemic/hypercholesterolemic ratio.

Tables 4.4. Dietary effects on PC scores of individuals belonging to different group for PC1 (*PUFA intake*) and PC2 (*n6/n3*).

	Groups				SEM	P *values*
item	CON	GS	LIN	MIX		Group
PC1	-5.7720^{d}	-0.2671^{c}	1.5283^{b}	4.5108^{a}	0.5905	***
PC2	-0.1499^{b}	1.8599^{a}	-2.0293^{c}	0.3193^{b}	0.3105	***

Means within a row with different superscripts (a, b, c) are different ($P < 0.05$).
[1]CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.
[2]SEM: standard error of the mean.
*** $P < 0.001$.

4.7 Figures

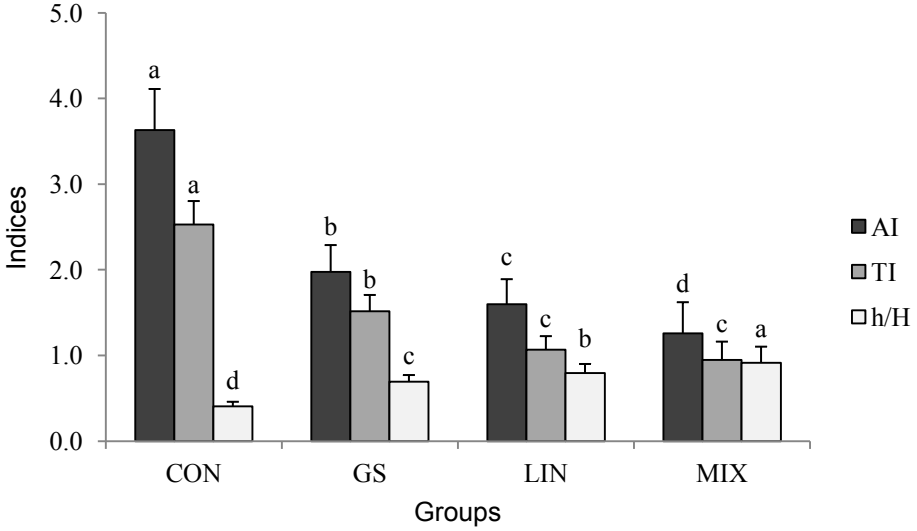

Figure 4.1. Effect of experimental diets on health milk lipid indices: atherogenic index (AI), thrombogenic index (TI) and hypocholesterolemic/hypercholesterolemic ratio (h/H). CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.

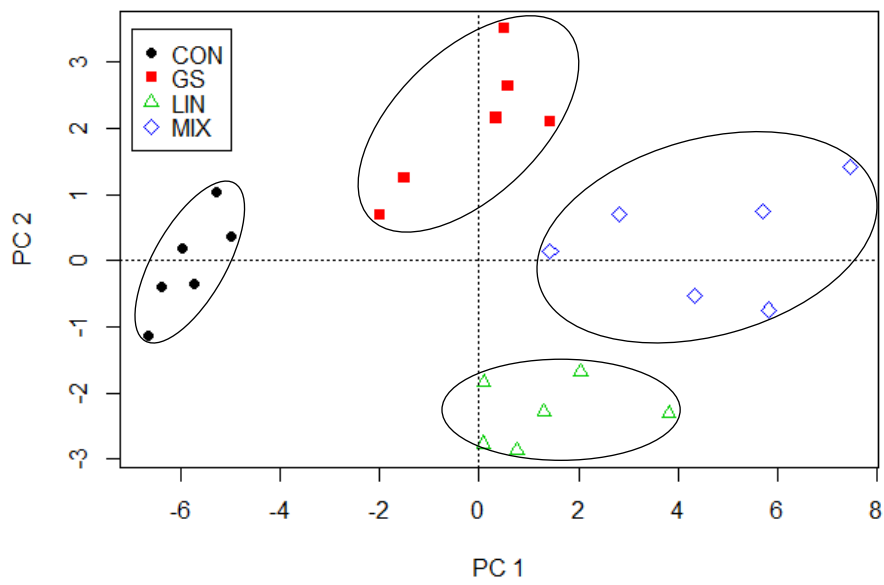

Figure 4.2. Plot of the scores of the first two principal components of individuals belonging to different experimental group. CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed.

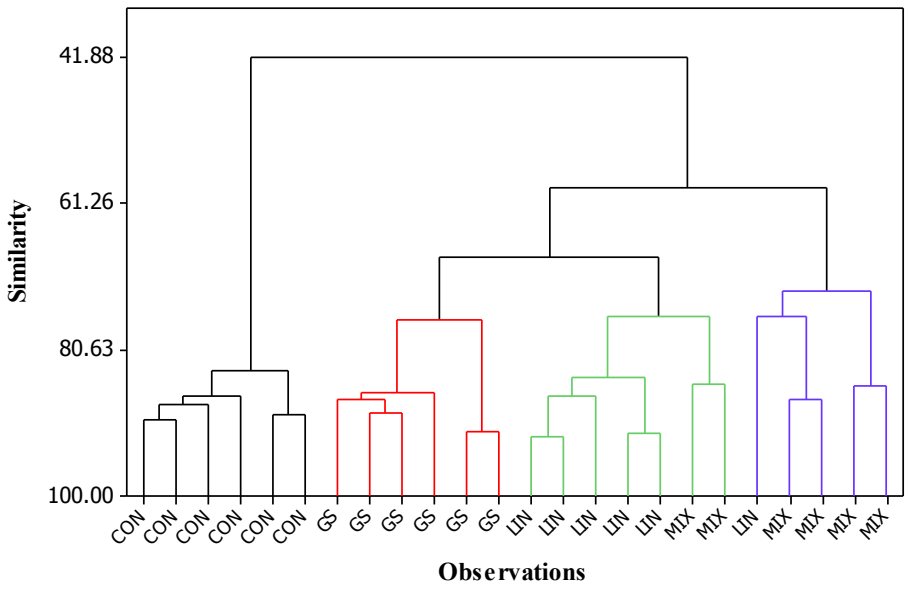

Figure 4.3. HCA results for milk of four dietary treatments. CON: control diet, GS: diet containing grape seed, LIN: diet containing linseed, MIX: diet containing both grape seed and linseed. (data from group of FA + health indices).

CHAPTER 5

Light-induced lipid oxidation in sheep milk: effects of dietary grape seed and linseed, alone or in combination, on milk oxidative stability

F. Correddu, A. Nudda, M. G. Manca, G. Pulina, and T. K. Dalsgaard

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5.1 Abstract

High levels of polyunsaturated fatty acids (PUFA) in milk are desirable for its nutritional quality, but make it vulnerable to oxidation. The present work aimed to investigate the milk oxidative stability when sheep diet includes a source of polyphenols (grape seed) and a source of PUFA (linseed), alone (GS and LIN groups, respectively) or in combination (MIX group). For this purpose the light-induced oxidation in milk was studied. The accumulation of lipid hydroperoxides after 24 hours of light exposure increased only in milk of both groups fed linseed (LIN and MIX). The higher fragility toward oxidative stress was reduced as the ratio between lipid hydroperoxides and unsaturated fatty acids in milk was lower in both groups fed grape seed (GS and MIX) than in CON and LIN groups. Furthermore, the ratio between hexanal and milk concentration of linoleic acid was lower in both groups fed grape seed (GS and MIX) than in CON group. In conclusion, dietary grape seed enhanced the oxidative stability of milk of lactating sheep.

5.2 Introduction

Over the last decades the presence of nutraceutical compounds in animal derived food has attracted growing interest. In order to improve the quality of milk and dairy products, particularly by enhancing the amount of healthy fatty acids, PUFA and CLA, several nutritional strategies have been studied, such as supplementing ruminant diet with different sources of PUFA (Nudda et al., 2014, Dewhurst et al., 2006, Toral et al., 2010). The increase of unsaturated fatty acids in dairy products makes them more susceptibility to the lipid oxidation, which can lead to degradation, reduction of the nutritional value and formation of undesirable off-flavors and potentially toxic compounds. The oxidation can occurs upon unbalanced condition between pro-oxidant and antioxidant factors; presence of enzymes (Østdal et al., 2000; Steffensen et al., 2002), metal ions (Timmons et al., 2001, Juhlin et al., 2010), oxygen (O'Brien and O'Connor, 2002) and exposure to heat (Pikul et al., 2013) and light (Wisiiner, 1964; Dalsgaard et al., 2011) could promote oxidative damage of dairy products. Photo-oxidation represents an important issue for the quality of dairy products, in particular for cheese, due to its exposition upon light in the supermarket.

The oxidation process starts with the interaction between light and naturally occurring photosensitizer compounds as flavins and chlorophylls; riboflavin (vitamin B2) represents the most important photosensitizer in dairy products (Bradley and Min, 1992). Interaction between light and riboflavin leads to lipid oxidation, but also to a loss of riboflavin.

Light exposition produces excitation of riboflavin electrons of the outer shell that reaches higher energy level, called singlet state; from this state the electron decays quickly to its excited triplet state. In this state riboflavin can react directly with a substrate, by donating or accepting a hydrogen molecule or an electron (Type I mechanism), or can transfer its energy by reacting with atmospheric oxygen, which reaches its excited singlet state (Type II mechanism) (Foote, 1968, Foote and Denny, 1968). In milk system, upon illumination, riboflavin excitation involves lipid oxidation. The Type I mechanism leads to the formation of lipid radical, while Type II leads to the formation of lipid hydroperoxides which results in the formation of volatile compounds and free radicals (Bradley et al., 2006). Due to its high reactivity and its capacity to

react directly with unsaturated fatty acids, singlet oxygen seems to be main responsible for the oxidation of lipids (Rawls and Van Santen, 1970).

In order to reduce the degradation of dairy products a number of antioxidant compounds have been evaluated. It has been reported that polyphenols can improve the oxidative stability of dairy products (O'Connell and Fox, 2001). Some agro-industrial by-products, particularly those of the Mediterranean area, are reported to be a suitable source of bioactive compound as polyphenols (Balasundram et al., 2006). Their use in animal feeding could be an alternative re-use of beneficial compounds and could reduce the expensive management and disposal.

The aim of the present study was to evaluate if grape seed (by-product) could be a suitable feed ingredient able to improve the oxidative stability of milk from sheep fed with linseed, as source of PUFA. For this purpose in this work the lipid oxidation of milk was investigated by following the accumulation of hydroperoxides, formation of volatile compounds and losses of α-tocopherol in sheep milk during light exposure.

5.3 Material and methods

Animal and diets

The details of experimental diets, the chemical analysis of feed and the sheep and feeding management were previously described in Chapter 2.

Milk samples

Individual morning milk samples, collected in the last four weeks of the trial, were stored at -20 °C until analysis. FA composition was analyzed on individual milk samples. For the analysis of riboflavin, α-tocopherol, hydroperoxides and lipid oxidation volatile compounds the weekly milk samples were combined for each ewe.

Experimental design

Milk fatty acid profile was determined in individual milk samples. To test the effect of light exposure on the milk oxidation stability, individual milk samples were transferred in 30 mL capacity flask, previously cleaned with chloroform, stored in a working cooler at 4 °C and placed under fluorescent light (400-600 nm) from a light source (TL-D 90 de Luxe Pro 18W/965 SLV, Philips, Frankfurt, Germany) with an intensity of 1500 lux and under continuous agitation by a rotator (Stuart rotator SB3, Bibby Sterilin, Stone,

UK). Control samples were stored in parallel, in the same condition and wrapped in an aluminum folium to protect the milk from the light exposure. Samples were collected at time 0, after 6 and 24 hours of light exposure.

Chemicals

Ascorbic acid (99.7%), α-tocopherol (all > 96%), citric acid monohydrate, ethylenediaminetetraacetic acid (99%), hexanal (98%), 1-hexanol (99.5%) and heptanal (> 92%) iron-(III)-chloride hexahydrate (97%), riboflavin (> 98%), were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Reagents such as HCl (37%), trichloroacetic acid (TCA) (99%), $BaCl_2$ (99%), iron-(II)-sulphate (99.5%), $NaH_2PO_4 \cdot 2H_2O$ (99.5%), $Na_2HPO_4 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$ (99%) and H_2O_2 (30%), NaOH (> 99%) were obtained from Merck (Darmstadt, Germany). NH_4SCN (99%) was from VWR BHD Prolabo (Leuven, Belgium). Sodium acetate, pentanal (97%) and 4-methyl-2-pentanone (99%) were purchased from Fluka Chemie GmbH (Buchs, Germany). All solvents of HPLC-grade (Acetonitrile, chloroform, hexane, 2-propanol and methanol) and heptane (99%) were supplied by Rathburn Chemicals (Walkerburn, Scotland).

Milk fatty acid profile

Milk fat extraction, fatty acid methyl esters (FAME) preparation and identification of individual FA were performed as previously described in Chapter 4, according to Nudda et al. (2005).

Riboflavin

Milk samples were added with 220 µl of 100% TCA to precipitate protein; after quick agitation were placed on ice for 10 min and centrifuged (10000 rpm, 10 min). About 1 mL of supernatant was transferred into a brown-colored vial through a micro-filter (cellulose, 0.45 µm) and injected into a HPLC system, equipped with a reverse phase column (Zorbax SB-C8 4.6 × 150 mm, 5 µm; Agilent Technologies, USA). Fluorescence detection was used to measure the riboflavin according with Silva et al. (2005) and an external riboflavin standard curve for the quantification (5.0, 2.0, 1.0, 0.5, 0.250, 0.1, and 0.05 µg/mL).

Tocopherols

Two milliliter of milk were added with equal volume of ethanolic ascorbic acid (1%) and 0.3 mL of saturated potassium hydroxide solution, and shaken for 10 s. The samples were incubated at 70 °C for 30 min, shaken for 10 s after 15 min from the beginning of the incubation, and then cooled on ice. Then 1 mL of Milli-Q water and 3 mL of heptane were added to the samples, mixed for 1 min and centrifuged at 1700 × g for 3 min. Thirty microliters of the upper phase were analyzed by HPLC to measure the concentration of α, β, and γ-Tocopherols. Chromatographic separation was performed using a Hypersil Silica column (4.6 × 200 mm, 5 μm; Thermo Fisher Scientific Inc., Waltham, USA) and hexane:2-propanol (98:2) at a flow rate of 1 mL/min as mobile phase. The elution was monitored by a fluorescence detector using emission and excitation wavelengths of 295 and 330 nm, respectively. An external standard curve was used for the quantitative determination.

Lipid hydroperoxides

Two milliliters of milk were transferred in 10 mL glass tube and added with 2 mL of methanol, 4 mL of chloroform and shaken for 30 s. After 10 min centrifugation at 3000 g, 1 mL of the chloroformic phase was mixed with 1 mL of iron-(II)-thiocyanate in a 10 mL glass tube. All glasses used were previously cleaned with chloroform. Absorbance was measured at 500 nm after 5 min, on a UV-Vis Spectrophotometer (Cary 60, Agilent Technologies, Palo Alto, USA), using chloroform for blank reading. Quantification was performed using an external standard curve made in the concentration 2, 5, 10, 15, 20, 40 and 60 μmol/L of hydrogen peroxide in chloroform:methanol (50:50).

Volatile compounds

The volatile compounds (hexanal, heptanal and benzaldehyde) were identified and quantified by gas-chromatography coupled with mass spectrometry head space analysis, according to Dalsgaard et al. (2010) with minor changes including the use of an isotopic internal hexanal standard. Two mL of milk were transferred to a 10 mL vial, added of deionized (18.2 MΩ) filtered water (0.22 μm) with an isotopic hexanal D12 (50 ng) from Fluka (Steinham, Germany). The samples were sealed with teflon coated lids before the headspace was analysed for volatile compounds using a Carboxen/PDMS SPME fibre with a film thickness of 30 μm from Supelco (Bellefonte PA, USA), which

was incubated at 60 °C for 30 min in the headspace of each sample. Desorption of the sample from the fibre was performed into the inlet of a GC 7890A from Agilent Technologies (Waldbronn, Germany) equipped with a HP5-MS column from Agilent J&W Scientific (Folsom, CA, USA) and coated with a non-metal 5%-phenyl 95%-dimethylpolysiloxane phase with the dimensions: 0.25 mm i.d., 0.25 µm, 30 m. Helium was used as carrier gas with a constant flow of 1.2 mL/m in. The splitless injector was kept at 250 °C. An SPME injection sleeve liner from Supleco, (Bellefonte, USA) with an inner diameter of 0.75 mm was applied. The column temperature was programmed to stay at 40 °C for 4 min followed by an increase from 40 to 120°C with a rate of 5 °C/min, a hold time of 5 min, and a subsequent temperature gradient from 120 to 300 °C with a rate of 20 °C/min. Mass spectral analysis was performed in SIM according to the ions determined by the use of a standard for each compound on a quadrupole MSD 5975 (Agilent Technologies, Germany) with a quadrupole temperature of 150 °C and a fragmentation voltage of 70 eV. The ion source temperature was 230 °C, and the interface was 280 °C. Quantification was performed relatively using and external standard curve with concentrations of 1-500 ng/mL milk of each compound including the isotopic hexanal to avoid in-between-sample variation on individual compounds.

Statistical analysis

Data of FA, riboflavin, tocopherol, hydroperoxides and volatile compounds were analyzed with the Generalized Linear Models GLM procedure of SAS (2002, SAS Institute INC., Cary, NC, USA). The model included the diet, time and their interaction as fixed effects. The mean values of FA in milk of each animal were used to calculate the ratio between lipid oxidation products after 24 h of light exposure and milk fatty acid composition; the data were analyzed with one-way ANOVA. Means were separated using Tukey test ($P < 0.05$).

5.4 Results

The milk fatty acids profile is reported in Table 5.1. The fatty acid composition of milk was highly affected by the diet. All groups receiving the experimental ingredients (grape seed and linseed alone or in combination) showed a higher level of stearic acid (C18:0, SA) and oleic acid (C18:1 *cis*-9, OA) than CON group ($P < 0.001$). The concentration of linoleic acid (C18:2 *n*-6, LA) increased ($P < 0.001$) in both groups fed grape seed (GS and MIX) compared with the CON and LIN groups. The level of α-linolenic acid (C18:3 *n*-3, LNA) increased ($P < 0.001$) in both group receiving linseed compared with CON and GS groups, with LIN group being the highest. The level of rumenic acid (CLA *cis*-9,*trans*-11, RA) was higher LIN and MIX groups than the CON group ($P < 0.001$); GS group showed a numerically higher concentration of CLA *cis*-9,*trans*-11 than LIN group, even though the differences did not reach the level statistical significance ($P < 0.10$). The total concentration of UFA increased in all groups receiving the experimental ingredients compared with the CON, with GS and MIX showing higher levels than CON and LIN, respectively ($P < 0.001$); the highest value was found in MIX, with GS and LIN being intermediate ($P < 0.001$).

The initial concentrations of riboflavin in milk from the 4 experimental groups (1.4 ± 0.4, 1.1 ± 0.4, 1.5 ± 0.3 and 1.1 ± 0.4 μg/mL for the group CON, GS, LIN and MIX, respectively) did not show any significant difference ($P > 0.05$). Figure 5.1A shows the degradation of riboflavin in milk samples exposed to fluorescent light at 4 °C. The level of riboflavin decreased ($P < 0.01$) by 8.9% after 6 hours of light exposure and by 35% after 24 hours in all groups, independently on diets, which showed no difference ($P > 0.05$) in the final concentration of riboflavin (0.83 ± 0.1, mean ± SD). Dark samples of each group (data not shown) did not show any significant reduction of riboflavin after 24 h ($P > 0.05$).

The α-tocopherol was the only tocopherol detected in the milk of the four experimental groups. In Figure 5.1B is shown the degradation of the α-tocopherol during light exposure. No difference was found ($P > 0.05$) in the initial level of α-tocopherol between the groups (0.38 ± 0.09 μg/mL, mean ± SD). The milk from all groups showed the same decrease tendency ($P > 0.05$) of vitamin E after 6 h (- 14.9%) and decreased ($P < 0.01$) after 24 h of light exposure (- 74.4%, respectively) without differences between

groups ($P > 0.05$), with α-tocopherol concentration being 0.1 ± 0.03 μg/mL (mean of all groups ± standard deviation).

The accumulation of lipid hydroperoxides in the milk from the 4 experimental groups during 24 h of fluorescent light exposure at 4 °C is shown in Figure 5.1C. The level of lipid hydroperoxides after 6 h of light exposition increased ($P < 0.05$), in all experimental groups except for the CON ($P > 0.05$). The concentrations of lipid hydroperoxides after 6 h were 1.49, 1.37, 1.81 and 1.68 mg/mL for CON, GS, LIN and MIX groups, respectively. After 24 h of light exposure the accumulation of lipid hydroperoxides increased significantly ($P < 0.05$) in both groups receiving the linseed supplementation, LIN and MIX (8.20 and 7.43 mg/mL, respectively); while no significant differences ($P > 0.05$) were found in the accumulation of hydroperoxides in the CON and GS groups (4.53 mg/mL and 4.88 mg/mL, respectively). The accumulation of lipid hydroperoxides after 24 h was numerically greater in the milk of both groups fed linseed (mean, 7.81 mg/mL) compared with that from CON and GS groups (mean, 4.71 mg/mL) even if did not reach a significant difference ($P > 0.05$). Dark samples of each group (data not shown) and the mean of all groups did not show any significant increase of lipid hydroperoxides accumulation after 24 h ($P > 0.05$).

Figure 5.2A shows the relationship between the accumulation of lipid hydroperoxides after 24 hours of illumination upon fluorescent light (1500 lx) at 4 °C and the concentration of UFA (g/100 g of total FAME) in milk samples from sheep of all groups. The level of hydroperoxides increased linearly ($P < 0.01$) with the concentration of total UFA in CON and LIN groups ($R^2 = 0.80$, $P < 0.01$). No correlation was found ($R^2 = 0.08$, $P > 0.05$) between the level of hydroperoxides and the concentration of total UFA in both groups feed grape seed (GS and MIX). Figure 5.2B shows the ratio hydroperoxides/UFA, calculated between the concentration of lipid hydroperoxides in milk after 24 hours of illumination upon fluorescent light (1500 lx) at 4 °C and the milk concentration of total UFA (g/100 g of FAME). The ratio was lower ($P < 0.05$) in GS and MIX (0.13 ± 0.05, mean ± SD) than in LIN group (0.26 ± 0.07, mean ± SD), with CON being intermediate (0.21 ± 0.06, mean ± SD).

The accumulation of volatile compounds (hexanal, heptanal and benzaldehyde) during 24 h of illumination at 4 °C is shown in Figure 5.3. The accumulation of hexanal (Figure 5.3A) was not dependent on the time of illumination for all groups ($P > 0.05$).

After 24 of light exposure the levels of hexanal numerically ($P > 0.05$) increased by 47.7, 83.0, 54.3 and 73.9% in CON, GS, LIN and MIX groups, respectively. No difference was found ($P > 0.05$) in the final level of hexanal between groups. Time of light exposure affected the accumulation of heptanal (Figure 5.3B) in the milk samples from MIX groups, which increased significantly ($P < 0.01$) in this group after 24 h of light exposure, whereas increased only numerically in the CON, GS and LIN groups after 24 h of illumination. The accumulation of heptanal did not differ ($P > 0.05$) between the experimental groups, although tended to be higher ($P < 0.10$) in the MIX group than CON and GS, after 24 h of illumination. The level of benzaldehyde after 24 h of light exposure (Figure 5.3C) increased ($P < 0.001$) in the CON group; no accumulation of this secondary product was found in the milk samples from the other groups ($P > 0.05$). The concentration of benzaldehyde after 24 h of illumination was higher ($P < 0.001$) in the CON group then the other groups. Dark samples of milk did not show ($P > 0.05$) any accumulation of secondary oxidation products after 24 h of storage (data not shown).

The ratio hexanal/LA (Figure 5.4A), calculated between the concentration of hexanal in milk after 24 hours of illumination upon fluorescent light (1500 lx) at 4 °C and the milk concentration of LA (g/100 g of FAME), was lower ($P < 0.05$) in GS and MIX (5.26 ± 1.81, mean ± SD) than in CON group (11.37 ± 1.18, mean ± SD), with LIN being intermediate (6.85 ± 1.47, mean ± SD).

Figure 5.4B shows the ratio between the milk concentration of heptanal after 24 hours of illumination and the sum of the milk concentration of OA, LA and RA. No significant difference was found between the experimental groups ($P > 0.05$); a numerically lower value can be noted for GS (0.89 ± 0.44; mean ± SD) compared with others groups (1.35 ± 0.07; mean ± SD).

5.5 Discussion

To our knowledge for the first time in this work the effect of fluorescent light on the lipid oxidation of sheep milk was investigated. Moreover the effects of grape seed, linseed alone or in combination included in the sheep diet, on oxidative stability of milk were studied. In this work the mean of the concentrations of riboflavin in ewe's milk was 1.3 µg/mL and no significant differences between the 4 groups were observed. The level of riboflavin in sheep milk founded in our experiment was similar to previous reports (1.58 and 1.77 µg/mL) (Pearson et al., 1946, Kneifel and Mayer, 1991) and was significantly reduced after the light exposure, independently of diets.

The degree of transfer of α-tocopherol from feed to the milk has previously been positively correlated with the level of linoleic and linolenic acids in milk (Goering et al., 1976, Atwal et al., 1990, Charmley and Nicholson, 1994). In our study the initial level of α-tocopherol seems to be independent from the concentration of LA and LNA; maybe the differences in the levels of these FA between groups were not enough to affect significantly the transfer α-tocopherol from feed to milk. The pattern of degradation of α-tocopherol in the present work was in agreement with other works that showed a loss of α-tocopherol in milk after fluorescent light exposure (Havemose et al., 2006, Jung, 2011). The loss of α-tocopherol did not affect the accumulation of lipid hydroperoxides within 24 h of light exposure; this finding disagrees with several works reporting capacity of α-tocopherol to quench singlet oxygen (Di Mascio et al., 1990, Yang et al., 2002). When α-tocopherol was included in cow's diet, reaching the concentration of 2.67 µg/mg in the milk, the oxidative stability of milk was improved (Focant et al., 1998). This discrepancy could be due to the different concentrations of α-tocopherol in milk; in the present work the mean of levels of α-tocopherol was seven time lower than to the previous cited work (Focant et al., 1998). Our result agrees with other studies where no relationship was found between the loss of α-tocopherol (at similar level to our finding) and the accumulation of lipid hydroperoxides, in cheese under light exposure (Dalsgaard et al., 2010; Jensen et al., 2011).

The accumulation of lipid hydroperoxides was used as a measure of the light-induced lipid oxidation in milk, and results can be related to the different diets of the four experimental groups. Although no statistical differences were found on the accumulation of lipid hydroperoxides between the 4 groups, it was evident that both

groups receiving linseed tended to show higher accumulation of lipid hydroperoxides, than the other groups. This finding could be explained as UFA concentration was significantly higher in the milk from group receiving linseed, LIN and MIX, compared with the milk from CON and GS groups. Frankel (1998) proposed the formation of lipid hydroperoxides in milk upon fluorescent light, as the consequence of the reaction between singlet oxygen, resulted from sensibilization of riboflavin, and the unsaturated lipids. The high amount of unsaturated fatty acids in milk samples made them more susceptible to the lipid oxidation (Durand et al., 2005). As unsaturated fatty acids are logically more susceptible to oxidation (Roman et al., 2013), a linear correlation between the concentration of these FA and the level of lipid hydroperoxides could be hypotized, according to the findings of Havemose et al. (Havemose et al., 2006) who showed that higher concentrations of LNA were correlated with higher accumulation of lipid hydroperoxides in milk, after 24 hours of fluorescent light exposure. In the present work, considering the level of total UFA found in milk, a good correlation was found between the accumulation of lipid hydroperoxides after 24 h of light exposure and the UFA (g/100g total FA) in the milk samples from CON and LIN groups, whereas no correlation was found in milk from sheep fed grape seeds (GS and MIX). This finding could be explained by the presence of polyphenols in grape seed, which are reported to be able to quenching singlet oxygen (Mukai et al., 2005). Our result agrees with the finding of Jung (2011) showing protective activity of catechins on the accumulation of lipid hydroperoxides in milk after fluorescent light exposure. Moreover, the lowest ratio between lipid hydroperoxides after 24 h and total UFA in milk samples from sheep receiving grape seed, suggests an important effect of grape seed in reducing the accumulation of hydroperoxides.

The higher, even though not significant, percentage of accumulation of hexanal in the two groups fed grape seed, than CON and LIN groups was not surprising, because of the higher concentration of LA in the milk from GS and MIX than the others groups. In fact, hexanal has been reported as typical volatile product derived from linoleic acid oxidation (Chavez-Servin et al., 2008, García-Martínez et al., 2009). It was also demonstrated that milk with high amount of linoleic acid was more susceptible to the lipid oxidation compared with milk rich in RA, OA and LNA, respectively (Timm-Heinrich et al., 2004). The same authors demonstrated that milk containing high amount

of linoleic acid showed the highest production of secondary volatile compounds, compared with other models of milk. In the present work, the lower ratio hexanal/LA calculated for the milk from GS and MIX compared with CON group supports the hypothesis that polyphenols from grape seed should be able to reduce the formation of secondary oxidation product hexanal. This finding agrees with several works reporting antioxidant capacity of grape seed polyphenols (Sanchez-Moreno et al., 1999, Berradre et al., 2013, Chouchouli et al., 2013) and is corroborated by other findings showing capacity of catechins to reduce the accumulation of lipid volatile aldehydes in cheese and milk after light illumination (Huvaere et al., 2011, Jung, 2011).

The higher rate of accumulation of heptanal in MIX than the other groups could be explained with the high levels of oleic, linoleic and rumenic acids founded in the milk of this group. In fact the formation of heptanal has been associated to the oxidation of these fatty acids (Zanardi et al., 1998, García-Martínez et al., 2009, Van Ba et al., 2013). According to this fact, in our work, the ratio between the accumulation of heptanal after 24 h of illumination and the sum of OA, LA and RA showed no difference between the experimental groups. The numerically lowest value of the ratio found in GS could be explained with the presence of grape seed polyphenols which could have reduced the formation of heptanal, according with previous works reporting ability of catechins to reduce the accumulation of lipid volatile aldehydes in cheese and milk after light illumination (Huvaere et al., 2011, Jung, 2011). However, this finding was not observed for MIX group, as expected.

Previous work evidenced a correlation between light exposure and benzaldehyde formation in cheese (Juric et al., 2003, Andersen et al., 2008). It has been suggested that this compound may result from the oxidation of benzene derivates (Ritter and Budge, 2012), which could be decomposition products of amino acids (Yvon and Rijnen, 2001, Giogios et al., 2009). Benzaldehyde also has been considered as volatile compound derived from lipid oxidation, in particular from the thermal degradation of 2,4-decadienal (Bruechert et al., 1988). However, this reaction occurs at high temperature (> 120 °C). Elmore (2005) found benzaldehyde formation in grilled meat from lamb fed lipid sources of LNA. Our experiment was conducted at 4 °C, and we can reasonably exclude that this volatile derives from lipid oxidation. The higher accumulation of benzaldehyde in the milk samples from CON could be related with the lower content of

UFA in this group compared with the others and thus indicating it to be formed as consequence of protein oxidation. We hypothesize that in the higher oxidability of milk with highest UFA concentration could reduce, to certain extent, the oxidation of protein and, consequently, the formation of benzaldehyde.

5.6 Conclusions

In conclusion this study illustrated the differences in the milk oxidative stability when sheep diet includes a source of polyphenols (grape seed) and a source of PUFA (linseed), alone or in combination. The inclusion of linseed in the diet of sheep increases the amount of PUFA in milk, advantageous for health-beneficial effects. However, high levels of polyunsaturated fatty acids (PUFA) in milk make it vulnerable to oxidation. The relationship and the ratio between lipid oxidation products after 24 h of light exposure and milk fatty acid composition helped to give a better interpretation of data. The inclusion of grape seed in the diet of lactating sheep can reduce the lipid oxidation in milk, leading a less formation and accumulation of lipid hydroperoxides and secondary lipid oxidation products.

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5.8 Tables

Table 5.1. Fatty acids composition of milk from different groups.

Fatty acid (g/100 g of FAME)	Group[1]				S.E.M.[2]	P *values*
	CON	GS	LIN	MIX		G[3]
C18:0	5.47[b]	8.73[a]	9.89[a]	10.03[a]	0.46	**
C18:1*cis*-9	13.39[b]	17.90[a]	19.66[a]	19.34[a]	0.64	**
C18:2 *n*-6 (LA)[4]	2.68[b]	4.65[a]	2.97[b]	4.86[a]	0.23	**
C18:3 *n*-3 (LNA)[4]	0.74[c]	0.58[c]	1.89[a]	1.43[b]	0.11	**
CLA *cis*-9,*trans*-11 (RA)[4]	0.70[c]	1.75[bc]	2.18[ab]	3.01[a]	0.01	**
UFA	25.11[c]	36.63[b]	41.14[b]	47.23[a]	1.81	**

[1]CON, control group; GS, group receiving grape seed; LIN, group receiving linseed; MIX, group receiving grape seed and linseed.
[2]S.E.M., standard error of mean.
[3]G, group.
[4]LA, linoleic acid; LNA, α-linolenic acid; RA, rumenic acid.
[a-d]Means with different letter within a row are significantly different, $P < 0.05$; ** $P < 0.0001$.

5.9 Figures

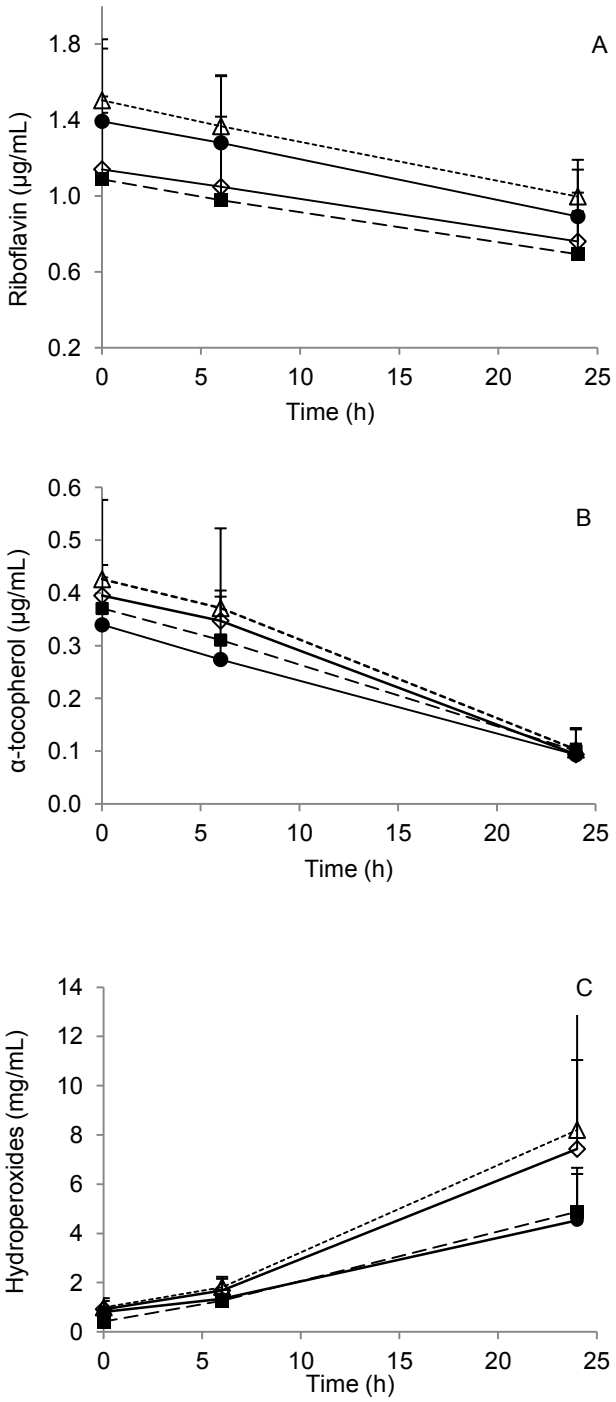

Figure 5.1. Decrease of riboflavin (A), degradation of α-tocopherol (B) and accumulation of lipid hydroperoxides (C) during illumination with fluorescent light (1500 lx) at 4 °C. Milk from control group (solid line, ●), group receiving grape seed (long-dish line, ■), group receiving linseed (dotted line, △) and group receiving both grape and linseed (solid line, ◊). Bars indicate standard deviations of the means of 6 animals in each treatment.

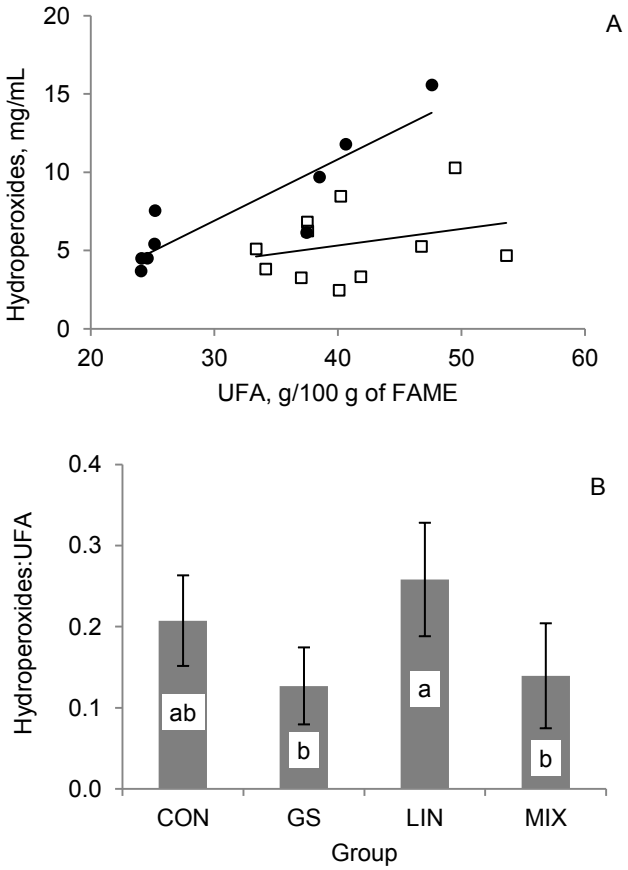

Figure 5.2. (A) Relationship between the accumulation of lipid hydroperoxides after 24 hours of illumination with fluorescent light (1500 lx) at 4 °C and total UFA in milk samples from sheep fed grape seed (□, GS and MIX) and others groups (●, CON and LIN). ($y = 0.1062x + 1.0588$; $r = 0.08$; $P > 0.05$; and $y = 0.3919x - 4.8692$; $r = 0.80$; $P < 0.01$, respectively). **(B)** Ratio between accumulation of lipid hydroperoxides after 24 hours of illumination with fluorescent light (1500 lx) at 4 °C and total UFA in milk samples from sheep of different experimental groups: CON: control group, GS: group receiving grape seed, LIN: group receiving linseed, MIX: group receiving both grape and linseed. Different letters (a, b) indicate significant differences ($P < 0.05$). Bars indicate standard deviations of 6 animals in each treatment.

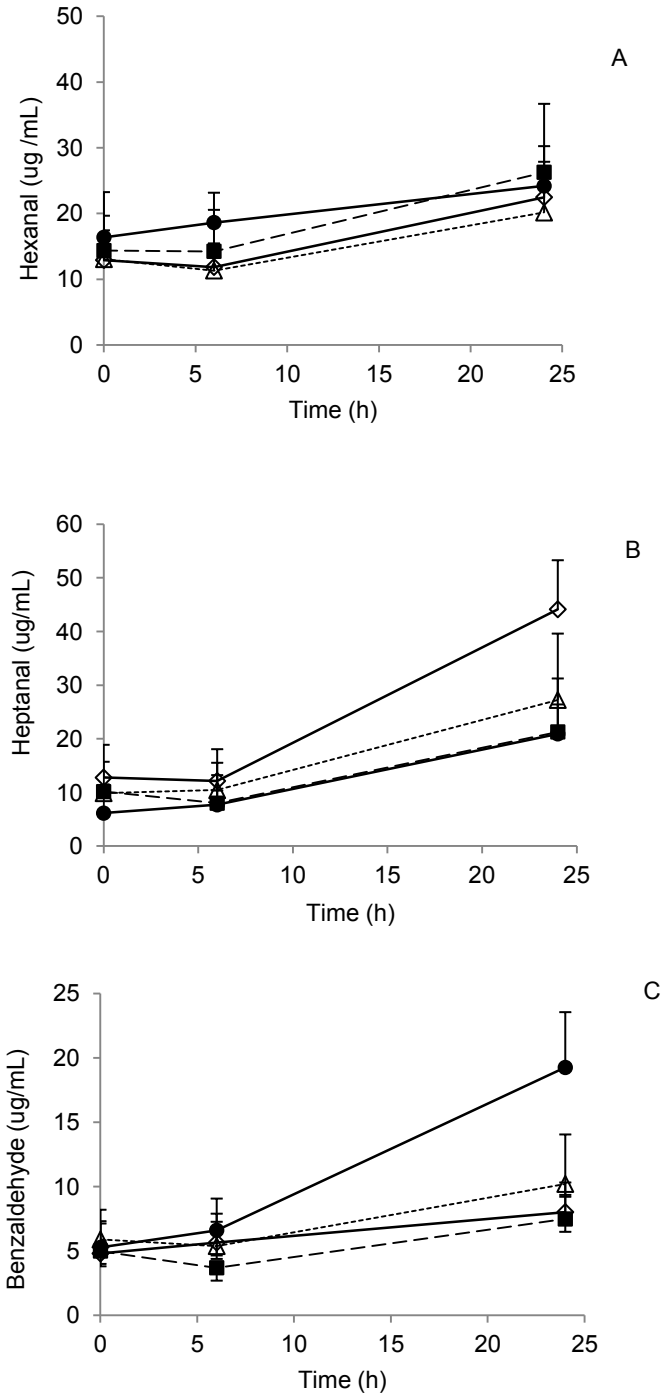

Figure 5.3. Accumulation of hexanal **(A)**, heptanal **(B)** and benzaldehyde **(C)** during illumination with fluorescent light (1500 lx) at 4 °C. Milk from control group (solid line, ●), group receiving grape seed (long-dish line, ■), group receiving linseed (dotted line, △) and group receiving both grape and linseed (solid line, ♢). Bars indicate standard deviations of the means of 6 animals in each treatment.

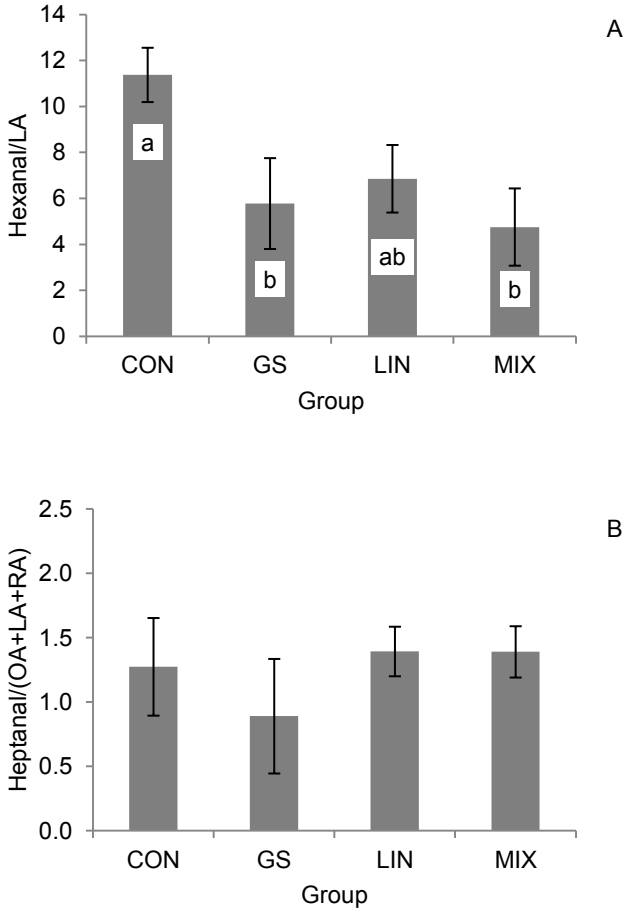

Figure 5.4. (A) Ratio between accumulation of hexanal after 24 hours of illumination with fluorescent light (1500 lx) at 4 °C and linoleic acid (LA) in milk sample from sheep of different experimental groups. **(B)** ratio between accumulation of heptanal after 24 hours of illumination with fluorescent light (1500 lx) at 4 °C and the sum oleic acid (OA), linoleic acid (LA) and rumenic acid (RA) in milk sample from sheep of different experimental groups. CON: control group, GS: group receiving grape seed, LIN: group receiving linseed, MIX: group receiving both grape and linseed. Different letters (a, b) indicate significant differences ($P < 0.05$). Bars indicate standard deviations of the means of 6 animals in each treatment.

FINAL CONCLUSIONS AND CONSIDERATIONS

In conclusion, this study evidenced that grape seed by-product can be included in diet of lactating ewes to improve the milk fat quality, as evidenced by the decrease of SFA and increase of PUFA and FA healthy indices, without adverse effects on milk production traits.

The use of grape seed, associated with linseed as source of PUFA *n*-3, leads to an improvement of the nutritional properties of milk fat, evidenced by the increase in CLA and VA concentration, and the FA healthy indices.

Finally, the inclusion of grape seed improve the oxidative stability of milk, especially when sheep diets are supplemented with source of PUFA in order to enhance nutritional properties of milk fat.

Further researches are needed to study the effects of grape seed by-product with higher content of polyphenols, on milk production traits and animal metabolism. Moreover, the effects of grape seed combined with other dietary source of vegetable oils in the diet of lactating ewes should be investigated.