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PhD Thesis

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“ Effect of saponins on lamb meat quality ”

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To my family

and Andrea

for always believing in me

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Preface

At the beginning of my PhD, I took part in an experiment about the effect of tannins on volatile compounds of lamb meat, so that I was co-author in this work published by *Food Chemistry*.

During the 2° year, I began studying the methods to determinate fatty acid and cholesterol content in animal products. I went to Pisa and in collaboration with the Faculty of Agriculture of Pisa, with Dr Marcello Mele and his research group, I learnt some of the methods used to analyse fatty acids and cholesterol content in animal products. Therefore, I elaborated a review about the advantages and disadvantages of these commonly used analytical methods. Moreover, my knowledge on fatty acid analysis gave me the opportunity to collaborate on a writing of a paper published by *Italian Journal of Animal Science.*

My research activity and individual study was also concentrated on the effect of another group of secondary plant metabolites on meat quality: the saponins. In collaboration with the National Institute of Agricultural Research of Tunisia (INRA), with Dr Ben Salem, I had the great opportunity to work on an experiment to evaluate the effects of saponins on lamb meat quality. The results were published by *Animal*.

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- Lanza M., Fabro C., Scerra M., Bella M., Pagano R., Brogna D.M.R., Pennisi P. 2011. Lamb meat quality and intramuscular fatty acid composition as affected by concentrates including different legume seeds. *Italian Journal of Animal Science 10, 87-94.*
- Brogna D.M.R., Nasri S., Ben Salem H., Mele M., Serra A., Bella M., Priolo A., Makkar H.P.S., Vasta V. 2011. Effect of dietray saponins from Quillaja saponaria L. on fatty aciod composition and cholesterol content in muscle Longissimus dorsi of lambs. *Animal 5, 1124-1130.*

At the beginning of my 3°year of PhD, I met Dr Juan Villalba and talking about his work at the Utah State University, in Logan (USA), I asked to him the possibility to conduct collaborative research on the project: "Primary Roles for Secondary Compounds: Enhancing the Health of Soil, Plants, Herbivores and People through Plant Biochemical Diversity". I went to Logan for three months to take part on a feeding trial about the effects of tannins and saponins on animal self-medication against nematode infection and lamb meat quality. At the end of the experiment, I analysed fatty acid composition, cholesterol content, volatile profile and oxidative stability on lamb meat. I conducted all of these analysis at the Department of Nutrition, Dietetics and Food Sciences, Utah State University, Logan, working with Dr Robert Ward, Dr Daren Cornforth and Rossarin Lynn Tansawat, a PhD student.

In this thesis I will review, in the first section, the biological properties of saponins in animal and human nutrition. In the second section, I will show the results obtained from the two experiments, made in Tunisia and in America, in

which dietary saponins were administered to lambs to evaluate their effects on meat quality.

SECTION I

Biological properties of saponins in animal and human nutrition

Introduction

The term "meat quality" includes many different attributes such as safety, nutrition value, flavour, texture, colour, lipid content and composition, oxidative stability. Moreover, environmental, ethical and animal welfare are other high standards of quality required in the production of meat. As a result, the meat quality is influenced not only by a singular factor but by a complex of interacting elements (Andersen et al., 2005).

Feeding strategy is considered an important tool which is used to improve meat quality. The use of antibiotic growth promoter (AGP) has been widely debated. In the United States, recommendations to reduce or eliminate the use of antimicrobials in feed were made in two reports by the Institute of Medicine (1980, 1989), a Council for Agricultural Science and Technology report (1981), and a Committee on Drug Use in Food Animals report (1998). The reports did not present data proving that the use of AGP in food animals caused antibiotic-resistant infections in humans. Nevertheless, the World Health Organization (WHO) published a report showing a link between the two causes (1997) and recommending national governments to reduce the use of antimicrobial in animals.

Thus, there has been increasing interest in using natural products as feed additives to solve problems in animal nutrition and livestock production (Wallace, 2004). These natural products include probiotics, prebiotics, enzymes, organic acids and plant secondary compounds (PSCs). The uptake and incorporation of these natural components in animal nutrition represent a tool to reach high quality standards.

Plant secondary compounds are organic compounds that are not directly involved with the essential or "primary" roles of photosynthesis, respiration, growth, development and reproduction of plants. They often play an important role in plant defence against microbial and insect attack (Swain, 1977). Many PSCs such as terpenes, alkaloids, essential oil, flavonoids, tannins and saponins from a wide range of plants have been identified and used in animal productivity and health as alternatives to chemical feed additives (Rochfort et al., 2008; Patra & Saxena, 2009; Provenza et al., 2010).

Saponins are a kind of PSCs mainly produced by plants and also by lower marine animals and some bacteria (Yoshiki et al., 1998). Their name derives from their ability to form stable foams in aqueous solutions. This detergent action was recognized several years ago when extracts of plants such as *Saponaria officinalis* were used to make soap (Osbourn, 1996).

Saponins are characterized by a sugar moiety, usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (named sapogenin) which may be triterpenoid (figure 1a) or steroid (figura 1b) in nature (Francis et al., 2002). Triterpenoid saponins have been detected in a lot of legumes such as soybeans, beans, peas, lucerne, etc. and also in alliums, tea, spinach, sugar beet, quinoa, liquorace, sunflower, horse chestnut and ginseng. Steroid ones are found in oats, capsicum peppers, aubergine, tomato seed, alliums, asparagus, yam, fenugreek, yucca and ginseng (Francis et al., 2002). There are some factors, such as physiological age, environmental and agronomic factors, which may affect the saponin content of plants (Yoshiki et al., 1998).

Several authors have studied the different biological properties of saponins in animal and human nutrition (Francis et al., 2002; Sparg et al., 2004; Wina et al., 2005).

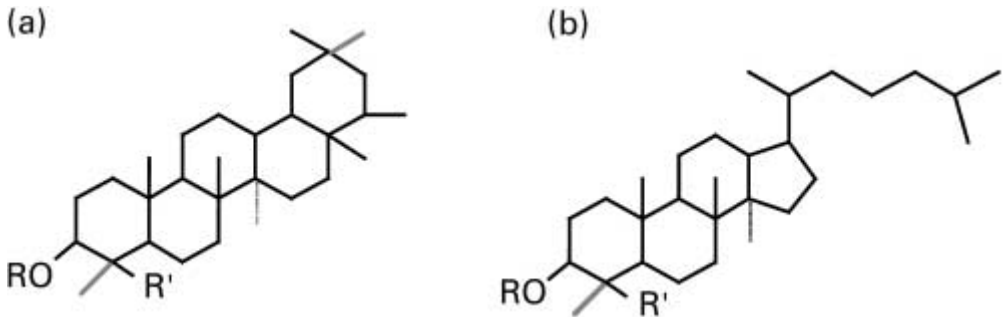

Figure 1: Basic structure of aglycone: triterpenoid (a) or steroid (b)

1. *Role of saponins in plants*

There is little information about the roles of saponins in vegetables. Some publications refer that saponins may be considered a part of plant defence system because they are known to inhibit mould, to be antimicrobial and to protect plants from insect attacks (Francis et al., 2002). They are usually found in tissue that are most vulnerable to fungal, bacterial and insect attack. *Avenacin*, for example, is a saponin found in large quantity in root tips and it is capable to act against the zoosporic fungus *Gaeumannomyces graminis* (Osbourn, 1996). The antifungal effect of saponins is due to their interaction with membrane sterols with consequent formation of pores and loss of membrane integrity (Price et al., 1987).

Saponins are generally included into two groups of protective plants named *phytoanticipins* and *phytoprotectans* (Morrissey & Osbourn, 1999). The first group include saponins that are activated by the plant enzymes in response to tissue damage or pathogen attacks. *Phytoprotectans* are saponins which have general anti-microbial and anti-insect activity (Gus-Mayer et al., 1994).

Many authors refer this antibacterial property of saponins. Applebaum et al. (1969) found that saponins were capable to inhibit development of the Azuni bean beetle, *Callosobruchus chinensis* L., a major pest of stored legume seeds. Moreover, Ohana et al. (1998) isolated, purified and characterized from *Pisum sativum* a glycosylated triterpenoid saponin as an inhibitor of diguanylate cyclase, a key regulatory enzyme in the synthesis of the cellulose synthesizing apparatus in the bacterium *Acetobacter xylinum*.

2. Biological effects of saponins

2.1 Effect of saponins on membrane properties

Saponins are known to have a lytic action on erythrocyte membranes. This has lead to the development of the hemolytic assays for detecting the presence of saponins in drugs or plant extracts. Many model have been proposed to explain the way in which saponins become incorporated into membrane (Price et al., 1987). The aglycone and the sugar moiety of the saponin molecule are both believed to have membraneolytic properties (Price et al., 1987).

The saponin aglycone should have an affinity for membrane sterols, particularly cholesterol (Glauert et al., 1962), with which saponins form insoluble complexes (Bangham & Horne, 1962). Nevertheless, it has been reported that the interaction between saponins and membranes is more complex. Saponins could affect membrane permeability even in absence of cholesterol (Hu et al., 1996).

It has been suggested that the hemolytic activity of saponins could increases with increasing number of polar groups in the aglycone moiety (Namba et al., 1973).

Carbohydrate chain length are also supposed to contribute to the effects on membranes: steroid and triterpenoid saponins with a single sugar chain (monodesmosides) have been found with more strong activity than those with two sugar chains (bidesmosides) (Fukuda et al., 1985). An increase in the number of sugar moieties can also enhance the effects of saponins on sarcolemmal membrane Ca^{2+} permeability (Yamasaki et al., 1987).

The specific interaction between saponin and membrane is very complex and it needs more elucidation. It seems likely that different mechanisms such as

the formation of saponin-cholesterol complex, alteration in the organization of sarcolemmal membrane phospholipids or saponin structure, are all involved in the action of saponins on membranes (Yamasaki et al., 1987).

2.2 Effect of saponins on nutrient metabolism

Saponins occur constitutively in both wild plants and cultivated crops and they are largely used in animal and human nutrition (Cheeke, 2000).

In ruminants and other domestic animals dietary saponins can have significant influence on all phases of metabolism from the ingestion of feed to the excretion of wastes (Cheeke, 1996).

It has been shown that saponins may be degraded by the saliva of sheep fed saponin-rich foods for a long time or there would also be an adaptation of the mixed microbial population of the rumen to saponins or saponin-containing plants (Odenyo et al., 1997; Newbold et al., 1997; Teferedegne, 2000). Rumen microbes are likely able to break the glycosidic bonds to release sugars and the aglycon, which might be considered as degradation of saponins. The aglycon molecule, being complex, will be difficult to break by rumen microbes. There is no evidence of complete breakdown of the aglycon part by the rumen microbes. These aglycons could have different effects and probably will not reflect the activity of the parent saponins (Gutierrez et al., 1959).

Recently, a number of studies have reported both beneficial and adverse effects of these compounds in a variety of animals (Sen et al., 1998; Francis et al., 2002).

Lu and Jorgensen (1987) showed in their study an increase in the digestion coefficient of organic matter, hemicellulose, and cellulose in the total digestive in

sheep fed concentrate diets containing saponins. Moreover, several saponin-rich plants, such as *Yucca schidigera*, have been found to improve growth, feed efficiency and health (Cheeke, 1996). *Quillaja saponaria* increased the efficiency of an *in vitro* rumen- microbial protein synthesis and to decrease degradability of feed protein (Makkar & Becker, 1996). The supplementation of feed with leaves of *Sesbania sesban*, known for its high saponin content, was able to improve protein flow from the rumen by suppressing protozoal action (Newbold et al., 1997).

The major effect of saponins on lipid digestibility seems to be exerted via effects on formation of micelles with bile acids (Sidhu & Oakenfull, 1986) and their capacity to solubilise monoglycerides improving fat emulsification and digestion (Barla et al., 1979). Saponins isolated from *K. scoparia* fruit and tea extracts have been suggested to inhibit the pancreatic lipase activity (*in vitro*) (Han et al., 2001; Han et al., 2006). However, direct effects of these plant secondary metabolites on plant lipases or lipases of rumen microbes have not yet been well studied.

Dietary saponins are also known to have negative effects on animal nutrition. Several studies showed that these negative effects refer to reduction of feed intake caused by the astringent and irritating taste of saponins (Sen et al., 1998), delay of intestinal motility (Klita et al., 1996) and protein digestibility (Shimoyamada et al., 1998), damage to the intestinal membrane and inhibition of nutrient transport (Francis et al., 2002).

It has been also reported that saponins can also form complexes with minerals (zinc, iron) rendering them unavailable for absorption (Milgate & Roberts, 1995).

Effects of saponins on vitamin metabolism it has been showed. Lowered plasma and liver concentrations of vitamins A and E have been noted in chicks fed fairly high levels (0.9%) of Quillaja saponin (Jenkins & Atwal, 1994). Fat-soluble vitamins form mixed micelles, necessary for their absorption; by binding bile acids; on the other hand, saponins impair micelle formation in the intestine and therefore vitamin absorption.

Dietary saponins are often suspected of having a role in causing bloat in ruminants (Cheeke, 1996; Sen et al., 1998). Production of slime from alfalfa saponins by rumen bacteria and alteration of the surface tension by rumen content were suggested as factors contributing to bloat formation (Sen et al., 1998).

2.3 Hypoglycaemic effect of saponins

Some authors (Lee et al., 2000; Yoshikawa et al., 2001) have demonstrated hypoglycaemic effect of saponins. It is probably due to suppression of the transfer of glucose from the stomach to the small intestine and the inhibition of glucose transport across the brush border of the small intestine (Matsuda et al., 1999). However, the mechanism is complex and not completely understood and other studies are necessary to highlight this aspect of saponins.

2.3 Anti-cholesterolaemic effect of saponins

A number of studies have shown that saponins when supplemented in diets reduce cholesterol content in blood and tissues in monogastric mammals, such as

rats, gerbils and humans (Sidhu & Oakenfull, 1986; Potter et al., 1993; Harris et al., 1997).

This cholesterol-reducing effect of saponins is attributed to their ability to form insoluble complexes (micelles) with sterols (Sidhu & Oakenfull, 1986), such as cholesterol and bile acids.

There are two school of thoughts emerging for the hypocholesterolaemic effect of saponins: (i) a direct binding between saponins and dietary cholesterol in the gut, which prevents its absorption from the small intestine and (ii) a binding between saponins and bile acids in the gut, which decreases the enterohepatic circulation of bile acids and increases cholesterol excretion with faeces (Sidhu & Oakenfull, 1986). These hypotheses have been formulated through studies with monogastric animals, there is few information about the effects of saponins on cholesterol metabolism in ruminants

Cholesterol-lowering properties of saponins in human is obviously interesting. However, there is little clinical trial information. Chapman et al. (1997) observed that the Masai people of East Africa have low serum cholesterol levels in spite of a diet rich in animal fats. The adding of saponin-rich herbs in milk and meat-base soups is the reason of this low cholesterol level.

The effect of *Yucca schidigera* and *Quillajia saponaria* extracts on cholesterol level in the human's blood and gastrointestinal functions was studied by Kim et al. (2003). Eighty-six patients, having more than 220 mg/dL triglyceride level in blood, received either the YQ2 (*Quillaja s.* and *Yucca s.* extracts in 6:4 v:v proportion) or placebo in a random selection. Taking 0.9 mg of

the YQ2 a day for 4 weeks resulted in the decreases in total and LDL cholesterol levels in blood plasma of hypercholesterolaemic patients.

Not all reports, however, agree on the anticholesterolaemic activity of saponins. Since cholesterol binding takes place in the intestinal lumen, factors such as quantity of saponins and cholesterol, and the presence of other ligands of both these compounds may play a role and these may have caused the observed discrepancies among the various results. Knowledge of the nature of the interaction between the particular saponin and cholesterol, and the nature of the cholesterol moieties and other ligands in the diet are essential to arrive at an effective dietary dose of that particular saponin that could have a significant hypocholesterolaemic effect (Francis et al., 2002).

2.4 Antioxidant effect

Some saponins have been found to have antioxidative or reductive effects. Yoshiki and Okubo (1995) reported that some legumes such as kidney beans, peanuts, chickpeas, clover and Japanese bush clover contain saponins with an antioxidant moiety attached to C_{23}. This sugar residue, called 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), allows saponins to scavenge superoxides by forming hydroperoxide intermediates, thus preventing bio-molecular damage by free radicals.

Ginseng saponins have been found to be specific inducers of the superoxide dismutase (SOD), a gene that codes for one of the major antioxidant enzymes (Kim et al., 1997).

Sur and coauthors (2001) also indicated significant antinflammatory and antioxidant properties of TS-1 and TS-2, two saponins isolated from tea root extract.

2.5 Anti-cancer effect of saponins

Anti-cancer effect of saponins take place through diverse and complex mechanisms. Saponins would be able to bind primary bile acids thus reducing their availability to form secondary bile acids (Rao & Sung, 1995). Secondary bile acids are indeed known to be cytotoxic and tumor-promoting. In addition to the bile acids, saponins also bind to cholesterol and prevent cholesterol oxidation in the colon. Oxidized cholesterol products are known to be promoters of colon cancer (Koratkar & Rao, 1997). Thus, dietary saponins may have beneficial effects against two major human health problems: coronary heart disease (by hypocholesterolaemic activity) and colon cancer (by sequestering bile acids).

2.6 Effects on immune system

Since saponins are part of the active immune system of plants research appears promising that dietary saponins can have the same effect when ingested and transferred to the human body. Thus, saponins might be suitable immunostimulators and they may be used as adjuvants in the preparation of vaccines against several types of fungal, bacterial and protozoal infections (Cheeke, 1996).

The saponin adjuvants widely used is a *Quillaja saponaria* fraction called Quill A which has been used to prepare an immunostimulating complex (ISCOM). This complex acts through the bind between the Quill A and

cholesterol in membranes macrophages or antigen-presenting cells of immune system (Dalgaard, 1978).

3. Effect of saponin on ruminal ecosystem

Several plants rich in saponins, such as, *Yucca schidigera* (Holtshausen et al., 2009), *Quillaja saponaria* (Holtshausen et al., 2009), *Medicago sativa* (Klita et al., 1996) and *Sapindus saponaria* (Abreu et al., 2004,) are used in ruminant nutrition mainly to inhibit methanogenesis in the rumen.

The rumen is a dynamic, continuous fermentation compartment in the alimentary canal of ruminants. It represents the primary site for microbial fermentation of ingested feed. The temperature is about 38-41°C and it is usually well-buffered by the presence of organic acids, produced during microbial fermentation, the buffering capacity of various feed and the flow of saliva, particularly rich in bicarbonates and phosphates. The rumen provides a suitable environment for the growth of numerous both strictly and facultative anaerobic microbes (Hoover & Miller, 1991) This microbial ecosystem consisting of bacteria, ciliate protozoa, anaerobic fungi and bacteriophages. The synergism and antagonism among the different groups of microbes and even among different genera of the same group is responsible for the bioconversion of feed into such form that is utilizable by the animal as a source of energy (short chain volatile fatty acids). Ruminal microbes can be influence by either natural or feed associated factor, such as saponins (Kamra, 2005).

3.1 Effect of saponins on rumen microbes

Protozoa. It has been shown both *in vivo* studies (Lu & Jorgensen, 1987; Wallace et al., 1994; Klita et al., 1996) and *in vitro* ruminal fermentation systems (Makkar et al., 1998; Wang et al., 1998) that saponins have pronounced antiprotozoal activity. One of the potential consequences of antiprotozoal effect is the reduction of the predation by protozoa on rumen bacteria, thus maximizing microbial protein synthesis and flow to the duodenum by reducing the recycling of microbial nitrogen in the rumen (Jouany, 1996). The susceptibility of rumen protozoa to the saponins and lack of susceptibility of rumen bacteria to saponins is probably explained by the presence of cholesterol in eukaryotic membranes (including protozoa) but not in prokaryotic bacterial cells (Klita et al., 1996).

Nevertheless, the antiprotozoal activity of saponins is only transient. This variability may be due to an adaptation of the microbial population to saponin or degradation of saponins in their two components (sugar and sapogenin) (Makkar & Becker, 1997; Wang et al., 1998; Teferedegne, 2000). Sapogenin is more resistant to degradation thus it has not the anti-protozoal property of the parent saponin (Gutierrez et al., 1959).

Bacteria. Some authors (Wallace et al., 1994; Wang et al., 2000) have observed that saponins negatively affected Gram-positive bacteria more than Gram-negative bacteria. Gram-negative bacteria possess two membranes in their cell walls that may protect them from substances like saponins (Wina et al., 2005).

Moreover, saponins may decrease cellulolytic bacteria rather than amylolytic bacteria (Wang et al., 1998; Wang et al., 2000). Nevertheless, in an *in*

vivo study, Diaz et al. (1993) observed a significant increase in cellulolytic and total bacteria in the rumens of sheep fed with *Sapindus saponaria* fruit.

Studies on the effect of saponins on methanogenic bacteria are also important because of methane emission, one of the greenhouse gases, by ruminants. As some methanogens (10-20% of total) live in association with protozoa (Newbold et al., 1995; Takura et al., 1997), it is expected that reducing protozoa would also reduce methanogens, thus decreasing methane production. Moreover, the availability of hydrogen to form methane may compete with the requirement of hydrogen to form propionate. So, if the concentration of propionate in the rumen increases in the presence of saponin, the methane emission would decrease (Wina et al., 2005). This saponin effect needs to be more elucidated.

In the study of Goel et al. (2008) no methane reduction was observed. One explanation could be an altered composition of methanogenic community and their increased efficiency of methane production. The two major groups of methanogens in rumen are methanobacteriaceae (89.3% of total archea in rumen fluid) and methanobacteriales (12.1%) (Sharp et al., 1998). The members of methanobaceriaceae contribute nearly 99.1% of total archea associated with protozoa, while free-living methanobacteriales account for only 0.05% in protozoal fraction (Sharp et al., 1998). Thus in this study, the species belonging to methanobacteriaceae declined with an increase in free-living members of methanobacteriales, while the similar methane levels could be explained on the basis of interspecies hydrogen transfer between increased population of both hydrogen-producing bacteria (*Ruminococcus flavefaciens* and *Fibrobacter*

succinogenes) and free-living methanobacteriales. Moreover, the no effect of saponins on methane reduction may be due to an adaptation of the microbial population to saponin or degradation of saponins (Makkar & Becker, 1997; Wang et al., 1998; Teferedegne, 2000).

Fungi. Anaerobic fungi are important in the rumen for digesting fiber, but they only comprise a small proportion of the total mass of the rumen microflora. There is little information on the effect of saponins on rumen fungi. Wang et al. (2000) demonstrated that the fungi, *Neocallimastix frontalis* and *Pyromyces rhizinflata*, are very sensitive to saponin from *Yucca schidigera*, and even at a low concentration of the saponin (2.25 μg/mL), the growth of both fungi was completely inhibited. The mechanism for the antifungal activity of saponins may be their interaction with membrane sterols.

3.2 Effect of saponins on fatty acids

The results on the effect of saponins on fatty acids are conflicting. Some authors report no difference in the short chain fatty acids (SCFA) production among control and saponin-containing diet (Makkar & Becker, 1996; Wang et al. 1998; Hess et al., 2003). On the other hand, Lila et al. (2003) reported increase in the total SCFA and other authors (Wina et al., 2005; Goel et al., 2008) found an increase in the propionate concentration. The increase of propionic acid is considered being caused by the competition between propionate and methane for the available hydrogen, and lower acetate and butyrate which are both major end products of protozoa. Therefore, defaunation shifts VFA patterns towards a higher propionate production (Jouany, 1996; Wina et al., 2005).

In a study conducted by Wallace et al. (1994), the administration of *Yucca schidigera* to heifers had not affect on the growth of *Selenomonas ruminantioum*, the most important rumen bacterium involved in the propionate production, whereas the growth of some other rumen bacterial species was inhibited. Thus, it was supposed that by inhibiting bacteria and protozoa not involved in propionate production in the rumen, species such as *Selenomonas ruminantioum* were promoted, thereby increasing the accumulation of propionate in the rumen.

Nevertheless, Lourenco et al. (2008) found that saponins from *Quillaja saponaria* did not affect rumen total microbial biomass or activity, as suggested from the similar total OBCFA proportions and total amounts of rumen VFA. Additionally, neither the FA metabolism nor the fermentation pattern were affected by triterpene saponins. The fast adaptation of the microbial population to saponins by its conversion to sapogenins (Teferedegne et al., 1999) could mask possible effects of the plant secondary metabolite on the rumen processes. A similar result was also found by Khiaosa-Ard et al. (2009). The inclusion of *Yucca schidigera* extract in *in vitro* system remained without major effect on any of the ruminal fermentation traits.

3.3 Effect of saponins on ruminal pH

The results about the effect of saponins on rumen pH are contrasting. Some authors (Nasri et al., 2011) did not found any effect on rumen pH, because animals, fed saponin- or control diet (without saponins), showed similar growth performances and carcass weight at the end of the experiment. It is known that meat ultimate pH is strongly affected by glycogen and carcass fitness (Priolo et al., 2001). On the other hand, in a study conducted by Klita et al. (1996), pH

decreased in response to increasing saponins. This result was explained considering the inhibition of VFA absorption by the ruminal epithelium or increased microbial VFA production.

3.4 Effect of saponins on ammonia concentration

Reducing of ammonia concentration in rumen is an important factor to reduce faecal odour. It has been shown both in *in vitro* (Wallace et al., 1994; Lila et al., 2003) and in *in vivo* (Hussain & Cheeke, 1995;) studies that saponins might reduce ammonia (NH3–N) concentration in rumen. It is thought that saponins may affect ammonia concentration, indirectly via their toxicity to rumen ciliate protozoa, with the consequent low predation of bacteria, or directly through their capacity to bind to ammonia. The observed low ammonia levels might derive from a balance between the two processes: degradation of feed proteins and uptake of ammonia for the synthesis of microbial protein. Moreover, *Yucca schidigera* extract and *Quillaja saponaria* saponins have been shown to reduce proteolysis (Wallace et al., 1994; Makkar & Becker, 1996) whose principal products are aminoacids, peptides and ammonia. *Yucca schidigera* plant also contains glycosylated components which are able to bind ammonia (Headon, 1991). These yucca extract can bind ammonia when the ruminal ammonia concentration is high and release of the bound ammonia when its concentration is low in the rumen, thus modulating diurnal fluctuations in ruminal ammonia concentrations to provide a continuously adequate amount of ammonia for microbial metabolism (Hussain & Cheeke, 1995). This would also spare energy otherwise needed for conversion of excessive ammonia to urea in the liver and its excretion.

SECTION II

Experimental results

EXPERIMENT ONE

Effect of dietary from *Quillaja saponaria* L. on fatty acid composition and cholesterol content in muscle longissimus dorsi of lambs

EFFECT OF DIETARY SAPONINS FROM QUILLAJA SAPONARIA L. ON FATTY ACID COMPOSITION AND CHOLESTEROL CONTENT IN MUSCLE LONGISSIMUS DORSI OF LAMBS[1]

Background

Many authors have studied the anticholesterolaemic effect of saponins and their effect on ruminal microbes, especially protozoa. Both of these effects are due to the ability of saponins to form insoluble micelles with sterols (Sidhu & Oakenfull, 1986). Several feedstuff used for livestock feeding, such as *Yucca schidigera* (Singer et al., 2008; Holtshausen et al., 2009), *Quillaja saponaria* (Holtshausen et al., 2009), *Medicago sativa* (Klita et al., 1996) and *Sapindus saponaria* (Abreu et al., 2004) contain saponins. Hitherto, there is no information with regard to the possible effects of dietary saponins on fatty acids (FAs) and cholesterol metabolism in ruminants and their effects on meat quality.

Dietary polyunsaturated FAs (PUFAs) are known reduce the risk of cardiovascular diseases and chronic pathologies (e.g. cancer, diabetes; Simopoulos, 1999). Besides PUFA, interest in conjugated linoleic acids (CLAs), especially in the C18:2 cis-9,trans-11 (rumenic acid, RA), mainly present in ruminant products, has incremented considerably over the last years because of its beneficial effects on human health (reduction of carcinogenesis, atherosclerosis and body fat; McGuire & McGuire, 2000). The CLAs are synthesized during

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biohydrogenation (BH) of dietary C18:2 cis-9,cis-12 (linoleic acid, LA; Kepler & Tove, 1967), a process which is carried out by the ruminal bacteria. One of the potential consequences of antiprotozoal effect of saponins is the reduction of the predation by protozoa on rumen bacteria, thus modifying ruminal ecosystem. Therefore, it is plausible to suppose an effect of saponin supplementation on the appearance of FAs synthesized by bacteria during the BH pathway. However, so far, there are no studies on the possible effects of saponin on BH, with consequent effect on muscle FA composition. In addition, considering the interaction between saponins and cholesterol in other animal species, including humans, we have hypothesized an effect of saponins on cholesterol content in muscle longissimus dorsi of lambs.

1. Aim of the experiment

The aim of this study was to evaluate the effects of increasing levels of sapogenins present in a preparation of *Quillaja saponaria* on FA profile and cholesterol level in lambs

2. Materials and methods

2.1 Experimental design, animals and management

The experiment was carried out from April to June 2008 at the National Institute of Agricultural Research of Tunisia (INRAT, Tunisia). A total of 24 male Barbarine lambs, 5- to 6-month-old and with average initial live weight 18.66 ± 1.98 kg, were assigned to four dietary groups, each consisting of six animals. All groups received the same basal diet consisting of oat hay *ad libitum* and 400 g of concentrate (80% barley, 17.5% soybean meal and 2.5% mineral and vitamin

supplement). Groups QS30, QS60 and QS90 were supplemented with 30, 60 or 90 ppm of a Q. saponaria preparation, respectively.

The Quillaja saponins were administered as a water solution drench, prepared by dissolving the opportune amount of Quillaja extract in tap water. The Q. saponaria extract used in this experiment was purchased from Sigma Aldrich (batch: 024K2505, Santiago, Chile, USA) and it contained 20% sapogenin. Therefore, the sapogenin content administered to the groups QS30, QS60 and QS90 was 6, 12 and 18 mg per kg of feed (concentrate 1 hay) consumed, respectively. The control group (C) did not receive the Quillaja water solution.

Lambs were allowed to the following experimental treatment: 15 days adaptation, 57 days growth trial, 4 days adaption to metabolic cages, 5 days digestibility measurement and 4 days resting (receiving the above diets). At the end of the experimental period, animals were slaughtered in an experimental abattoir. After carcass refrigeration at 4°C for 24 h, the muscle longissimus dorsi was excised, wrapped in aluminum foil, vacuum packed and stored at -20°C until analysis.

2.2 FAs analysis in the longissimus dorsi muscle

Total lipids of longissimus dorsi were extracted with Folch's method (1957) from 8 g of minced meat. FA methyl esters (FAMEs) were prepared by the base-catalyzed trans-methylation (Christie, 1982). Before methylation, C9:0 and C23:0 FAMEs were added together as internal GC 8000 Top standards.

The FAMEs were determined by a ThermoQuest (Milan, Italy) gas-chromatograph (GC) apparatus equipped with a flame ionization detector (FID) and a high polar fused silica capillary column (WCOT fused silica CP-Select CB

for FAMEs Varian, Middelburg, Netherland; 100 m × 0.25 mm i.d.; film thickness 0.25 mm). Helium was used as the carrier gas at a flow of 1 mL/ min. The split ratio was 1:80. The GC conditions were: the oven temperature was programmed at 150°C and held for 1 min, then increased up to 175°C at a rate of 0.8°C/min, held for 14 min, then increased up to 188°C at 2°C/min, held for 18 min, and then increased up to 230°C at a rate of 2°C/min, held for 13 min. The injector and detector temperatures were set at 270°C and 300°C, respectively. Meat FAs' results are expressed as percentage of total identified FAs.

2.3 Feedstuff analysis

Total lipids of hay and concentrate were extracted with chloroform–methanol, according to Folch's method (1957) The FAMEs were prepared by the base-catalyzed trans-methylation as described by Christie (1982). Feed FAs analysis was performed as described for meat samples. Feed FAs'profile is reported in Table 1 and the results are expressed as percentage of total identified FAs.

Table 1. Fatty acid composition of control diet (% FA)

	Feed	
FA	**Hay**	**Concentrate**
C10	1,59	0,19
C12	1,49	0,20
C14	3,43	0,57
C15	0,70	0,11
C16	29,38	18,99
C16-1c7	0,08	ND
C16-1c9	0,48	0,11
C18	4,79	2,85
C18-1c9	15,63	28,77
C18-1c11	0,64	0,93
C18-2n6	20,26	41,77
C18-3n3	7,12	0,15
C20	1,22	0,28
C21	0,11	0,51
C20-4n6	1,92	0,27
C22	1,28	0,28
C24	0,78	0,19
C24-1	0,12	0,10

FA=fatty acid; ND=not detectable

2.4 Cholesterol analysis

For cholesterol analysis, we have adopted the method described by Sander et al. (1989). The extracted total lipids (100 mg) were weighed in a 20 mL test tube, dried under nitrogen flow and then kept in vacuum and in the dark overnight.

To this 500 mL of Betulin in chloroform (1 mg/mL) was added as an internal standard, and then 10 mL of 1N KOH in methanol was added and the mixture was vortexed for 30 s to make it free of dispersed fat particles. The tubes were shaken at room temperature for 18 to 20 h to allow the formation of a saponifiedfraction. Distilled water (10 mL) was then added to the saponified mixture, which was transferred to another test tube. The unsaponifiable fraction was extracted three times with 10 mL of diethyl ether, and the pooled diethyl ether extracts were washed twice with 5 mL of 0.5 N KOH and twice with 5 mL of saturated NaCl solution. After drying with anhydrous sodium sulfate for 1 h at 4°C, the extract was filtered, dried under vacuum using a rotary evaporator and then the dried material was kept for one night in the dark, in a glass bowl containing hygroscopic salts and under vacuum: this procedure allows the complete dryness of the samples before measuring the weight of the extracted lipids.

The unsaponifiable fraction was then recovered in a solution of hexane : 2-propanol mixture (4:1 v/v) and stored at 220°C until analysis. Cholesterol was determined after silylation of unsaponifiable matter. Silylation solution was composed of a pyridine solution of hexamethyldisilazane and trimethylchlorosilane (5/2/1 v/v/v, respectively; Sweeley et al., 1963) of the silylated sample. Derivatization was completed in 20 min at 40°C.

About 0.4 mL was injected at a split ratio of 1:100. Trimethysilyl derivatives were identified and quantified by using a GC equipped with FID and an apolar 25 m × 0.25 mm i.d. capillary column according to Boselli et al. (2005). The injector and detector temperatures were set up at 325°C. The oven temperature was programed at 250°C and increased to 325°C at 3°C/min, with final temperature of 325°C held for 15 min. Helium was used as the carrier gas with a flow rate of 0.75 mL/min.

2.5 Statistical analysis

Data were analyzed by ANOVA as a completely randomized design and included in the model treatment effects and experimental error. Individual animals were considered as experimental units. When ANOVA was significant ($P < 0.05$) means were separated by the Pairwise comparison. The data were analyzed by the software MINITAB version 14.0.

3. Results

3.1 Effect of saponin supplementation on muscle FA composition and cholesterol content

Tables 2a and 2b, show the effects of increasing levels of saponins on the FA profile and on the desaturation indexes (DIs) of lamb meat. The concentration of C14:0, C16:0 and C18:0 in meat was similar for the four groups of lambs (Table 2a).

The concentration of total saturated FAs (SFAs) was unaffected by dietary treatments (Table 2b). The concentrations of iso-BCFA (branched chain FAs), anteiso-BCFA and total BCFA were not influenced by saponin supplementation

($P > 0.05$). None of the individual BCFA was affected ($P > 0.05$). The iso-BCFA/anteiso-BCFA ratio was similar in the meat of all experimental groups ($P = 0.225$; Table 2b).

When saponins were added to the diet, the concentration of C14:1 cis-9 in meat was lower ($P = 0.001$), whereas C16:1 cis- 9 and C18:1 cis-9 were not affected by saponin supplementation ($P > 0.05$; Table 2a).

None of the FAs arising from ruminal BH was affected by saponin supplementation ($P > 0.05$). Neither the total trans C18:1 nor the individual trans C18:1 were influenced by saponin supplementation ($P > 0.05$). The concentration of RA was similar between the four groups of animals (Table 2a). The sum of the C18:1 trans and C18:2 with at least one double bound in trans configuration was not influenced ($P > 0.05$) by saponin supplementation (Table 2b).

The concentration of LNA (a-linolenic acid) and LA did not differ among the four treatments ($P > 0.05$). The ratio LNA/LA was lower ($P < 0.05$) in the group receiving 60 ppm of Quillaja than the C group (0.053 v. 0.083, respectively), and was found at intermediate value in the meat of lambs from the QS30 and QS90 groups (0.067 and 0.079, respectively; Table 2b). The concentration of C20:4n-6 in the meat of animals receiving 60 ppm of Quillaja was twice (2.22% of total FA) compared with that of meat from the C and QS30 groups (0.99% and 1.29% of total FA, respectively). The concentration of this FA in the meat of animals from the QS90 group was intermediate of the values (1.33% of total FA) of the other three groups. The concentration of C20:3n-6, C20:5n-3, C22:4n-6 and C22:5n-3 was unaffected by Quillaja supplement (Table 2a).

The concentrations of total UFAs (unsaturated FAs), PUFAs, medium-chain FAs and long-chain FAs were not influenced by saponin supplementation. Saponin supplementation did not affect ($P > 0.05$) the PUFA/SFA ratio (Table 2b).

The total DI, calculated as described by Kelsey et al. (2003) (product of Δ^9-desaturase/[product of Δ^9-desaturase + substrate of Δ^9-desaturase]), was not affected by the dietary treatments ($P > 0.05$). The DI C14:1 cis-9/(C14:0 1 C14:1 cis-9) was lower ($P < 0.005$) in QS30, QS60 and QS90 groups than in C group. The DI C16:1 cis-9/(C16:0 1 C16:1 cis-9), the DI C18:1 cis-9/(C18:0 1 C18:1 cis-9) and the DI RA/(vaccenic acid; VA 1 RA) were not influenced by saponin supplementation ($P > 0.05$; Table 2b).

Table 2A. Effect of saponin supplementation on longissimus dorsi muscle individual fatty acids (% of total fatty acids)

	Treatments *					
Fatty acid	**C**	**QS30**	**QS60**	**QS90**	**† s.e.m.**	***P*-value**
C10:0	0.21	0.18	0.18	0.18	0.008	0.367
C12:0	0.17	0.15	0.14	0.14	0.014	0.831
C14:0	2.20	1.99	1.78	1.83	0.151	0.233
C15:0	0.37	0.34	0.37	0.35	0.012	0.757
C16:0	23.70	22.52	21.63	22.86	0.337	0.181
C17:0	1.042	1.028	1.068	1.016	0.016	0.71
C18:0	21.46	22.84	22.16	24.36	0.567	0.325
C20:0	0.13	0.16	0.17	0.15	0.013	0.814
C14:0 *iso*	0.031	0.011	0.014	0.027	0.003	0.092
C15:0 *iso*	0.13	0.12	0.12	0.12	0.004	0.961
C16:0 *iso*	0.14	0.13	0.12	0.13	0.005	0.808
C17:0 *iso*	0.50	0.55	0.61	0.55	0.019	0.257
C18:0 *iso*	0.122	0.155	0.141	0.127	0.009	0.612
C1:0 *anteiso*	0.151	0.130	0.167	0.144	0.006	0.209
C17:0 *anteiso*	0.52	0.51	0.52	0.51	0.011	0.979
C14:1 *cis*-9	0.057 a	0.032 b	0.031 b	0.030 b	0.003	0.001
C16:1 *trans*-9	0.03	0.03	0.04	0.06	0.010	0.248
C16:1 *cis*-7	0.31	0.28	0.32	0.28	0.011	0.457
C16:1 *cis*-9	1.07	0.99	0.92	0.91	0.028	0.196
C16:1 *trans*-14	0.027	0.008	0.012	0.007	0.004	0.16
C17:1 *cis*-9	0.56	0.54	0.58	0.48	0.019	0.256
C18:1 *cis*-9	36.86	36.79	34.97	34.05	0.493	0.105
C18:1 *cis*-11	0.89	1.01	1.03	0.91	0.041	0.559
C18:1 *cis*-12	0.18	0.16	0.27	0.19	0.018	0.135
C18:1 *cis*-13	0.036	0.004	0.031	0.047	0.007	0.221
C18:1 *cis*-15	0.027	0.010	0.008	0.032	0.004	0.097

(continued)

C18:1 *trans*-6,*trans*-8	0.22		0.24		0.27		0.24		0.009		0.349
C18:1 *trans*-9	0.228		0.237		0.244		0.264		0.006		0.241
C18:1 *trans*-10	0.304		0.298		0.390		0.343		0.020		0.362
C18:1 *trans*-11	0.90		0.78		0.81		1.03		0.041		0.116
C18:1 *trans*-12	0.31		0.33		0.36		0.35		0.014		0.675
C18:1 *trans*-15	0.112		0.146		0.126		0.127		0.007		0.401
C18:1 *trans*-16	0.182		0.155		0.176		0.223		0.011		0.162
18:2 *trans*-8,*cis*-13	0.110		0.039		0.059		0.119		0.013		0.057
C18:2 *cis*-9,*trans*-12	0.028		0.000		0.009		0.026		0.005		0.11
C18:2 *trans*-11,*cis*-15	0.09		0.07		0.06		0.08		0.013		0.417
C18:2 *cis*-9,*trans*-11	0.28		0.24		0.26		0.30		0.016		0.686
C18:2 *cis*-9,*cis*-12	4.22		4.91		6.51		4.68		0.579		0.057
C18:3 *cis*-9,*cis*-12,*cis*-15	0.36		0.33		0.34		0.39		0.026		0.868
C20:1 *cis*-11	0.054		0.023		0.041		0.073		0.009		0.21
C20:3 *n*-6	0.095		0.159		0.184		0.149		0.014		0.143
C20:4 *n*-6	0.99	b	1.29	b	2.22	a	1.33	ab	0.262		0.027
C20:5 *n*-3	0.090		0.113		0.266		0.166		0.030		0.154
C22:4 *n*-6	0.060		0.027		0.063		0.072		0.011		0.494
C22:5 *n*-3	0.22		0.26		0.43		0.36		0.033		0.088

† Mean of standard error

***** Treatment: C = lambs receiving control diet; QS30 = lambs receiving control diet plus 30 ppm of *Quillaja*; QS60 = lambs receiving control diet plus 60 ppm of *Quillaja*; QS90 = lambs receiving control diet plus 90 ppm of *Quillaja*;

[a, b] Different letters within the same row indicate significant differences ($P < 0.05$)

[A, B] Different letters within the same row indicate significant differences ($P < 0.01$)

Table 2B. Effect of saponin supplementation on longissimus dorsi muscle fatty acid indices (% of total fatty acids)

	Treatments *											
Fatty acid	**C**		**QS30**		**QS60**		**QS90**		**†SEM**	***P*-value**
SFA [1]	51.01		50.76		49.18		52.61		0.725	0.443
BCFA [2]	1.69		1.65		1.76		1.70		0.035	0.759
iso-BCFA [3]	0.92		0.97		1.02		0.96		0.024	0.607
anteiso-BCFA [4]	0.77		0.67		0.74		0.75		0.021	0.428
iso-BCFA / *anteiso*-BCFA	1.20		1.49		1.40		1.28		0.053	0.225
total *trans* C18:1 [5]	2.25		2.18		2.37		2.57		0.082	0.37
total *trans* [6]	2.53		2.23		2.48		2.83		0.096	0.18
LNA / LA	0.083	a	0.067	ab	0.053	b	0.079	ab	0.004	0.023
UFA[7]	48.71		49.00		50.56		47.09		0.718	0.422
PUFA [8]	6.55		7.23		10.21		7.68		0.538	0.074
MCFA [9]	31.09		29.18		28.36		29.46		0.402	0.098
LCFA [10]	68.68		70.60		71.46		70.34		0.407	0.095
PUFA / SFA	0.130		0.142		0.211		0.151		0.012	0.081
Total DI [11]	0.44		0.44		0.44		0.41		0.006	0.32
DI C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)	0.025	a	0.016	b	0.017	b	0.016	b	0.002	0.002
DI 16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)	0.043		0.042		0.041		0.039		0.001	0.638
DI C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)	0.63		0.63		0.61		0.58		0.008	0.202
DI RA[12]/(VA[13] + RA)	0.238		0.245		0.242		0.220		0.011	0.848

[*] Treatment: C = lambs receiving control diet; QS30 = lambs receiving control diet plus 30 ppm of *Quillaja*; QS60 = lambs receiving control diet plus 60 ppm of *Quillaja*; QS90 = lambs receiving control diet plus 90 ppm of *Quillaja*;

[†] Standard error of the means

[a, b] Different letters within the same row indicate significant differences ($P < 0.05$)

[A, B] Different letters within the same row indicate significant differences ($P < 0.01$)

[1] SFA = sum of saturated fatty acids: C8:0; C10:0; C12:0; C14:0; C15:0; C16:0; C17:0; C18:0; C20:0

[2] BCFA = sum of branched chain fatty acids *iso* and *anteiso*: *iso*-BCFA; *anteiso*-BCFA

[3] *iso*-BCFA = C14-*iso*; C15-*iso*; C16-*iso*; C17-*iso*; C18-*iso*

[4] *anteiso*-BCFA = C15-*anteiso*; C17-*anteiso*

[5] Total *trans* C18:1 = sum of *trans* C18:1: C18:1 *trans*-6,*trans*-8; C18:1 *trans*-9; C18:1 *trans*-10; C18:1 *trans*-11; C18:1 *trans*-12; C18:1 *trans*-15; C18:1 *trans*-16

[6] Total *trans* = sum of total *trans* fatty acids: C16:1 *trans*-14; C18:1 *trans*-6,*trans*-8; C18:1 *trans*-9; C18:1 *trans*-10; C18:1 *trans*-11; C18:1 *trans*-12; C18:1 *trans*-15; C18:1 *trans*-16; C18:2 *trans*-8,*cis*-13; C18:2 *cis*-9,*trans*-12

[7] UFA = sum of unsaturated fatty acids: C14:1 *cis*-9; C16:1 *trans*-9; C16:1 *cis*-7; C16:1 *cis*-9; C16:1 *trans*-14; C17:1 *cis*-9; C18:1; C18:1 *trans*-6,*trans*-8; C18:1 *trans*-9; C18:1 *trans*-10; C18:1 *trans*-11; C18:1 *trans*-12; C18:1 cis-9; C18:1 *trans*-15; C18:1 *cis*-11; C18:1 *cis*-12; C18:1 *cis*-13; C18:1 *trans*-16; C18:1 *cis*-15; C18:2 *trans*-8,*cis*-13; C18:2 *cis*-9,*trans*-12; C18:2 *trans*-11,*cis*-15; C18:2 *cis*-9,*cis*-12; ; C18:3 *cis*-9,*cis*-12,*cis*-15; C20:1 *cis*-11; C20:3 *cis*-8,*cis*-11,*cis*-14; C20:5 *cis*5,*cis*-8,*cis*-11,*cis*-14,*cis*-17; C22:4 *cis*-7,*cis*-10,*cis*-13,*cis*-16; C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19

The effect of saponin supplementation on meat cholesterol content is shown in Table 3. The supplementation of saponins did not influence muscle cholesterol content ($P > 0.05$). The cholesterol content in meat ranged between 47.91 and 61.32 mg/100 g of muscle.

Table 3. Effect of saponins on longissimus dorsi muscle cholesterol

	Treatments*					
	CO	S30	S60	S90	s.e.m	*P*-value
cholesterol mg/100 g muscle	61,32	53,12	47,91	52,46	2,48	0,295
cholesterol g/100 TL	4,248	4,303	4,207	3,782	0,208	0,825

*Treatments: C 5 lambs receiving control diet; QS30 5 lambs receiving C diet plus 30 ppm of Quillaja; QS60 5 lambs receiving C diet plus 60 ppm of Quillaja; QS90 5 lambs receiving C diet plus 90 ppm of Quillaja.

4. Discussion

4.1 Effect of saponins on muscle FA composition

In our study, we have focused on meat FA profile with particular emphasis on FAs arising from metabolism of rumen bacteria (e.g. BCFA) or those FAs, which are intermediates in the process of ruminal BH.

The concentration of BCFA was not affected by the dietary treatments. BCFAs are formed by ruminal bacteria from the propionate that originates in the rumen by the fermentation of dietary carbohydrates (Scaife et al., 1978) and their synthesis is enhanced when animals are fed concentrate-based diets (Vlaeminck et al., 2004). The concentration of BCFA in the rumen also depends on ruminal protozoal population (Or-Rashid et al., 2007), which is sensitive to saponins

(Wallace et al., 1994; Makkar et al., 1998). Therefore, in our study, we would have expected an interaction between saponin supplementation and the BCFA in the muscle. Probably the levels of saponin supplementation chosen for this study were not sufficient to have an impact on those microorganisms responsible for BCFA synthesis.

The concentrations of C16:1 cis-9 and C18:1 cis-9 were not affected by Quillaja supplementation; these FAs derive both from feed and from the desaturation of C16:0 and C18:0, operated by Δ^9-desaturase enzyme in the muscle (Choi et al., 2001). Conversely, C14:1 cis-9 is exclusively synthesized endogenously in muscle by Δ^9-desaturase enzyme (Palmquist et al., 2004) and therefore the DI C14:1 cis-9/(C14:0 1 C14:1 cis-9) has been proposed as an evaluation index of Δ^9-desaturase enzyme activity in lamb muscle (Palmquist et al., 2004). The lowering effect of saponins on the concentration of C14:1 cis-9 and of its DI was an unexpected result. Nevertheless, saponin supplementation did not affect the other DIs. The fact that the DI C14:1 cis-9/(C14:0 1 C14:1 cis-9) was the only DI affected by saponins might be explained by considering that Δ^9-desaturase enzyme has higher affinity for 16 and 18 carbon atoms' FAs than FAs with a shorter carbon chain (Enoch et al., 1976). Even though we did not observe a dose-response effect of saponin supplementation, maybe in the presence of saponins, Δ^9-desaturase enzyme activity was weaker for C14:0, which is not among the Δ^9-desaturase enzyme preferred substrates of action. We did not observe changes between the four treatments in C16:1 and C18:1 accumulation probably because the high concentration of these FAs could have masked a variation of Δ^9-desaturase activity. In addition, it is plausible to suppose that

saponins (or their aglycone) may influence directly or indirectly Δ^9-desaturase enzyme activity. In fact, an enzyme-inhibitory effect of saponins has been previously described, for example, *in vitro* for elastase and hyaluronidase enzymes (Facino et al., 2006) and *in vivo* for the cytochrome P450 enzymatic system in rats (Kim et al., 1997). In a previous study, we have found that supplementing lambs with tannins (another group of plants' secondary compounds, which are not absorbed through the digestive tract) reduced the Δ^9-desaturase enzyme protein expression (Vasta et al., 2009a). Further research is needed to elucidate it in context to saponins.

In addition, RA is endogenously synthesized from the desaturation of VA by Δ^9-desaturase enzyme. According to our hypothesis of a possible inhibition of Δ^9-desaturase enzyme by saponins, we would have expected a lower concentration of RA in the meat of the saponins-supplemented lambs, but this was not the case. Palmquist et al. (2004) have shown that up to 90% of the RA in lamb intramuscular fat originates through endogenous synthesis. Ruminant products are significant sources of dietary trans FAs, which originate from BH of dietary UFAs by the activity of ruminal microbes (Mosley et al., 2002).

In this study, none of the FAs that originated during the BH was affected by saponin supplementation. For most of the trans FAs the exact synthetic pathways are still unclear. The conversion of LA and LNA to VA is carried out by 'group A' bacteria (mostly Gram-positive bacteria), whereas the following step of the BH is carried out by 'group B' bacteria (mostly Gram-negative bacteria; Kemp and Lander, 1984). As Wang et al. (2000) noted, the Gram-negative bacteria are more resistant to saponins than Gram-positive bacteria. Therefore, we would have

expected an effect of dietary saponins on ruminal BH, but at the inclusion levels of saponins chosen for this study we did not observe any interference of saponins on ruminal BH. Gutierrez et al. (1959) reported that *Butyrivibrio fibrisolvens* – one of the bacteria involved in the BH of PUFA in the rumen – strains isolated from cattle rumen was able to degrade alfalfa saponins. Therefore, it is possible that in this study saponins were ineffective because they were degraded by ruminal microorganisms. C20:4n-6, whose concentration in this study was higher in the meat of QS60 lambs compared with that of C and QS30 lambs, is mainly derived from LA through elongation and desaturation reactions (Brenner, 1989).

Arachidonic acid is preferentially stored in cell membrane phospholipids, rather than in triglycerides (Aurousseau et al., 2004). There was a tendency to have a leaner meat in QS60 group compared with the other groups (1.16 for QS60 v. 1.5 for C, 1.25 for QS30, 1.43 for QS90 g of total intramuscular fat/100 g of muscle; P , 0.1). Leaner meat contains greater proportions of phospholipids than triglycerides (Aurousseau et al., 2004) and this would explain the higher concentration of C20:4n-6 in the meat of the QS60 lambs compared with the other groups.

4.2 Effect of saponins on muscle cholesterol composition

A large number of studies have shown an hypocholesterolemic effect of saponins in mammals (Sidhu & Oakenfull, 1986). However, so far, there is no information on the effects of saponins on cholesterol metabolism in ruminants. About the two school of thoughts above mentionated about the hypocholesterolemic effect of saponins, considering that ruminants' diets do not contain cholesterol, the first of the hypotheses is not valid. Moreover, saponins are

structurally modified by ruminal microorganisms (Makkar & Becker, 1997; Wang et al., 1998) and it is likely that the aglycone remaining after saponin degradation is not as effective as the entire saponin in binding bile acids in the gut. This would explain why we did not observe any hypocolesterolemic effects of saponins in lamb. It is also likely that the inclusion level of saponins was not enough for interfering with cholesterol metabolism.

5. Conclusions

Saponin supplementation did not affect the overall FA profile of lamb meat. However, we have observed that saponin supplementation reduced the concentration of C14:1 cis-9 and the respective DI (C14:1 cis-9/(C14:0 1 C14:1 cis-9)). We hypothesize an effect of saponins on Δ^9-desaturase enzyme activity, although the other DIs were not affected by saponin supplementation. None of the FAs arising from ruminal BH was affected by Q. saponaria. Supplementing lambs with 60 ppm of Quillaja increased C20:4n-6 in meat. Thus, an influence of saponin on LA metabolism is possible. The concentration of cholesterol in meat was not influenced by saponin supplementation. This result could be attributed to insufficient dose of saponin supplemented in the diet or to structural modification of saponins carried out by ruminal microorganisms. Further investigations should be undertaken to evaluate the effect of saponins on ruminal BH, with emphasis on Δ^9-desaturase enzyme activity, and on cholesterol levels of lamb meat.

EXPERIMET TWO

Effect of tannin and saponin extract in single ratio or as choice of them on lamb meat quality

EFFECT OF TANNIN AND SAPONIN EXTRACT IN SINGLE RATION OR AS CHOICE OF THEM ON LAMB MEAT QUALITY

Background

In animal nutrition, the use of antibiotic growth promoters has been practiced for about 50 years in the United States and in several other countries (Dibner & Richards, 2005). However, Europe and US legislators have moved to prohibit the use of these chemical compounds from the end of 2005 because of an increase awareness of hazards (e. g. transmissible resistance factors) associated with their use (Wallace, 2004). Thus, there has been an increasing interest in the use of plants and their extracts to solve problems in animal nutrition and livestock production (Wallace, 2004). Among the natural products, many researchers have paid their attention on plant secondary compounds (PSC).

Plant secondary compounds are a diverse group of molecules that constitute the "plant defence system" and are not involved in the primary biochemical pathways of cell growth and reproduction (Wallace, 2004). Some of the major PSC that occur in plants include tannins and saponins, which have been identified and used in animal productivity and health as alternatives to chemical feed additives (Rochfort et al., 2008; Provenza et al., 2010).

Tannins are phenolic compounds which have the capability to link to dietary proteins and reduce the activity of ruminal microbes (Vasta et al., 2010a). Several studies have demonstrated that dietary tannins are able to strongly affect meat colour (Luciano et al., 2009a), flavour (Priolo et al., 2009; Vasta et al., 2006;

Vasta et al., 2010b) and fatty acid composition (Vasta et al., 2009a; Vasta et al., 2007). It has been shown that tannins are able to reduce ruminal biohydrogenation (BH) in *in vitro* studies (Khiaosa-Ard et al., 2009; Vasta et al., 2009b) and increase muscle Δ^9-desaturase protein expression in sheep (Vasta et al., 2009a).

Saponins have been largely used in animal nutrition to reduce methanogenesis in the rumen (Patra & Saxena, 2010). Moreover, they have pronounced antiprotozoal activity, due to their ability to bond to cholesterol present in the protozoa membranes (Makkar et al., 1998), thus reducing the predation of protozoa on rumen bacteria. Saponin ability to form micelles with sterols, such as cholesterol and bile acid, could explain their cholesterol-reducing effect found in blood and tissues of monogastric mammals (Potter et al.,1993; Harris et al., 1997). Nevertheless, this effect has not been still found in ruminants In a study conducted by Brogna et al. (2011), dietary saponin supplementation reduced the concentration of C14:1 cis-9, a fatty acid synthesized by Δ^9-desaturase enzyme, in lamb meat, thus suggesting a possible effect of saponins on this enzyme. Some studies have also demonstrated an antioxidant activity of saponins (Rodrigues et al., 2005), but there are no studies about the effect of dietary saponins on meat color stability.

Research of natural compounds able to provide ruminant meat rich in healthy fatty acids, poor in cholesterol and with a long shelf life represents an important objective.

1. Aim of the experiment

This study is a part of project named "Primary Roles for the Secondary Compounds: *Enhancing the Health of Soil, Plants, Herbivores and People through Plant Biochemical Diversity*", which aim is to investigate the effects of PSCs on parasitized animals. The results about the effects of PSCs on infection are reported in a companion paper Copani et al. (submitted).

The aim of this study was to evaluate the effects of a diet-containing tannins (extract of quebracho) or a diet-containing saponins (extract of *Quillaja saponaria*) when given in single ration or as choice of them, on volatile profile, fatty acid composition, cholesterol content and oxidative stability in lamb meat.

2. Materials and methods

2.1 Animals and dietary treatments

The study was conducted at the Green Canyon Ecology Centre, located at the Utah State University in Logan, Utah. Twenty-eight commercial Finn-Columbia-Polypay-Suffolk crossbred lambs (2 months of age) were placed randomly in individual pens and assigned to four dietary groups, each consisting of seven animals. Three experimental diets were prepared every 2 days in a batch of 30 kg. The control diet (P) consisted of beet pulp added of 1,5 % vegetable oil. The tannin-rich diet (T) was the P diet added of 8% tannins (27 kg beet pulp + 2,4 kg tannins) (tannins from quebracho, Industria Argentina ATO, UNITAN S.A.I.C.A., Buenos Aires, Argentina); the saponin-rich diet (S) consisted of P diet plus 1.5% saponins (29 kg beet pulp + 0,45 kg saponins) (saponin from *Quillaja saponaria* bark S7900, SIGMA). The fourth diet (C) was a choice between tannin-

and saponin-rich diets. Each lamb had free access to fresh potable water and trace mineral salt blocks.

Lambs were allowed to the following experimental design (fig. 1): at day 0 lambs were weighed and drenched with a combination of antiparasitic agents: Stronid® T (Pyrantel pamoate) 25 mg/kg, and Valbazen (Albendazole) 7.5 mg/kg; from day 0 to 10, animals were fed a diet consisting of alfalfa pellets *ad libitum* and 300 g of rolled barley per head per day; at day 10, faecal samples of each animal were taken to assess fecal egg counts (FEC); from day 10 to day 22 lambs were familiarized with the experimental diets (adaption period); at day 23 each animal was infected orally with a normal syringe containing 30 ml of water solution with a single dose of 5,000 L_3 of *Haemonchus contortus*; from day 23 to 49, the animals were kept on a diet of *ad libitum* allowance of alfalfa pellets. At day 49 FEC was assessed again but the level of infestation was inappropriate because the number of eggs in faeces was lower than the expected level for an infection. Thus, animals were re-infested at day 50. The second infestation was done following a similar procedure, but using a single dose of 8,000 L_3 of *Haemonchus contortus.* Following the second larval infestation, animals were kept on a diet of *ad libitum* allowance of alfalfa pellets until day 73. From day 73 to 85, animals received the experimental diets. After the experimental period, lambs received alfalfa pellets until slaughter.

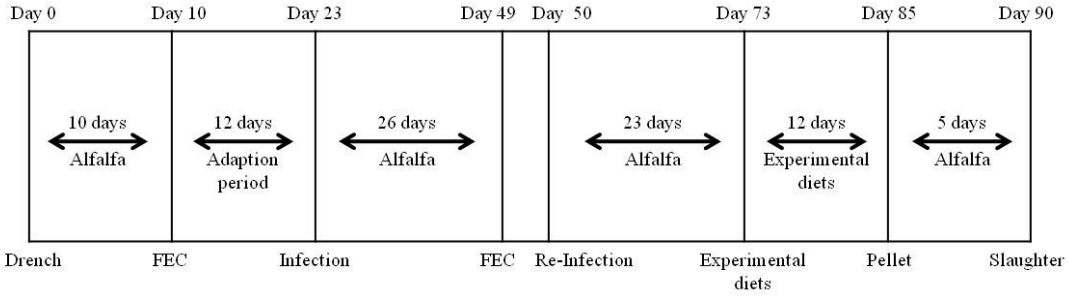

Figure 1: Experimental design

2.2 Slaughter procedures and muscle sampling

Lambs were slaughtered in an experimental slaughter house. After carcass refrigeration at 4°C for 24 h, the longissimus dorsi muscle (LM) was sampled from the carcass. One portion (about 10 g) of LM was immediately vacuum-packaged and frozen at -30°C for about two weeks before analyzing intramuscular fatty acid composition and muscle volatile compounds. The remaining portion of LM was used fresh for determination of meat colour stability and lipid oxidation over a 12-day period of refrigerated storage, as well as for measurement of metmyoglobin reducing activity (MRA) and of the resistance of myoglobin to nitrite-induced oxidation.

2.3 Muscle analyses

2.3.1 Colour stability measurements

Slices of fresh LM (3 cm thickness) were placed in a styrofoam tray, over-wrapped with polyvinyl chloride (PVC) and stored at 4°C until colour measurement was taken. Colour reading were performed following 2 hours of blooming (day 0) and, subsequently, after 2, 4, 6, 8, 10 and 12 days of storage at 4°C. A HunterLab Miniscan portable colorimeter (Reston, VA) with a 5 mm

diameter aperture, set to use illuminant D-65 was used to measure meat colour parameters lightness (L*), redness (a*) and yellowness (b*). Hue angle (H*) values were calculated as $\tan^{-1}(b^*/a^*) \times (180/л)$. The colorimeter was calibrated through a single layer of PVC film using both white and black standard tiles. Triplicate readings were made on non overlapping areas of the sample and values were then averaged.

2.3.2 Metmyoglobin Reducing Activity (MRA)

Muscle slices stored for 0 and 12 days as described above were used for determination of MRA following the method described by Mancini et al. (2008). A 3 × 3 × 2 cm sample of LM that did not contain any visible fat or connective tissue was prepared. Samples were submerged in 0.3% $NaNO_2$ solution for 20 min at room temperature to induce metmyoglobin (MMb) formation. Then, they were removed from beaker and blotted to remove the excess of oxidizing solution.

Reflectance spectra were recorded at the meat surface using a HunterLab Miniscan portable colorimeter (Reston, VA) with a 5 mm diameter aperture, set to use illuminant D-65. Percentage of MMb formed after the oxidizing treatment were calculated as described by Mancini et al. (2008). Furthermore, MMb formation at the meat surface following treatment with $NaNO_2$ was estimated by calculating the $(K/S)_{572} \div (K/S)_{525}$ ratio (Stewart et al., 1965). Samples were then placed up in an impermeable bag, vacuum packaged and incubated for 2h at 30°C in order to allow MMb reduction. Following incubation, the metmyoglobin reducing activity (% of MMb reduced) was calculated as: MRA = [(Initial %MMb - Final %MMb)÷Initial %MMb] ×100.

2.3.3 Lipid oxidation measurement (TBARS assay)

Slices of LM stored for 0, 4, 8 and 12 days, as described for colour stability determination, were used for measurement of lipid oxidation using the thiobarbituric acid and reactive substances (TBARS) assay described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 mL of 0.375% TBA - 15% TCA - 0.25 N HCl solution. The mixture was heated for 10 min in a boiling water bath to develop a pink colour. Samples were then cooled with tap water and centrifuged at 5500 rpm for 25 min in a Beckman centrifuge (Model F0850/Allegra X-22 Series, Palo Alto, CA). The supernatant was measured at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). Lipid oxidation was calculated as milligrams of malonaldehyde per kg of meat.

2.3.4 Volatile compounds analysis

Volatile profile of lamb meat was determinated as described by Vasta et al. (2010b). The frozen LM of each animal was trimmed of external fat and finely sliced (thickness < 1 mm). Six g of raw sliced meat was placed in a 20-mL glass vial and capped with a PTFE septum.

Solid phase micro-extraction (SPME) technique was used to extract headspace volatile compounds. Two microliters of 1,2 dichlorobenzene (7.7469 μg/mL) was added to the vial containing the sample as internal standard. The vial was then placed in a heat bath set at 60 °C for 10 min. A 2 cm-50/30 DVB/CarboxenTM/PDMS fiber (Supelco, Bellefonte, PA) was then exposed to the headspace over the sample at 60 °C for 30 min to absorb the volatile compounds.

The fiber was then removed from the vial and immediately inserted into the GC (GC/MS-QP 2010S, Shimadzu Co., Kyoto, Japan). The injector was set up at 250 °C, in splitless mode and provided with a 0.75 mm inlet liner (Supelco, Bellefonte, PA). Helium was used as carrier gas with a flow rate of 1.0 mL/min. Volatile compounds were separated using a Supelco DB-5ms column (30 m × 0.250 mm × 0.25 mm). The GC oven temperature was programmed as follows: 40°C for 2.4 min; then to 325 °C with a rate of 6°C/min, with a total program time of 43.16 min. The GC/MS interface was heated at 290 °C. The acquisition was performed in ionization voltage mode (70 eV) at 5 microscans/s, scanning the mass range 33-350 m/z.

Compound identification was performed using AMDIS (version 2.62, 1999-2000) for de-convolution and compared with mass spectra of the NIST Mass Spectral Library (version 2.0, 2005), by data reported in literature, by comparison with linear retention indexes (LRI). The LRI was calculated by previous injection of standards of n-alkanes from 7 to 40 carbon atoms (Supelco, Bellefonte, PA). The software program spectconnect was also used to detect components that were conserved across samples. Volatile compounds are expressed as back transformed values of concentration (μg/kg of meat).

2.3.5 Fatty acid analysis in muscle

Fatty acids were analyzed using the method developed by O'Fallon et al. (2007). Fatty acid methyl esters (FAMEs) were obtained directly from meat sample without passing through the conventional organic solvent extraction.

Freeze-dried samples (1 g) were placed into 16 × 125 mm screw-cap Pyrex culture tubes to which 1 mL of C17:1 internal standard (0.2826 mg/mL of

C17:1/mL of MeOH), 0.7 mL of 10 N KOH in water and 5.3 mL of MeOH were added. The tubes were incubated in a 55°C water bath for 1.5 h, hand-shaking vigorously for 5 s every 20 min to dissolve and hydrolyze the sample.

After cooling in a cold bath, 0.58 mL of 24 N H_2SO_4 in water was added. Tubes were again incubated as described above. After FAMEs synthesis, tubes were cooled in a cold bath. Two millilitres of hexane were added and tubes were centrifuged for 5 min at 500 rpm at 4°C. The upper phase, containing FAMEs, was placed into GC vials and store at -20°C until analysis.

The FAMEs were determinate by using a gas chromatograph (GC) (2010S, Shimadzu Co., Kyoto, Japan) apparatus equipped with a quadrupole (QP) detector and a HP-88 (100 m × 0.25 mm × 0.20 μm) high polar capillary column (Supelco). The injector was set up at 250°C and in split mode (1:10). Helium was used as carrier gas with a flow rate of 1.0 mL/min. The GC condition were: the oven temperature was programmed at 35°C and held for 2 min, then increased up to 175°C at a rate of 4°C/min, held for 4 min, then increased up to 250°C at a rate of 3.5°C/min and held for 25 min, with a total program time of 55.93 min. Fatty acid are expressed as mg/100 g of fresh meat.

2.3.6 Fatty acid analysis of feed

The FAMEs analysis in feedstuff (control diet, tannin-diet, saponin-diet and pellet of alfalfa) was performed as described for meat samples. Feed fatty acid composition is reported in table 1 and the results are expressed as mg/100 g of feed.

Table 1. Fatty acid composition of diets[a]

	Treatments[b]			
Fatty acid	***T***	***S***	***P***	***PE***
C16:0	599.00	343.05	353.07	320.11
C16:1 trans-10	11.64	7.13	7.39	4.68
C18:0	65.62	41.42	42.95	48.16
C18:1 trans-11	10.04	5.43	6.16	1.33
C18:1 cis-9	1854.11	1172.33	1243.15	4.92
C18:2 cis-9, cis-12	1332.70	775.30	824.42	196.61
C18:3 cis-6, cis-9, cis-12	16.60	10.72	11.14	3.15
C18:3 cis-9, cis-12, cis-15	322.15	198.78	208.75	325.15

[a] Expressed as mg /100 g of diet
[b] The treatments were: beet pulp plus tannin extract (T); beet pulp plus saponins extract (S); beet pulp without tannin or saponin extracts (P); pellet (PE)

2.3.7 Cholesterol analysis

The Amplex® Red Cholesterol Assay Kit was used to determinated cholesterol content in meat samples. Meat samples were previously homogenated using liquid N_2 and their cholesterol content was determinate following the manual provided by the Amplex Red reagent kit (INVITORGEN). Cholesterol concentration was then determinated by using a fluorometer (TECAN, Infinite M200 Series) and the results are expressed in µM.

2.4 Statistical analysis

Data of colour stability, MRA and TBARS were analyzed with a GLM procedure with repeated measures. The model included the dietary treatment (Diet: T, S, P and C), the time of storage (Time: days 0, 2, 4, 6, 8, 10, 12 for colour; 2, 4, 8, 12 for TBARS; 0, 12 for MRA) and the diet × time interaction as

fixed effects, while individual lamb was included as random effect. The Tukey's test was used for comparing mean values.

Volatile compounds and fatty acids data were analyzed by One-Way ANOVA as a completely randomized design, including in the model the effect of the dietary treatment (Diet: T, S, C, P) and experimental error. Individual animals were considered as experimental units. When ANOVA was significant ($P < 0.05$) means were separated by pairwise comparison using the Tukey's test.

The Anderson-Darling normality test was used to test the normal distribution of data. When not normally distributed (significance of the test: $P < 0.05$) a $\log_{10}$ transformation of the data was performed before analysis of variance; back transformed value of concentration are presented in table. All the statistical tests were performed using the software MINITAB version 14.0.

3. Results and discussion

3.1 Meat colour stability and metmyoglobin reducing activity (MRA)

The main effects of diet and time and their interaction on oxidative stability parameters of LM are reported in Table 2.

Colour is the main criterion consumers use to judge meat quality and acceptability. Little is known about the effect of saponins on meat colour, while it has been shown that the inclusion of quebracho tannins in sheep diets improved meat color stability over extended refrigerated storage both in high oxygen modified atmosphere (Luciano et al., 2009a) and in aerobic conditions (Luciano et al., 2011).

In our study, meat colour stability descriptors (L*, a*, b*, H*) were strongly affected by the time of storage ($P < 0.0005$), but there was no effect of the diet. Nevertheless, a diet × time interaction was found for a*, b* and H* values ($P = 0.001$, $P = 0.012$ and $P = 0.001$, respectively), but not for L* values ($P = 0.211$). After 10 days of refrigerated storage, redness (a* values) were lower in the meat from lambs fed saponin-containing diet (S) as compared to the control diet (P; $P = 0.01$; Fig. 2a). Hue angle (H* values) is another indicator of meat discolouration (Lee et al., 2005). Between 4 and 6 days of storage, hue angle increased in meat of lamb fed S-diet ($P = 0.01$) but did not change in the lamb meat from T, P and C-diets (Fig. 2b).

Meat discoloration is due to the oxidation of myoglobin (Mb) to metmyoglobin (MMb) over time of storage (McKenna et al., 2005). It has been shown that including quebracho extract into a conventional concentrate could exert a protective effect against meat discoloration (Luciano et al., 2009b), while few information is able about the effect of saponin extract on Mb oxidation. In the present study we measured the ability of muscle samples to reduce MMb (metmyoglobin reducing activity; MRA) and neither effect of the diet nor of the time of storage was detected (Fig. 3a). The method adopted for measuring MRA involved an initial step in which MMb formation was induced by treatment with $NaNO_2$ followed by a second step in which MMb is reduced. However, the initial levels of MMb depend on the inherent resistance of myoglobin to oxidation, which may vary between different samples. Therefore, the initial amount of MMb formed after the treatment with $NaNO_2$ have been proposed as a measure of the resistance of the sample to form MMb (Mancini et al., 2008; Luciano et al., 2011).

Luciano et al. (2011) found that dietary quebracho tannins improved the resistance of myglobin to nitrite-induced oxidation in lamb meat compared to a tannin-free control diet. In the present study, the accumulation of MMb in meat after $NaNO_2$ treatment was estimated by calculating the $(K/S)_{572} \div (K/S)_{525}$ ratio: higher are the values of the ratio, lower the MMb level (Stewart et al., 1965). The $(K/S)_{572} \div (K/S)_{525}$ values were affected by the time of storage, whereby lower values (higher MMb%) were found in meat stored for 12 days compared to meat at day 0 of storage (1. 021 vs. 0.961, respectively; $P = 0.004$; Fig. 3b). This indicates that, in meat, the inherent resistance of myoglobin to oxidation decreased along time of storage. However, no diet nor diet × time effects were detected.

The weak effect of PSCs on colour stability, MRA and resistance of myoglobin to nitrite-induced oxidation could be explained considering that, in our experiment, lambs received PSCs only for 12 days during the long experimental period (90 days), whereas in the study conducted by Luciano et al. (2011) lambs were fed with tannins for 60 days. Moreover, lambs ate saponins from alfalfa during the whole experimental period. It is known that saponins can bind tannin promoting chemical interactions that inhibit their absorption from the intestinal tract and thus their effects (Freeland et al., 1985). It is also plausible to suppose that there was an adaptation of rumen microbes to saponins because eaten for a long time (Wang et al., 1998).

Table 2. Main effects of diet and storage time and their interaction on indices of oxidative stability in LM

	P-values		
	Diet[a]	***Time***[b]	***Diet x Time***
Lightness (L* value)	0.662	<0.0005	0.211
Redness (a* value)	0.861	<0.0005	0.001
Yellowness (b* value)	0.546	<0.0005	0.012
Hue angle (H* value)	0.929	<0.0005	0.001
MRA values (%MMb reduced)	0.166	0.795	0.602
K/S_{572}/ K/S_{525}	0.694	0.004	0.649
Lipid oxidation (mg of MDA/kg of meat)	0.106	<0.0005	<0.0005

[a] The diets were: beet pulp plus tannin extract (T); beet pulp plus saponins extract (S); beet pulp without tannin or saponin extracts (P); choice between T and S diets
[b] Time of storage: days 0, 2, 4, 6, 8, 10, 12 for colour a*, b*, L*, H*; days, 0, 12 for MRA values; days 0, 4, 8, 12 for lipid oxidation

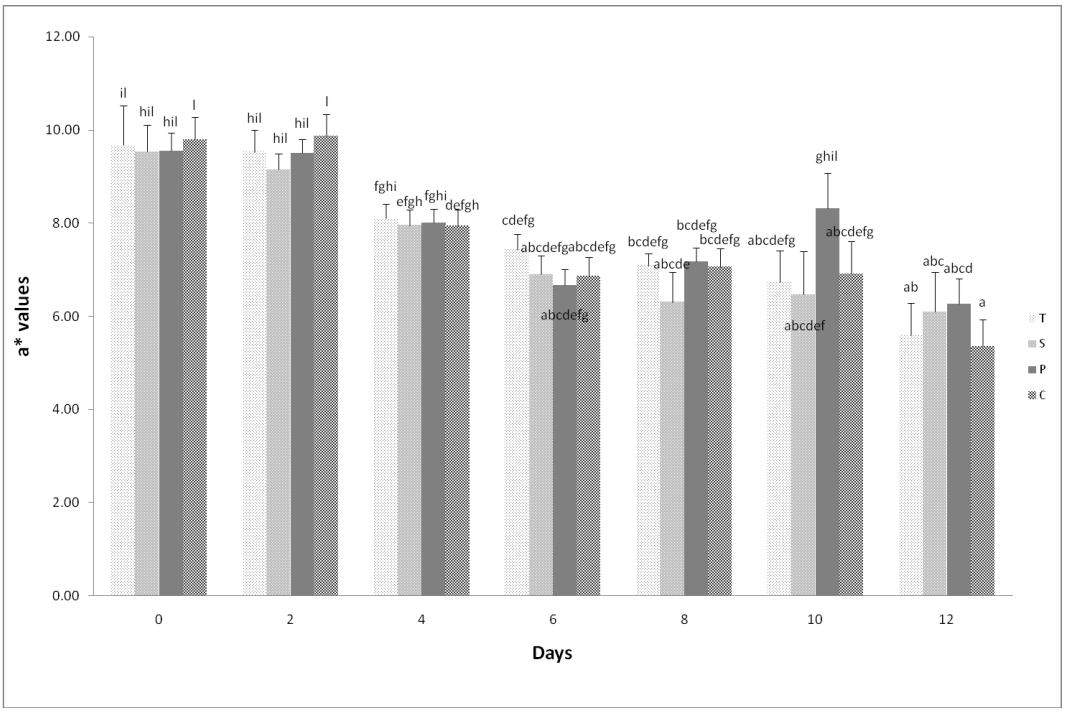

Figure 2a: Effect of diet, time and diet × time interaction on redness (a*) values of lamb meat

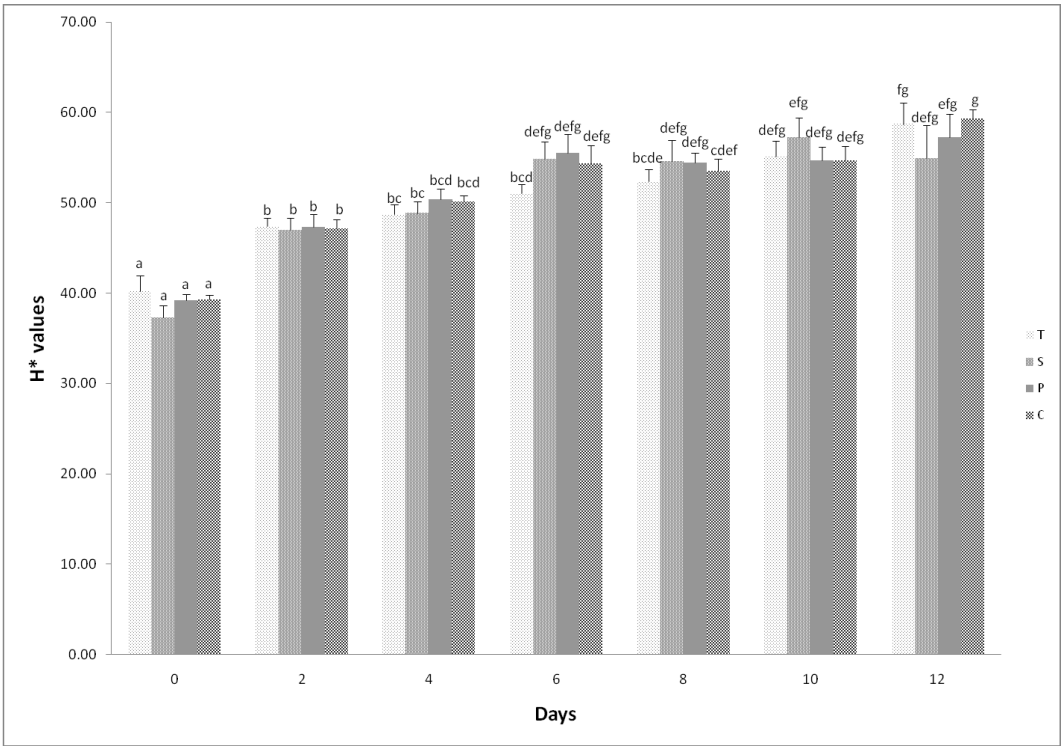

Figure 2b: Effect of diet, time and diet × time interaction on Hue angle (H*) values of lamb meat

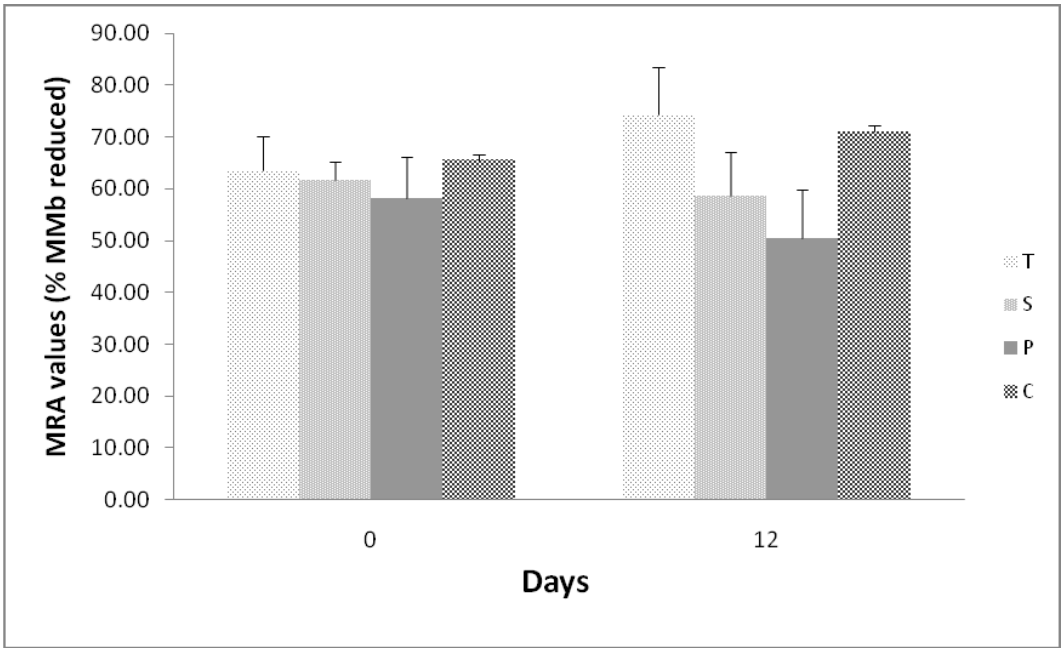

Figure 3a: Effect of diet, time and diet × time interaction on MRA values of lamb meat

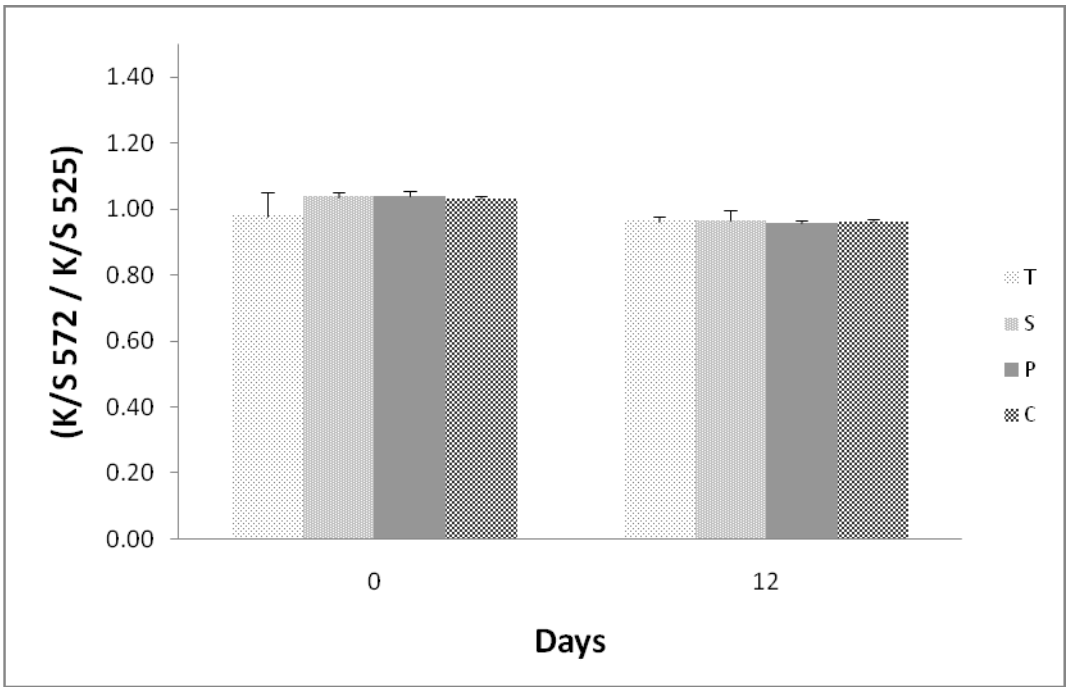

Figure 3b: Effect of diet, time and diet × time interaction on $K/S_{572}/K/S_{525}$ ratio of lamb meat

3.2 Lipid oxidation in the L. dorsi muscle

As reported in Table 4, lipid oxidation increased across the 12 days of storage period ($P < 0.0005$). Moreover, the diet affected in tendency ($P = 0.106$) lipid oxidation, and a strong diet × time interaction was also found ($P < 0.0005$). At days 8 and 12 lambs fed saponin-containing diet (S) had TBARS values lower than lambs fed tannin-diet (T) ($P < 0.0005$, P=0.03, respectively; Fig. 4).

This result could be due to the antioxidant effect of saponin extract used in this experiment. A similar results was found in a study conducted by Ibrahim et al. (Ibrahim et al., 2010) in which saponin extract from ginseng added to lamb patties was more effective in reducing lipid oxidation than jatropha, ginger and jojoba extracts, respectively rich in phenolic compounds and flavonoids.

Supplementation of dietary tannins had no effect on lipid oxidation of lamb fed T diet. Luciano et al. (2009a) found a similar result in their study, in which quebracho tannins had no appreciable effect on TBARS values during storage at 4°C.

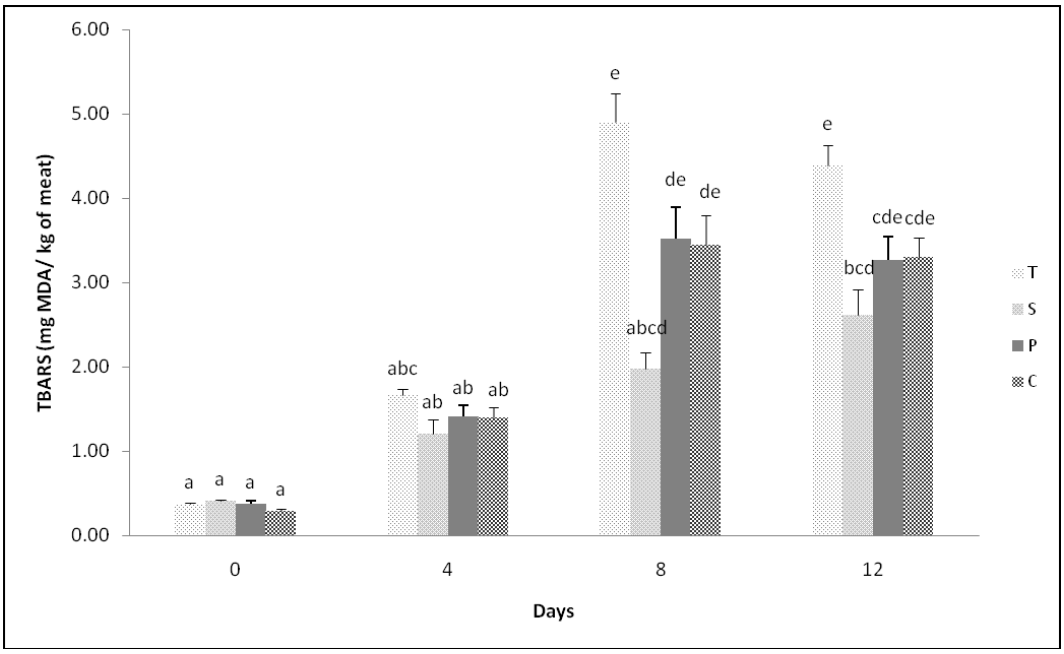

Figure 4: Effect of diet, time and diet × time interaction on TBARS values of lamb meat

3.3 Volatile profile of L. dorsi muscle

Meat volatile compounds have been studied for their favourable and unfavourable effects on flavour and for their potential use in tracing the animal feeding system (Vasta et al., 2006). Animal diet is one of the main factors that can influence meat volatile profile (Vasta et al., 2006). Little is known about the effect of tannin- and saponin-containing diets on lamb meat volatile profile. In the present study, supplementation of tannin and saponin extracts had not a strong effect on lamb meat volatile. Only few volatile compounds were significantly affected by the diet (Tab. 3).

The concentration of furan, 2-pentyl was significantly lower (P = 0.001) in the muscle of lambs fed saponin-diet (S) than P and C diets. It is proposed that this compound is formed by autoxidation of linoleic acid (Krishnamurthy et al., 1967). The content of this fatty acid was slightly higher, even if not significantly

(P = 0.45; Tab. 3), in the muscle of lambs fed S diet than the other groups. This result could be explained considering the antioxidant effect of saponins, which avoided the oxidation of linoleic acid and consequently a minor furan, 2-pentyl formation. The concentration of 2-hydroxypropanoic acid, also known as lactic acid or lactate, was lower in the muscle animals fed S diet than T diet. In the rumen, it is well established that some bacterial species, such as *Megasphaera*, *Selenomonas* and *Veillonella*, are able to convert lactate in acetate, propionate and butyrate (Counotte et al., 1981).

Moreover, it has been found that lactate-using bacteria often increase after defaunation (Kurihara et al., 1968). This means that saponin extract used our in experiment might have had a negative effect on protozoa with consequent increase of lactate-using bacteria, thus explaining the lower concentration of 2-hydroxypropanoic acid in the S group.

3.4 Fatty acid composition of L. dorsi muscle

Tannins are known to reduce the proliferation of ruminal bacteria and to inhibit their biohydrogenation activity, thus reducing SFA formation (Khiaosa-Ard et al., 2009). Moreover, the antiprotozoal capacity of saponins to modify ruminal ecosystem (Patra & Saxena, 2009) could alter BH process. In our experiment, supplementation of tannin and saponin extracts had not a strong effect on meat fatty acid profile. In the study conducted by Brogna et al. (2011) an expected result was found. The concentration of C14:1 cis-9 and its desaturation index (DI), calculated as described by Palmquist et al. (2004), were lower only in the groups of lambs fed saponin-containing diet.

This fatty acid is exclusively synthesized in the muscle by the action of Δ^9-desaturase enzyme (Palmquist et al., 2004), thus suggesting a possible effect (direct or indirect) of saponins on this enzyme expression or activity. In our study, the concentration of C14:1cis-9 in muscle of lamb fed S diet was similar to the other groups; also, DI was not significantly affected by diet ($P = 0.07$; Tab. 4). It is possible that there was an adaptation of rumen microbes to saponins (Wang et al., 1998), or they were degraded by the saliva of lambs because eaten for a long time (Teferedegne, 2000).

In the muscle of lambs fed T diet the concentration of C14:1 cis-9 was higher than lambs fed P and C diets (Tab. 4). The more concentration of C14:1 cis-9 in the muscle of lamb fed T diet could be explained considering that tannins would be able to increase Δ^9-desaturase protein expression (Vasta et al., 2009a) and thus more production of enzyme product.

Nevertheless, neither Δ^9-desaturase enzyme products, such as RA, nor intermediates of ruminal BH, such as vaccenic acid (VA), were not affected by tannin-containing diet. This result is different from that found by Vasta et al. (2009c), in which tannin supplementation increased the concentration of RA and VA. The difference could be explained considering that our experiment lasted 90 days and lambs received a diet containing 8% of fresh feed of tannin extract only for 12 days. On the other hand, in the experiment conducted by Vasta et al. (2009c), quebracho tannin supplementation was 10 % of dry matter (DM) and given for 60 days. Moreover, the presence of saponins, administered as alfalfa pellet for all experimental period, might have masked the effect of tannin (Freeland et al., 1965).

Table 3. Volatile profile of muscle longissimus dorsi from lambs fed different diets[a]

Compounds	***LRT[c]***	***Method of identification[d]***	***Treatments[b]***								***SEM[e]***	***P-value[f]***
			T		***S***		***P***		***C***			
Alcohols												
1-Pentanol	764	MS, LRI	0.338		0.323		0.510		0.581		0.062	0.376
1-Hexanol	864	MS, LRI	0.222		0.228		0.284		0.311		0.036	0.425
3-Heptanol	893	MS, LRI	0.085		0.089		0.123		0.109		0.098	0.938
1,2-Propanediol	905	MS	0.747	a	3.401	ab	2.620	ab	5.430	b	0.117	0.050
3,4-Dimethyl-3-hexanol	938	MS	0.131		0.122		0.142		0.170		0.095	0.962
Heptanol	968	MS, LRI	0.249		0.161		0.365		0.405		0.070	0.164
1-Octen-3-ol	977	MS, LRI	0.714		0.938		0.994		1.007		0.045	0.635
1-Hexanol, 2-ethyl-	1026	MS, LRI	1.738		2.427		1.878		2.521		0.045	0.519
1-Octanol	1068	MS, LRI	1.036		1.280		1.394		1.460		0.034	0.452
1-Propanol, 2-(2-hydroxypropoxy)	1240	MS	0.109		0.052		0.055		0.223		0.114	0.163
2-Tridecen-1-ol	1502	MS	0.405		0.463		0.341		0.573		0.043	0.302
Phenol, 3,5-bis(1,1-dimethylethyl)	1502	MS	0.177		0.318		0.612		0.231		0.084	0.117
1-Hepten-4-ol	1540	MS	0.017		0.021		0.025		0.039		0.072	0.350
Aldehydes												
Heptanal	897	MS, LRI	0.232		0.205		0.337		0.321		0.046	0.273
Benzaldehyde	956	MS, LRI	0.164		0.154		0.276		0.212		0.058	0.412
Tetradecanal	1609	MS, LRI	0.203		0.329		0.363		0.345		0.055	0.355
Furans												
Furan, 2-pentyl	986	MS, LRI	0.188	ab	0.019	b	0.679	a	0.241	a	0.159	0.001
2(3H)-Furanone, 5-ethoxydihydro	1210	MS	0.444		0.536		0.667		1.273		0.094	0.348
Hydrocarbons												
Heptane, 2,2-dimethyl	775	MS	0.097		0.196		0.230		0.149		0.129	0.775

(continued)

3,4-dimethylbenzamide	785	MS	0.108		0.120		0.079		0.253		0.132	0.597
Heptane, 2,5-dimethyl-	806	MS	0.181		0.187		0.306		0.277		0.120	0.883
Octane, 2,2-dimethyl-	871	MS	0.294		0.151		0.178		0.282		0.065	0.314
Heptane, 2,5,5-trimethyl	981	MS	0.072		0.104		0.128		0.125		0.093	0.780
2-Pentene, 5-(pentyloxy)-	981	MS	0.194		0.242		0.288		0.315		0.046	0.408
Propane, 1-(1,1-dimethylethoxy)-2-methyl	1028	MS	1.645		1.773		1.644		2.412		0.038	0.365
Benzene, 1,3-bis(1,1-dimethylethyl)	1248	MS	0.412		0.407		0.337		0.616		0.045	0.209
Ethene, methoxy	1694	MS	0.047		0.087		0.094		0.350		0.135	0.133
Methyl tetradecanoate	1719	MS, LRI	0.391		0.327		0.331		0.450		0.030	0.316
2-Hexadecene, 3,7,11,15-tetramethyl	1839	MS	0.111		0.143		0.112		0.172		0.058	0.617
Ketones and Hydroxyketones												
2-Pentanone, 4-hydroxy-4-methyl-	845	MS, LRI	0.277		0.075		0.083		0.155		0.131	0.401
Butyrolactone	907	MS, LRI	0.485		0.501		0.604		0.894		0.054	0.286
Ethanone, 1-(4,5-dihydro-2-thiazolyl)	1100	MS, LRI	0.318		0.280		0.405		0.308		0.072	0.893
4-Methyl-5-nonanone	1298	MS	0.485		0.815		0.642		0.652		0.035	0.159
Organic acid												
Butanoic acid	780	MS, LRI	0.327		0.404		0.191		0.261		0.100	0.715
2-Hydroxypropanoic acid	904	MS	0.709	a	0.086	b	0.375	ab	0.612	ab	0.129	0.038
Hexanoic acid, methyl ester	919	MS, LRI	0.311		0.422		0.383		0.333		0.049	0.797
Aminocaproic Acid	999	MS	0.641		0.326		0.392		0.546		0.059	0.293
Heptanoic acid, methyl ester	1020	MS, LRI	0.207		0.200		0.205		0.252		0.042	0.837
Octanoic acid, methyl ester	1120	MS, LRI	0.454		0.655		0.558		0.655		0.040	0.464
Octanoic Acid	1164	MS, LRI	0.229		0.320		0.308		0.368		0.045	0.448
Nonanoic acid, methyl ester	1220	MS, LRI	0.576		0.722		0.751		0.891		0.047	0.581
Nonanoic acid	1261	MS, LRI	0.442		0.615		0.537		0.722		0.057	0.623
Decanoic acid, methyl ester	1319	MS, LRI	0.391		0.343		0.353		0.387		0.035	0.928
Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	1348	MS	0.080		0.100		0.066		0.105		0.054	0.558

(continued)

n-Decanoic acid	1357	MS, LRI	0.321		0.522		0.482		0.761		0.059	0.156
Propanoic acid, 2-methyl-, butyl ester	1370	MS	0.615	a	0.250	ab	0.048	b	0.133	ab	0.146	0.044
3,4-Dimethyl-2-(3-methyl-butyryl)-benzoic acid, methyl ester	1504	MS	0.054		0.079		0.115		0.084		0.084	0.609
Dodecanoic acid, methyl ester	1519	MS, LRI	0.610		0.675		0.794		0.754		0.038	0.743
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1855	MS	0.931		1.043		0.884		1.009		0.037	0.911

[a] Values (back transformed values of concentration expressed as µg/kg of muscle) are means of seven lambs per dietary treatment

[b] The treatments were: beet pulp plus tannin extract (T); beet pulp plus saponins extract (S); beet pulp without tannin or saponin extracts (P); choice between T and S diets (C)

[c] Linear retention index

[d] MS: mass spectrum identified using AMDIS and NIST Mass Spectral Library; LRI: Linear Retention index, obtained with DB5 column, in agreement with data reported in literature

[e] Standard error

[f] P-value: [a, b] different letter in the same row denote significant differences ($P < 0.05$)

Table 4. Muscle fatty acid composition and cholesterol content of muscle longissimus dorsi of lambs fed different diets[a]

	Treatments[b]									
Fatty Acid	***T***		***S***		***P***		***C***		***SEM [c]***	***P-value[d]***
Total FA	2114.940		1665.560		1632.080		1907.330		88.749	0.182
C10:0	4.827		3.213		2.936		3.512		0.271	0.058
C12:0	5.578		3.543		2.356		2.567		0.474	0.054
C14:0	71.117	a	45.892	ab	36.382	b	43.689	ab	4.533	0.029
C14:1 cis-9	4.127	a	2.416	ab	1.551	b	1.781	b	0.294	0.003
C16:0	564.035		419.293		406.912		498.133		26.842	0.127
C18:0	308.922		239.138		259.950		315.204		16.658	0.302
C18:1 trans-11	35.852		91.711		35.359		39.775		14.475	0.462
C18:1 cis-9	743.179		515.036		575.701		670.583		39.866	0.187
C18:2 cis-9, cis-12	122.324		122.944		106.889		104.790		5.094	0.454
C18:3 cis-6, cis-9, cis-12	1.150		1.085		0.988		1.224		0.051	0.432
C18:3 cis-9, cis-12, cis-15	40.568		36.413		31.258		36.536		1.314	0.089
C18:2 cis-9, trans-11	6.560		5.118		5.015		5.948		0.302	0.226
C18:2 trans-10, cis-12	0.614		0.555		0.476		0.535		0.024	0.233
C20:4 n6	41.080		41.310		39.941		39.020		0.964	0.839
C20:5 n3	11.751		12.316		10.046		10.407		0.357	0.066
C22:5 n3	17.283		17.068		14.971		14.249		0.602	0.193
C22:6n3	4.445		5.342		4.806		4.876		0.164	0.293

(continued)

SFA	990.318	739.553	735.948	895.235	46.631	0.144
MUFA	875.232	680.156	678.097	788.494	38.405	0.207
PUFA	249.390	245.855	218.034	223.605	6.825	0.273
DI C14:1 cis-9/(C14:0 + C14:1 cis-9)	0.0531	0.0477	0.0413	0.0366	0.002	0.072
Cholesterol (μM)	18.564	16.923	18.159	17.678	0.603	0.814

[a] Fatty acids (expressed as mg/100 g of fresh meat) and cholesterol (expressed as mg/100 g of muscle) are means of seven lambs per dietray treatments

[b] The treatments were: beet pulp plus tannin extract (T); beet pulp plus saponins extract (S); beet pulp without tannin or saponin extracts (P); choice between T and S diets (C)

[c] Standard error

[d] P-value: [a, b] different letter in the same row denote significant differences ($P < 0.05$)

3.5 Effect of PSCs on cholesterol content of muscle

Saponins are known to bind sterols, such as cholesterol and bile acids, and this capacity could explain their anticholesterolaemic effect found in monogastric mammals (Potter et al., 1993; Harris et al., 1997). In the light of above, we supposed that saponins could reduce cholesterol content in ruminant meat. No significant effect on cholesterol content in the muscle of lambs fed saponin-containing diet was found (Tab. 4). This result is similar to that found by Brogna et al. (2011), which failed to observe any effect of saponins on lamb meat cholesterol content. They suggested that saponins could have been degraded by ruminal microflora or the chosen levels of inclusion (30, 30, 90, mg/kg of feed) were ineffective. In our study, the inclusion of saponin extract was more than the experiment above-mentioned (15×10^3 mg/kg of feed), but it was ineffective. The explanation could be an adaptation of ruminal microbes to saponins (Wang et al., 1998), or their degradation by the saliva of lambs (Teferedegne, 2000).

4. Conclusions

Tannin- and saponin-rich diet, given in single ration or as a choice of them, did not strongly affected lamb meat quality. However, we observed an antioxidant effect of saponins considering the low concentration of furan, 2-pentyl and TBARS values. Moreover, tannin supplementation increased the concentration of C14:1 *cis*-9 in the meat of lamb fed T diet, suggesting a major Δ^9-desaturase enzyme activity than the other groups. The no effect on colour stability, MRA values and cholesterol content could be explained considering that the experimental diets were administered for only 12 days. Moreover, lambs received

alfalfa saponins during all the trial (90 days) which probably caused an adaptation of ruminal microbes to these PSCs or they might have masked tannin effect.

In the light of above, more investigation is necessary to understand which is the period of time during which PSCs can express their positive effect and if their interaction could negatively affect meat quality.

Concluding remarks

The two experiments presented in this thesis show that tannin and saponin-rich diets can have an influence on lamb meat quality. The lower and higher concentration of C14:1cis-9 in the meat of lamb found, respectively in the first and the second experiments, suggest a possible effect of these PSCs on Δ^9-desaturase enzyme activity. Moreover, saponin may have an antioxidant effect on lipid oxidation, as shown in the second experiment, but more elucidation is necessary. The no effect of tannin and saponin extract on cholesterol content, colour stability and MRA values, could be explained considering that some factors such as dose of PSCs supplemented in the diet, structural modification of them carried out by ruminal microbes and duration of the experiment may affect negatively these parameters.

In the light of the results showed in this thesis, further research is certainly needed to understand which are the better conditions in which PSCs can express their effect on meat quality and if their interaction represents a loss of their specific effects.

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