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XXIX CYCLE

Plant secondary compounds in ruminant feeding: implication and effect on meat fatty acid.

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Knowledge is knowing that a tomato is a fruit.
Wisdom is not putting it in a fruit salad.

B. O'Driscoll

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Thanks for everything and see you space cowboy…

Abstract

According to FAO, about 9 billion of people will ask for food by 2050. The agriculture challenge is to increase food production by preserving food quality and natural resources.
According to WHO, red meat can favor the onset of cardiovascular disease and colon-rectal cancer; however, they are also an important source of vitamin, protein and functional fatty acids (CLA, EPA, DHA).
Future animal breeding techniques aims will be improving food nutritional profile and reducing environmental impact at the same time.
Rumen lipolysis and bio-hydrogenation allow the synthesis of a pool of fatty acids; this process is affected by animal diet. Pasture feeding increase omega-3 and CLA content in food; however, the problems related to pasture availability ask for innovative techniques that promote their synthesis even if green herbage is not present. The use of plants, or plant-extracts, containing bioactive compounds represents an achievable prospect.
The aim of this PhD was to deepen how different plants bioactive compounds affect fatty acid composition in lamb meat.
In the first experiment, the use of *Trifolium pratensis* (containing polyphenol oxidase enzyme, PPO) and *Onobrychis viciifolia* (containing condensed tannins, CT) silages were tested. The effect of PPO and CT on rumen lipid profile was comparable and even additive when silages were supplied as a mixture. In this case, meat fatty acid profile was similar to pasture-fed animals.

In the second experiment, three plants extract were added to lambs' diet: hydrolizable tannins from chestnut (*Castanea sativa*), CT from mimosa (*Acacia dealbata*) and from gambier (*Uncaria gambir*). The effect of the different source of tannins on meat fatty acid composition was not very strong; however, multivariate analysis allowed discriminating the different groups.

Sommario

La FAO prevede che entro il 2050 si debbano sfamare 9.1 miliardi di persone. La sfida per l'agricoltura è quella di aumentare le produzioni rispettando la qualità dei prodotti e le risorse naturali.
Secondo la WHO, le carni rosse possono favorire l'insorgenza di malattie cardiovascolari e del cancro al colon-retto; tuttavia, sono un importante fonte di vitamine, proteine e acidi grassi funzionali (CLA, EPA e DHA).
L'obiettivo da perseguire nelle tecniche di allevamento deve puntare a migliorare il profilo nutrizionale dei prodotti di origine animale e l'impatto ambientale.
I processi di lipolisi e bioidrogenazione ruminale consentono la sintesi di un pool di acidi grassi variabile in funzione della dieta aniamle. L'alimentazione al pascolo aumenta il livello di omega-3 e CLA nei prodotti; i problemi legati alla sua disponibilità richiedono tecniche innovative per favorire la sintesi di questi composti anche in assenza di erba. L'utilizzo di piante, o di loro estratti, contenenti metaboliti bioattivi rappresentano una prospettiva perseguibile.

L'obiettivo di questo dottorato è stato quello di investigare gli effetti di composti biologicamente attivi sulla composizione lipidica della carne ovina.
Nel primo esperimento è stato testato l'impiego dell'insilato di Trifolium pratensis (enzima polifenolossidasi, PPO) e di quello di Onobrychis viciifolia (tannini condensati, CT). L'effetto di PPO e CT sul profilo lipidico ruminale è stato simile e additivo, quando i due insilati sono miscelati. In questo caso, la composizione lipidica della carne è risultata confrontabile a quella di animali al pascolo.
Nel secondo esperimento sono stati testati tre diversi estratti addizionati alla dieta degli agnelli: tannini idrolizzabili (HT) di Castagno (*Castanea sativa)* e CT di mimosa (*Acacia dealbata*) e gambier (*Uncaria gambir).* L'effetto sulla composizione lipidica della carne non è stato incisivo; tuttavia, l'analisi multivariata dei dati ha permesso di discriminare i gruppi in prova.

1. Introduction

One of the most controversial matters that the researchers have widely discussed in the last years was the role of meat in human diet.

Meat represents the principal source of protein in the human diet, with a forecast global annual consumption that is going to reach an average value of 45.3 kg by 2030 (Kuovari et al., 2016). Meat is an important source of high biological value proteins and important micronutrients such as B vitamins, iron and zinc (Pereira & Vicente, 2013), but it also represent a font of saturated fatty acid and cholesterol.

Concerning this issue, in the last two decades researchers studied and discussed the correlation of red meat consumption and the onset of colorectal cancer and heart diseases, in reason of the correlation of these pathologies and the level of plasma low density lipoprotein (LDL) concentration (Micha et al., 2010; Mozaffarian et al,. 2010).

In 2015 the World Health Organization (WHO) published a report entitled "Carcinogenicity of consumption of red and processed meat" where the International Agency for Research on Cancer (IARC) issued a warning relative to the cancinogenicity effect of red and processed meat. The magnitude of this report created a "world wide alert" in reason of the impact given by some media and social media, with a consequential reduction of consumption and also a negative effect on the meat global market.

However, it would be necessary a more diligent approach for explaining these results. IARC classified consumption of processed meat as "carcinogenic to humans" (group 1) considering an average intake of 50 g per day, and

consumption of red meat as “probably carcinogenic to humans” (group 2A) considering an average intake of 100 g per day (Working im, 2015).

Therefore, a wise interpretation should not be the total replacement of red meat with other source of protein, like someone had suggested after the publication of WHO report, but just a more conscientious consumption of this food, as was already suggested in the Mediterranean diet (Willett et al., 1995).

The report also explain the criteria how red meat and processed meat are distinguished (Working im, 2015):

- Red meat: refers to unprocessed mammalian muscle meat, for example beef, veal pork, lamb, mutton, horse, and goat, including minced or frozen meat. It is usually consumed cooked.
- Processed meat: refers to meat that has been transformed through salting, curing, fermentation, smoking, or other processes to enhance flavor or improve preservation. Most processed meats contain pork or beef, but might also contain other red meats, poultry, offal (eg, liver) or meat byproducts such as blood.

If, from one side, it is correct to inform consumers about the effect of an unhealthy diet and the risk that may incur from this, on the other hand, it would be desirable provide the background of knowledge about their diet.

Concerning meat fat quality, a lot of researches in meat science assess that fresh red meat product from ruminant may also contain some key nutrient factor, such as omega-3 fatty acids ($\omega 3$) (Palmquist, 2009). This fatty acid group was strongly linked with improved resilience against coronary disease and diabetes, better mental status and infant development and the maintenance of neural and

optical tissue (Ponnanpalam et al., 2016). Another beneficial compound is conjugated linoleic acid (CLA). Among the several isomers that have been identified in ruminant products, the most abundant is the isomer *cis*-9 *trans*-11 (rumenic acid - RA). It was identified as health promoting because of its biological properties, including antitumor and anticarcinogenic activity (Scollan et al., 2006).

Considering long chain PUFA with $\omega 3$ configuration, it is important to cite C20:5 n-3 (eicosapentaenoic acid, EPA) and C22:6 n-3 (docosahexaenoic acid, DHA). These fatty acids have a critical role in the prevention of several diseases like atherosclerosis, heart attack, depression and cancer. Particularly DHA is highly sought in brain: low levels have been linked to low brain serotonin levels, which are connected to an increased tendency for depression (Brenna et al., 2002; Ponnampalam et al., 2006).

The proportion of any fatty acid that results in meat is the direct consequence of the feeding strategy applied in animal breeding. This is because different feedstuffs can affect the chemical, physical and nutritional composition of the final product through different metabolic way that may involve rumen bacteria metabolism and/or tissue activity (Priolo et al., 2001, Laurenço et al., 2007, Mele et al., 2011).

The aim of this research activity was to analyze how innovative feeding strategies affect fatty acid modulation in ruminants and influence, as a consequence, meat fatty acids quality.

2. Lipids metabolism in ruminant

Increasing proportion of ω3 PUFA with the consequence reduction of SFA and ω6 fatty acid was the target of the animal science researchers in the last 20 years.
It is possible to identify two different steps in ruminant lipid metabolism. A ruminal one, which is consequence of two different biochemistry processes: lipolysis and biohydrogenation (Buccioni et al., 2012), and other process definable as "post-ruminal metabolic activities" that are largely cause by enzymes present in different tissues (Smith et al., 2006).

2.1. Lipolysis and biohydrogenation

Dietary esterified lipids, after ingestion, are hydrolyzed at free fatty acid (FFA) and glycerol by the high specific action of a limited number of microorganisms involved in lipolysis (LP) process (Buccioni et al., 2012; Fai et al., 1990). Particularly, the action of *Butyrivibrio fibrisolvens* lipase is associated with phospholipids hydrolysis, while *Anaerovibrio lipolytica* hydrolyzes di- and try-glycerides. Both microbes hydrolyze also the ester bonds, but with a different efficiency in terms of hydrolysis power (Buccioni et al., 2012).
FFA may also arise from hydrolysis of plant galactolipids and phospholipids catalyzed by several bacterial galactosidases and phospholipases (e.g., phospholipase A and phospholipase C) produced by rumen microbes (Jenkins, 1993).
After FFA releasing, the unsaturated fatty acid (UFA) are rapidly hydrogenated into the saturated configuration by

rumen microorganism. As a matter of fact, this reaction occurs as a self-defense mechanism by bacteria to protect themselves from the toxicity effect of UFA (Dehority et al., 2004). Indeed, in whole microbial population, bacteria are involved in bio-hydrogenation (BH) process more than the others microorganisms. These bacteria attack the UFA merged on feed particles surface easily, and with more efficiency, than other disperse in liquid phase (Buccioni et al., 2012).

Considering unsaturated fatty acids with double bonds in *cis-12* position, firth step of BH consist into an enzymatic isomerization reaction that convert the *cis-12* bond into *trans-11* one. This reaction is strongly connected with the presence of free carboxyl produced during lipolysis. In reason of this, lipolysis is considered a "rate determinate step" that determinate the kinetics of the whole process (Antongiovanni et al., 2003). After the formation of *trans-11* bond, microbial reductase operates the hydrogenation in *cis-9* position.

Vaccenic acid (VA) represents a particularly important intermediate of the BH process; this intermediate is partly transformed into stearic acid (SA). To determinate the ratio of VA which is reduced to SA it should be considered a combination of two different aspects: the rumen physiological condition and the amount of linoleic acid (LA) content in the rumen that can inhibit the process permanently (Harfoot et al., 1973). However, just a large amount of unesterified LA affect and block the second step of BH process, the same process would not occur with LA in esterified form (Moore et al., 1969).

Activity of bacteria is also conditioned by the fibre/starch ratio in the diet. Indeed, a low fiber content and a high level of concentrates diet cause a reduction of the number of

cellulolytic bacteria in the rumen, and possibly also their activity during both step of LP and BH (Buccioni et al., 2012; Gerson and King, 1985, Loor et al., 2004). As a matter of fact, a high lipids content in the diet could be an easily way for lipid passage across the rumen without UFA being reduced, especially for oleic acid (OA) and LA (Chilliard et al., 2007).

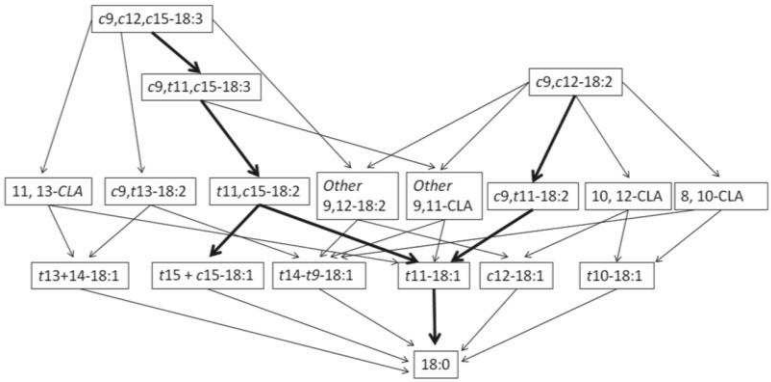

Figure 1 BH pathways in rumen (Chilliard et al., 2007)

However, also in this diet condition, other alternative BH process arise with the appearance of some *trans* fatty acids (TFA) (fig. 1), characterized by a *trans* double bond in the carbon chain (Griinari and Bauman, 2006).

Not only the quantity, but also the quality of fat included into the diet can affect the lipid transformation during BH in the rumen. Fat-rich feed source that contain a high amount of LA, like soybean oil, inhibit the reduction process to SA, in favor of an accumulation of *trans* C18:1 intermediates, and this action is strong when LA is given as free-acid form (Antongiovanni et al., 2003, Moore et al., 1969). Also other oil seeds such as sunflower, canola and linseed mainly

contain C18 PUFA and MUFA. Moreover, marine oils are rich of other compound like EPA and DHA. All these FAs are hydrogenated in the rumen and induce synthesis of BH intermediates such as conjugated and *trans* isomers (Chilliard et al., 2007).
Other dietary factors that cause a possible effect on LP and BH are worth of mention: a low level of nitrogen in the diet, the use of mature forage or the use of too finely ground feeds (Antongiovanni et al., 2003).

2.2. Microbial fatty acids synthesis

In relation to FA fraction from rumen bacteria, part of these are degraded from the feedstuffs, but a small amounts are synthesized ex novo by activities that take place within the microbial cell. Once again, this mechanism remains strongly connected with diet fat content (Antongiovanni et al., 2003, Jenkins et al., 1993). In lipid rich diets, small droplets that are formed in cytoplasm favor the transport of lipids inside the bacteria cell. In this step de novo synthesis of FA leads mainly to the formation of stearic (SA) and palmitic (PA) acid in 2:1 ratio (Bauchart et al., 1990). Microorganism in this step tend to use FA, and a small amount of free non-esterified FA (NEFA), to create membrane phospholipids, but at the same time are not able to store triglycerides (Viviani et al., 1970).
In the last years, rumen bacteria population involved in LP and BH process was divided into three main groups: bacteria associated with the liquid phase (LAB), bacteria either loosely (SAB1) and bacteria firmly (SAB2) attached to feed particles (Buccioni et al., 2012, Legay-Carmier and Bauchart, 1989). Concerning the FA composition of these groups of bacteria, it is possible to clearly distinguish a

difference between LAB and SAB. LAB had a lower content of FA but a higher proportion (g/kg FA) of odd and branched chain FA (OBCFA) compared to SAB groups. On the other side, SAB bacteria present an higher content of C18 BH derived compounds like *trans* C18:1 and CLA, particularly in SAB2 group where a higher concentration of 18:2 n-6 and 18:3 n-3 compared to SAB1 (Bessa et al., 2009) has been observed.
On average, 87% of the total ingested FA arrive in duodenum, while microbial synthesis contributes to an average of 15 g of lipid per kg of fermented DM in the rumen (1.5%) (Antongiovanni et al., 2003).
This balance is strongly affected by the amount of lipid included in the diet that could represent one of the key factors that influences lipid microbial synthesis.

3. Effect of feeding strategies on meat fatty acid composition

Considering how ruminant metabolism is affected by dietary treatment, and how this modifies products FA profile, scientists in the last years tried to manipulate diet content with the aim of improving "healthy" FA components as to provide the best product, in terms of lipid profile, to the consumer.
As previously mentioned, an important group of compounds that appear to have positive effect on human health are CLA isomers of LA (Belury et al., 2002; Mc Guire and Mc Guire, 1999).
The most important and representative one is rumenic acid (RA, cis9, trans11 C18:2 isomer). This particularly

configuration is naturally abundant in the products of ruminants, such as milk, milk products and meat, and is thought to represent about 75% of the CLA (Howes et al., 2015). Particularly in meat, CLA content varies greatly in a ranges from 0.2% to 2% of fat tissue, which, on its turn, depends on animal's diet (Khanal and Olson 2004). RA is synthetized in small amount in the rumen by the isomerization of LA by linoleic isomerase, but reaches its peak of synthesis in mammary gland tissue by Δ9 desaturation of VA in lactating ruminants (Bernard et al., 2009; Buccioni et al., 2012, Mosley et al., 2006; Shingfield and Griinari, 2007). Other studies show that this enzymatic activity can also occur in lamb's adipose tissue (Faria et al., 2012; Palmquist et al., 2004; Raes et al., 2004; Vasta et al., 2009c).

Like most of the rumen activity, environmental factor can interact with rumen CLA metabolism. Martin and Jenkins (2002) showed that rumen pH condition has a great influence on production of *trans* C18:1 and CLA isomers, with a threshold value that should be above 6.0. On the other hand, rumen pH is strongly correlated with the animal diet. Actually, when animal receives a diet with high ratio of concentrate to forage, i.e. a small content of fiber, saturation of LA may divert in favor of *trans* 10 C18:1 instead of VA, with a shift effect of this FA (Fig. 2) that occurs during fermentation process (Grinari et al., 1998). In support of this thesis, French and others (2000) suggested that grass diets could favor the growth and activity of the bacteria *Butyrivibrio fibrisolvens*, which leads to protein breakdown, fiber and hemicellulose degradation and biohydrogenation, which subsequently influences the production of CLA. The effects of pasture feeding have been widely studied in the past (Priolo et al., 2002; Scollan et al., 2006); Wood et al.,

2004) and it is clear how this feeding technique strongly influence lipid composition, both in medium chain and long chain FA. Grass-based compared to concentrate-based diet reduce the percentage of SFA like palmitic acid (PA) and increase alpha-linolenic acid (α-LNA) and CLA; in particular for CLA content, it can also be 2 to 3 times higher as compared to the concentrate diet (Alfaia et al., 2009; Daley et al. 2010; Aurousseau et al., 2004; French et al., 2000).

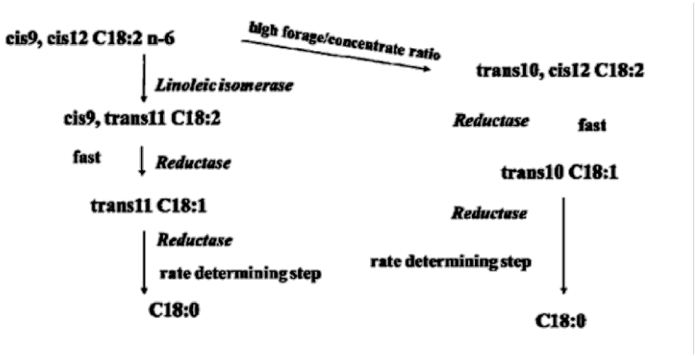

Figure 2 Shift of LA in ruminant (Buccioni et al., 2012)

Other FA that might be of particular interest for human health are eicosapentaenoic acid (C20:5n-3 EPA) and docosahexaenoic acid (C22:6n-3, DHA).

Synthesis of these FA occurs in the muscular tissue and takes place thanks to the activity of Δ6 - desaturase on LA and LNA. Typically a greater concentration of LA in cellular pools, result in a greater conversion to longer chain n-6 PUFA. Because Δ6 - desaturation is the rate limiting step in the pathway, high dietary intakes of n-6 PUFA have been proposed to be a limiting factor in the conversion of LNA to EPA and DHA (Palmquist et al., 2009).

Grass-feed diet management represents an opportunity to improve product quality in terms of proportion of PUFA and $\omega 3$ FA elongation in meat and dairy production, with no effect on $\omega 6$ content, which then reduce $\omega 6/\omega 3$ ratio (Daley, Abbott, Doyle, Nader, & Larson, 2010). Moreover, pasture particularly rich in LNA content result in a higher proportion of EPA and DHA in the muscle and adipose tissue (Scollan et al., 2006).
Moreover, pasture affects also other important parameters like vitamin E, colour and shelf life (Daley et al., 2010); (Howes et al., 2015; Luciano et al., 2013; Priolo et al., 2002).

In the light of the healthy nutritional properties of products arising from pasture-raised livestock, is it possible require that all the farmers apply grass feeding management in any farms all around the world? The answer is no.
There are several reasons. The main reason is that pasture for livestock management requires a lot of grazing land, and not all the countries have enough land to be able to support meat and milk demand. Moreover, the research of new pasture has already led to an important negative environmental impact in terms of deforestation, as for example in Central and South-America.
In addition, pasture is subjected to rotation, to avoid overgrazing and subsequent land degradation, and seasonality. This means that herbage availability and quality could be strongly affected by season, in correlation to temperature and rainfall, which affect herbage growth (Walter et al., 2013).
At last, grazing systems are usually extensive systems and, hence, characterized by low level of productivity; the low productivity arise from both the local breeds and the rearing

systems usually adopted in extensive system. So, they probably cannot be able to support the whole meat and milk demand.
In reason of that, it is necessary to explore the possibility to develop alternative feeding techniques able to compensate the shortage of grass feed while, in the same time, conferring similar nutritional properties to animal's products.

3.1. Alternative feeding strategy and effect on meat FA

In order to preserve or even improve the meat nutritional value and the animal performance, scientist explored different techniques and compounds to fed livestock. In these last years, researches aim at inducing ruminant metabolism towards to the increase of the proportion of healthy compounds through the manipulation of rumen activity. Indeed, ruminal BH manipulation represents an important opportunity to improve nutritive value of ruminant production (Morales and Ungerfeld, 2015).
However, in modern ruminant intensive systems, diets are usually formulated with a high-energy ratio in advantage of animal performance, at the expense of ω6/ω3 ratio and SFA and PUFA percentage (Wood et al., 2004).
As a matter of fact, during fattening phase ruminant are finished with cereal rich concentrate diets that promote intramuscular fat (IMF) deposition and stearoyl-CoA desaturase (SCD) activity (fig.3). Nevertheless, this feeding strategy negatively affects *trans*-11 C18:1 (VA) outflow that is frequently replaced by *trans*-10 C18:1 (t10-shift), which is not convertible to CLA (Bessa et al., 2015). Indeed, one of the schemes to promote content of RA in meat must be

preserving VA formation in the rumen and its uptake to duodenum (Vasta and Luciano, 2011).

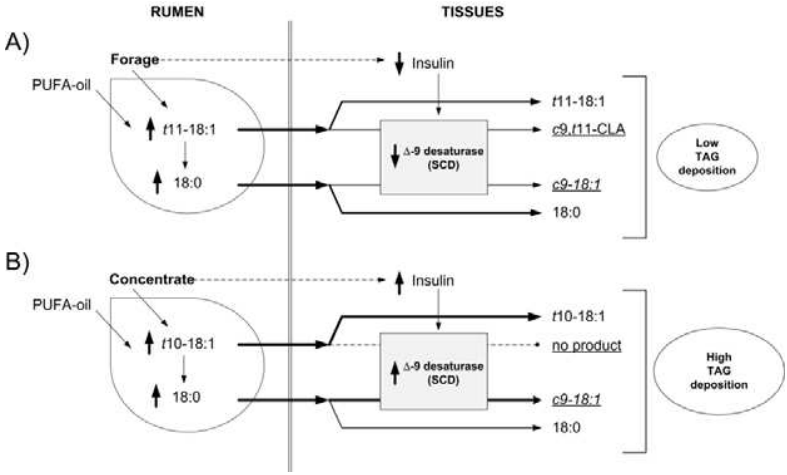

Figure 3 Patterns of *cis* 9 *trans* 11 CLA deposition in meat of ruminant (Bessa et al., 2015)

Strategy to preserve VA includes the use of feedstuffs rich in PUFA lipid such as fresh forage, some legumes silage, oil-rich cereals, oilseeds, fish oil, marine algae, chia see, lupine and so on (Laurenço et al., 2007, 2010; Woods and Fearon, 2009).

However, FA composition of feedstuff is not the only option to condition microbial behavior and rumen metabolism; indeed, plant secondary compounds and some specific plant enzymes can also influence BH.
Secondary compounds are complex chemicals synthesized by plants that are not essential to the life of the plant. They are thought to be produced primarily as defensive factor against animal grazing, but also as attractants, like pigments or scents, for pollinators and seed-dispersing animals, and as

allelopathic agents that influence competition among plant species (Lewis et al., 2001). Secondary compounds are the adaptation response of plants to their environmental challenges, and may reflect a sort of evolutionary arms race between plants and grazers that has led to the rapid evolutionary diversification of some of these substances (Heinstein, 1985).

The interest about the effect of secondary compound on the quality of animal production has been highlighted successively to studies on ruminant production in marginal or dry areas, particularly with sheep and goats that result easily adapted to rural low-input environments (Vasta and Luciano, 2011). Feeding effect of alternative resources (bushes, shrubs, by-product and forages) containing secondary compounds, represent a new approach for ruminant nutrition and has been subject of several studies in the last years (Ben Salem et al., 2012; Cabiddu et al., 2010; Lee, 2014; Vasta et al., 2008).

From these studies it is clear how plant secondary compound, naturally present in the feedstuff or administered such a pure extract, resulted in conditioning animal metabolism and then the quality of final products (Vasta and Luciano, 2011).

Plant secondary compounds, in fact, can interact with microbial population in the rumen and influence BH (Morales & Ungerfeld, 2015). Among these compounds a particular attention has been given to the biological effect of phenolic compounds, tannins in particular, but also saponins and essential oil (Fransis et al., 2002; Frutos et al., 2004; Laurenço et al., 2010; Makkar et al., 2003).

Recent researches have put into evidence that also other plant components, i.e. some specific plant enzymes such as polyphenol oxidase, can interact with ruminal BH; as a

consequence, a possible effect on meat fatty acid could be expected.

3.2. Polyphenol oxidase effect on fatty acid metabolism

Polyphenol oxidase (PPO) is a group of copper metallo-enzymes which include: catecholase, laccase and cresolase. Catecholase is the most dominant PPO in forage crops and usually, using PPO is intended to refer to this (Lee, 2014).
PPO activity can reduce quinones to phenol using molecular oxygen, and then bind to lipases; the complex PPO-lipases can affect and inhibit LP step by inhibiting plant lipases (Cabiddu et al., 2010; Lee et al,. 2007; Van Ranst et al., 2011).
This enzyme is normally stored in the plant in latent form (90%); its activation requires a neutral pH and oxygen. During grazing windows, the mastication, that chops and blows air in the herbage, creates the ideal condition for the enzyme activation, so the percentage of PPO in latent form decrease (Lee et al., 2008).
Availability of PPO enzyme, and the interaction with ruminant digestion, is different in reason of the botanical species; differences have been observed in grass species compared with legumes species (Lee et al., 2006). A recent study suggests that legumes species, particularly red clover with a high content of PPO, cause a significant reduction in BH providing greater protection of PUFA in the rumen (Buccioni et al. 2012).
However, the exact mechanism that causes the effect of PPO in red clover on BH is still totally unknown. Indeed, two different ways has been hypothesized: an indirect influence on ruminal lipolysis and biohydrogenation through a shift in the ruminal microbial population or, otherwise, a direct

protection of PUFA through encapsulation in protein–phenol matrices (Huws et al., 2010, Lee et al., 2010).

Nevertheless, even if this process needs to be better specified, protection activity of PPO on lipids against their degradation is desirable because it could help to improve the fatty acid profile of ruminant products.

3.3. Phenolic compounds effect on fatty acid metabolism

Tannins are polyphenolic compounds having wide prevalence in plants; after lignins, they are the second most abundant group of plant phenolics.
These compounds have a range of effects on various organisms from toxic effects on animals to microorganism's growth inhibition; however the biological potential depends more on the chemical structure than on the concentration (Makkar et al., 2003; Waghorn and McNabb, 2003). Both these factors are strongly connected to the plant species and the ecosystem where the plants grow. Nevertheless, also phenological stage of the plant results crucial for defining both of these characteristics (Berar et al., 2011; Patra and Saxena, 2011).
Hydrolysable (HT) and condensed tannins (CT) are the two major classes of tannins; they differ in molecular weight that is between 500 to 3000 g mol^{-1} for HT and up to 20000 g mol^{-1} for CT (Cieslak et al 2013).
HT are made up of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids (mainly gallic and hexahydroxydiphenic acid). CT are oligomers and polymers of flavanols units, which are most frequently linked either via C4 C6 or C4 C8 bonds (B- type proanthocyanidins). The most common condensed tannins occurring in plant tissues are procyanidins, which derive from catechin or epicatechin

and gallic acid esters. (Bath et al., 1998; Frutos et al 2004; Karonen et al., 2004; Mueller-Harvey, 2001; Romani et al., 2008).
In terms of voluntary feed intake tannins are generally considered astringent and unpalatable; this effect could be caused through a reaction between the compound and the salivary muco-proteins, or through a direct reaction with the taste receptors, provoking an astringent sensation (McLeod, 1974; Frutos et al. (2004), in a review, reported different experiment on HT and CT and described that palatability of both tannins generally depends on the amount included in the diet. Generally, an inclusion > 50 $g*kg^{-1}$ of DM resulted in a significantly reduction of voluntary feed intake, both for the CT and for the HT. However, when the inclusion of tannins is < 20 $g*kg^{-1}$ of DM, this reduction of palatability usually not occur (Patra and Saxena, 2011).
In terms of digestibility, most of the effects caused by tannin intake are principally associated with a modification in the ruminal fermentation pattern, along with changes in intestinal digestibility.

In rumen environment, tannins form hydrogen bond with plant proteins, reducing the availability of these compounds also in function of the molecular weight. Indeed, tannins with a lower molecular weight have a higher interaction capacity with the protein (Frutos et al., 2004; Patra and Saxena, 2011).
Rumen microbes do not dissociate the CT protein complex, whereas the HT protein complex is degraded consequently to a depolymerisation of tannin polymers; this occurs through the cleaving of the ester linkages between glucose and the phenolic subunits by the enzymes secreted by the rumen microbes (Patra and Saxena, 2011).

The stability of links with protein, and their disposal in terms of dietary protein for microbial population and then for the host animal, also depends on rumen pH. A wide rumen pH range from 3.5 to approximately 8 is required for making stable hydrogen bond (Frutos et al., 2004; Hagerman et al., 1992; Mueller-Harvey and McAllan, 1992).

In order to avoid the link of tannins with protein, several studies were performed employing polyethylene glycol (PEG) as tannins bind agent. PEG binds to tannin and prevent the formation of tannin-protein complexes, thus making them inactivate (Frutos et al., 2004; Makkar et al., 2003; Priolo et al., 2010). PEG has been used to control tannins treatment in experiment on ruminal fermentation, with the aim of neutralizing their effect on protein and then, without affecting the digestibility of the diet (Waghorn et al., 2008).

The effect of tannins was widely studied for their influence on ruminal BH. These compounds seem to have an effective role in fatty acid metabolism, whose action appears to impair rumen BH, in particularly the last step that reduce VA to SA; this results in the accumulation of PUFA and VA (Khianosa-Ard et al., 2011).

In vitro and in vivo studies were performed in the past years with the purpose of verifying the effect of tannins on LA and LNA hydrogenation; the expected benefit is a reduction of SA formation in advantage of VA accumulation and RA production trough metabolism, and their consequentially impact on quality of production (Min et al., 2003; Morales and Ungerfeld 2015).

According to this hypothesis, researchers tested the effect of tannins directly presents in the feedstuff, like forage species rich in tannins (sulla - *Hedysarum coronarium,* sainfoin -

Onobrychis viciifolia, ecc) or leaves from redberry juniper (Juniperus *pinchotii*) or *Cistus ladanifer.* But also plant tannin extracts from quebracho (*Schinopsis lorentii L.*), acacia (*Acacia mearnsii*), grape seeds (*Vitis vinifera L.*) and others plants sources were supplemented to animal diet.
Results in this field did not ever confirm the hypothesis and scientific background, but generally give interesting results.
In vitro studies on quebracho tannins supplemented in to diet confirm an inhibition of LNA in early BH step (Kronberg et al., 2007). Vasta et al. (2009a) showed that purified tannins from acacia, carob and quebracho impair ruminal BH, obtaining a higher PUFA and VA accumulation at the expense of SA. Also, the effect of tannins on linoleic acid isomerase did not suggest a direct inhibition on this enzyme, but an effect on microorganism that interact with this. Using quebracho and chestnut tannin extract Buccioni et al. (2011) observed an initial decrease of RA content, in both treatments, followed by an increase from 2 to 3.4 times compared to the control at the end of the incubation period. Both extracts produced a less SA accumulation, confirming an inhibitory effect on the last step of BH. Recently Ishlak et al. (2015), in an in vitro experiment with quebracho, obtained a reduction of SA together with a significant increase of LNA and RA, confirming this effect.
In vivo researches have been performed with tannins containing plants and with tannin extracts addition to the feed. Effect of tannins on meat fatty acid profile was based on the theory that protection of VA during BH resulted in a higher content of this acid in muscle (Priolo and Vasta, 2007). However, lambs fed sulla rich in CT had a greater proportion of LNA in IMF, without any effect on RA and VA. In the same experiment, the addiction of PEG to sulla,

reduced LNA concentration, a sign of inhibition of early BH steps by CT (Priolo et al., 2005).
Vasta et al. (2007), in a feeding trial with a 2.7% inclusion of CT (*kg DM) from carob, observed a reduction of RA and VA accumulation in IMF of *L. dorsi* muscle. Vasta et al. (2009b) found that including 4% (*kg DM) of quebracho tannins extract in fresh forage or high concentrate lamb diet increased PUFA and reduced SFA content in IMF of *L. dorsi* in both treatment. Tannins supplement had, in agreement with this result, produced a higher proportion of VA and RA in ruminal fluid.
Few studies were performed to clarify the possible different mechanism of HT and CT on meat fatty acid profile proportion.
Supplementation of tannins was also studied concerning other aspects of animal science, which still involves the interaction between these compounds and rumen microbial biological process. Most of them regard mitigation technique for reducing methane enteric emission from ruminant farms, but also concern effect of tannins on milk quality.
The clearer expression of tannins potential on rumen metabolism can be found in terms of reduction of methane emission from enteric fermentation. Several studies approach this aspect in reason of ruminant breeding impact on GHG emission, with the aim of mitigating this side effect of ruminant metabolism, through new feeding strategies. Inhibitory effects of tannins on rumen methanogenesis have been associated with a direct effect on methanogenic archaea, protozoal-associated methane production and indirectly through a depression of fibre digestion in the rumen (Patra and Saxena, 2010). Tannin-containing forages and tannin extracts have been proved to reduce enteric

methane production both in vivo and in vitro trials (Carulla et al., 2005; Jayanegara et al., 2015; Min et al., 2005; Patra and Saxena, 2010; Sliwinski et al., 2002).
In the light of the above mentioned studies, the use of tannin containing feedstuff could represent an opportunity to improve food quality and, in the meantime, to mitigate the impact that the production of high quality food have on the environment.

4. Objectives

Keeping in mind the past investigation and the available bibliography, the purpose of the research activity of this PhD was to modify fatty acid in the lamb's meat by using different plant compounds.
Two in vivo experimental trials have been planned by using fattening lambs. In both the experiments, the effect of diet on fatty acid intra-muscular fat has been investigated.

The purpose of the first experiment was to evaluate the effect of feeding plants containing PPO or tannins, offered in different mixes, on fatty acid proportion in *l. dorsi* muscle of lambs. The aim was to verify i) if the biological effect of a single bioactive component affects fatty acid profile, and ii) if an additive effect from both components mixed together can be obtained.

The second experiment aimed at comparing the effect of tannin extracts containing CT or HT on fatty acid profile on meat, and performance in lambs finished on a concentrate-feeding condition. Previous studies didn't clearly explain the interaction between tannins and fatty acid production in meat; so further investigations are required, especially considering new tannins extract not yet tested.

5. Experiment 1

Fatty acid composition of ruminal digesta and *longissimus* muscle from lambs fed silage mixtures including red clover, sainfoin and timothy *

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5.1. Introduction

Increasing the levels of PUFA and reducing the levels of SFA in ruminant meats is an important objective because of the implications that this has on human health. Allowing animals to graze green herbage is an excellent strategy to attain this goal (Aurousseau et al., 2004). However, in several areas, cold winters and/or dry summers make this possible only for a limited duration through the year. Therefore, preserving forages is an alternative to overcome this problem. Among the methods of conservation, the forage ensiling has the advantage of maintaining a fatty acid (FA) composition very close to that of the original green herbage (Halmemies-Beauchet-Filleau et al., 2013).

Polyphenol oxidase (PPO) from red clover (*Trifolium pratense;* RC) is an enzyme that reduces the ruminal biohydrogenation (BH) of PUFA, thus resulting in a greater PUFA content in meats (Lee et al., 2009a). The activation of PPO requires the cell damage and aeration that typically occur in the ensiling process while, when RC is grazed, PPO is poorly activated due to the anaerobic nature of the rumen (Lee et al., 2009b). It has been shown that other plant compounds, such as tannins, may impair ruminal BH (Vasta et al., 2009a; Carreño et al., 2015). The effects of PPO and tannins on ruminal BH are exerted by different mechanisms (Vasta and Luciano, 2011; Buccioni et al., 2012). Red clover silage given as a sole diet has been studied (Lee et al., 2009a). However, it has been demonstrated that animals grow better in mixed diets (Phelan et al., 2015). Indeed combining RC and grass silages increased the digestible matter intake (Niderkorn et al., 2015).

Therefore, with the aim of investigating if the effects of PPO and tannins on ruminal and i.m. FA are additive, in this

work lambs were fed silage mixtures of a plant containing PPO (RC), a plant containing tannins (sainfoin; *Onobrychis viciifolia;* SF) and a grass species not containing these compounds (timothy; *Phleum pratense*; T).

5.1 Materials and methods

The study was conducted indoors, between December 2013 and March 2014, in the facilities of the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes Center in central France (45°42'N, 03°30'E). Animals were handled by specialized personnel who took care of animal welfare in accordance with European Union Directive No.63/2010 and French Directive on the use of animals for experimental purposes (Statutory order No. 87-848, guideline April 19, 1988). The experimental procedure had been reviewed by the local ethics committee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne").

5.1.1. Experimental Design, Animals, and Diets

Forty 4-month-old castrated male Romane lambs (initial BW 30.7 ± SE 0.68 kg) were used. At weaning, 5 weeks before the onset of the trial, all animals were drenched with an antiparasitic agent (albendazole, 0.75 mg/kg BW). In a complete randomized design, lambs were divided into five groups (n = 8) that were balanced for initial BW and offered one of the following five types of silages (treatments): (1) pure timothy grass silage (100% *Phleum pretense* cv. Liglory; T), (2) binary mixture of timothy and tannin-containing sainfoin (*Onobrychis viciifolia* cv. Perly; T-SF; 50:50), (3) binary mixture of timothy and PPO-containing red clover (*Trifolium pratense* cv. Mervius; T-RC; 50:50),

(4) ternary mixture of timothy, sainfoin and red clover containing both tannins and PPO (T-SF-RC; 50:25:25, respectively), (5) binary mixture of tannin-containing sainfoin and PPO-containing red clover (SF-RC; 50:50). No additive was used during the ensiling process and the fermentation lasted 31 weeks.

The 10 week experimental trial began after a 7-day pre-experimental period (week 0). During the experimental period, all the animals were transferred to individual adjacent pens (2 m^2) bedded with sawdust. For the ten following weeks (referenced to as week 1 to week 10), lambs were individually fed with their respective silage, still *ad libitum*, with a restricted supplementation of barley and straw. The amount of barley was calculated on the basis of the individual BW and weekly adjusted in order to cover maintenance requirements of energy. Small fixed amounts of straw were also daily distributed to all lambs as an additional source of fiber. The quantity of straw (60 to 80 g DM on average) was adjusted to minimize refusals and was the same for all the animals. Silage and barley were offered at 0900 and 1600 h, after weighing of refusals at 0800 h. Straw was distributed in the morning, due to the small amounts administered. Animals had free access to salt blocks and fresh water. Lambs were weighed weekly and individual intakes were measured according to the refusal left by each animal

5.1.2. Slaughter Procedure and Samplings

At the end of the experimental period, all the lambs were slaughtered according to European Union welfare guidelines at the experimental slaughterhouse of the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes Center. All the animals had access to their respective diets until

approximately 30 min before slaughter. Once transported by truck to the slaughterhouse, which was less than 1 km distance from the experimental farm, lambs were stunned by a captive bolt then exsanguinated. Lambs were slaughtered by blocks (balanced for treatment) over three consecutive days (13 lambs/day), with the heaviest animals being slaughtered first.

The rumen content was sampled individually just after slaughter. Rumen was cut opened and the whole content was transferred into a bucket and homogenized with a spoon. An aliquot of approx. 150 mL of rumen content was taken and stored at -20°C. Subsequently the samples were freeze-dried and packed under vacuum.

The carcasses were immediately weighed to obtain hot carcass weight and stored at +4 °C for 24 h, after which the LM from the left side was excised from the 13th thoracic rib and vacuum sealed then stored at -80°C until analysis.

5.1.3. Fatty Acid Analysis - Feedstuffs and Ruminal Digesta

Fatty acid methyl esters (FAME) of lipid in freeze-dried samples of offered feedstuffs (i.e., silage mixtures, straw and barley grain) were prepared in a 1-step extraction-transesterification procedure using chloroform (Sukhija and Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al., 2003), and *cis*-12 tridecenoate (Larodan Fine Chemicals AB, Malmö, Sweden) as an internal standard. Lipids in 200 mg of freeze-dried rumen digesta were extracted using a mixture of hexane and 2-propanol (3:2, v/v) and converted to FAME by sequential base-acid catalyzed transesterification (Toral et al., 2010). Methyl esters were separated and quantified using a gas chromatograph (Agilent 7890A GC System, Santa Clara, CA) equipped with a flame-ionization detector and a 100-m

fused silica capillary column (0.25 mm i.d., 0.2-μm film thickness; CP-SIL 88, CP7489, Varian Ibérica S.A., Madrid, Spain) and hydrogen as the carrier gas (207 kPa, 2.1 mL/min). Total FAME profile in a 2 μL sample volume at a split ratio of 1:50 was determined using the following temperature gradient program (Shingfield et al., 2003): the oven temperature was held at 70 ºC for 4 min, increased up to 110 ºC at a rate of 8 ºC/min, then increased up to 170 ºC at a rate of 5 ºC/min, held at 170 ºC for 10 min, and increased at 4 ºC/min to a final temperature of 240 ºC that was held for 14.5 min. Isomers of C18:1 were further resolved in a separate analysis under isothermal conditions at 170 ºC (Shingfield et al., 2003). The injector and detector temperatures were maintained at 255 ºC. Peaks were identified based on retention time comparisons with commercially available authentic standards (Nu-Chek Prep., Elysian, MN; Sigma-Aldrich, St. Louis, MO; and Larodan Fine Chemicals AB), cross referencing with chromatograms reported in the literature (Shingfield et al., 2003; Toral et al., 2010) and comparison with reference samples for which the FA composition was determined based on gas chromatography analysis of FAME and gas chromatography-mass spectrometry analysis of corresponding 4,4-dimethyloxazoline derivates (Toral et al., 2010).

5.1.4. Fatty Acid Analysis - Intramuscular Fat

Intramuscular fat in 10 g of finely minced LM (taken at the level of the 6th thoracic rib) samples was extracted with a mixture of chloroform and methanol (2:1, v/v; Folch et al., 1957) and 30 mg of lipids were converted to FAME by base catalyzed transesterification (Christie, 1982), using 0.5 mL of sodium methoxide in methanol 0.5 *N* and 1 mL of hexane

containing 19:0 as an internal standard. Gas chromatographic analysis was performed as described by Mele et al. (2014), using a Trace Thermo Finnigan GC equipped with a flame-ionization detector (ThermoQuest, Milan, Italy) and a 100-m fused silica capillary column (0.25 mm i.d., 0.25 μm, film thickness; SP 24056, Supelco, Bellefonte, PA) and helium as the carrier gas (1 mL/min). Total FAME profile in a 1 μL sample volume at a split ratio of 1:80 was determined using the following temperature gradient program: the oven temperature was programed at 150 °C and held for 1 min, then increased to 175 °C at 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 2 °C/min and held for 13 min. The injector and detector temperatures were 230 °C and 250 °C, respectively. Peaks were identified based on retention time comparisons with commercially available authentic standards (Nu-Chek Prep, and Sigma-Aldrich).

5.1.5. Statistical Analysis

Data on FA composition of rumen digesta and i.m. fat, and FA intake were analyzed by one-way ANOVA using the MIXED procedure of the SAS software package (version 9.4, SAS Inst. Inc., Cary, NC), with a model that included the fixed effect of experimental silage. Animals were nested within the treatment and used as the error term to contrast the effect of the silage. Means were separated using the 'pdiff' option of the 'lsmeans' statement of the MIXED procedure, and least square means are reported. Differences were declared significant at $P \leq 0.05$ and considered a trend towards significance when $0.05 < P \leq 0.10$.

5.2. Results

Data on animal growth performance and voluntary feed composition and intake have been reported by Copani et al., (in press). Briefly, the use of ensiled bioactive legumes improved grass silage fermentation and conservation and allowed, even with a restricted supplementation, high ADG, due to the association of high intakes with high feed conversion efficiency. Lambs fed the RC-containing silages had greater DMI than those receiving T and T-SF, as well as the greatest ADG, feed conversion efficiencies, BW and carcass weights.

5.2.1. Fatty Acid Composition of Feedstuffs and Fatty Acid Intake

Table 1 reports the FA composition of the five dietary treatments. The intake of individual FA was affected ($P < 0.05$) by the dietary treatment (Table 2). Particularly, the intake of linoleic acid (*cis*-9 *cis*-12 18:2) was greater in the groups receiving RC in comparison with T-SF treatment ($P = 0.001$). Similarly, the intake of α-linolenic acid (*cis*-9 *cis*-12 *cis*-15 18:3) was greater for T-SF-RC and SF-RC groups compared with T-SF treatment ($P = 0.003$).

Table 1. Fatty acid (FA) profile of the different 5 silage mixtures and of the barley grain and straw

	Silage[1]						
	T	T-SF	T-RC	T-SF-RC	SF-RC	Barley	Straw
Total FA, mg/g of DM	19.8	18.4	19.7	19.2	20.1	29.1	4.94
FA, g/100 g of total FA							
14:0	1.18	1.04	1.08	0.82	0.86	0.98	9.29
16:0	18.9	18.7	19.7	19.0	20.9	23.1	40.9
18:0	3.68	3.31	4.23	2.98	3.70	2.14	6.04
cis-9 18:1	3.10	2.60	2.64	2.51	2.20	11.8	8.49
cis-11 18:1	0.55	0.48	0.40	0.43	0.42	0.90	0.83
cis-9 *cis*-12 18:2	16.6	16.2	17.4	17.0	16.1	53.5	12.8
cis-9 *cis*-12 *cis*-15 18:3	51.6	52.9	49.6	52.8	49.7	6.13	5.92
20:0	0.68	1.01	0.94	0.97	1.33	0.22	3.02
22:0	1.39	1.30	1.26	0.82	1.74	0.25	4.37
24:0	0.70	0.85	0.98	0.91	1.15	0.17	2.42

[1]Silages were based on timothy (T), timothy + sainfoin 1:1 (T-SF), timothy + red clover 1:1 (T-RC), timothy + sainfoin + red clover 2:1:1 (T-SF-RC), or sainfoin + red clover 1:1 (SF-RC).

Table 2. Total fatty acid (FA) intake from each feed and intake of individual FA (data are expressed as g/d)

	Silage[1]						
	T	T-SF	T-RC	T-SF-RC	SF-RC	SEM	*P*-value
Total FA intake	21.7^{bc}	19.8^{c}	24.3^{ab}	23.8^{ab}	25.7^{a}	0.52	0.001
FA intake from silage	14.9^{bc}	13.2^{c}	17.3^{ab}	16.7^{ab}	18.6^{a}	0.46	<0.001
FA intake from barley	6.48	6.31	6.73	6.78	6.76	0.477	0.213
FA intake from straw	0.31^{b}	0.31^{b}	0.34^{a}	0.32^{b}	0.31^{b}	0.028	<0.001
14:0	0.27^{ab}	0.23^{c}	0.29^{a}	0.24^{bc}	0.26^{abc}	0.005	0.001
16:0	4.50^{bc}	4.11^{c}	5.18^{ab}	4.95^{ab}	5.68^{a}	0.119	<0.001
18:0	0.72^{b}	0.60^{b}	0.91^{a}	0.67^{b}	0.87^{a}	0.023	<0.001
cis-9 18:1	1.27^{ab}	1.13^{b}	1.30^{a}	1.26^{ab}	1.25^{ab}	0.019	0.029
cis-11 18:1	0.14^{a}	0.12^{b}	0.13^{ab}	0.14^{ab}	0.14^{a}	0.002	0.031
cis-9 *cis*-12 18:2	6.06^{ab}	5.61^{b}	6.74^{a}	6.59^{a}	6.75^{a}	0.114	0.001
cis-9 *cis*-12 *cis*-15 18:3	8.24^{ab}	7.50^{b}	9.16^{ab}	9.42^{a}	9.87^{a}	0.229	0.003
20:0	0.13^{c}	0.16^{bc}	0.19^{b}	0.19^{b}	0.28^{a}	0.009	<0.001
22:0	0.24^{bc}	0.20^{cd}	0.25^{b}	0.17^{d}	0.36^{a}	0.012	<0.001
24:0	0.13^{c}	0.13^{c}	0.19^{b}	0.17^{b}	0.24^{a}	0.008	<0.001

[a-c]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Silages were based on timothy (T), timothy + sainfoin 1:1 (T-SF), timothy + red clover 1:1 (T-RC), timothy + sainfoin + red clover 2:1:1 (T-SF-RC), or sainfoin + red clover 1:1 (SF-RC).

5.2.2. Fatty Acid Composition of Rumen Digesta

As shown in Table 3, different experimental silages induced different FA profiles in the rumen digesta of lambs, whereas changes in total FA concentration were of relatively small magnitude (8% less in T-SF than the mean of T, T-SF-RC and SF-RC; $P = 0.005$). Partial or total replacement of T grass with forage legumes was associated with greater concentrations of PUFA ($P < 0.001$) and lesser of MUFA ($P < 0.001$). However, the percentage of total saturates only tended to be decreased in the SF-RC treatment ($P = 0.055$), in agreement with variations in the content of 18:0 in the rumen ($P = 0.003$). In general, the T silage resulted in greater proportions of FA with 16 or less carbons, and lesser of ≥ C20 FA, compared with the other diets ($P < 0.05$), with the exception of the values of 16:0 and 20:4 n-3 ($P < 0.001$). Changes in the 18:1 and 18:2 isomers did not follow a common trend, showing different variations depending on the particular FA (Table 4).

The inclusion of forage legumes in the silage favored the accumulation of 18:3 n-3, *trans*-13+14, *trans*-15 and *trans*-16 18:1, and *cis*-12, *cis*-15 and *cis*-16 18:1 in rumen digesta ($P < 0.001$; Table 4), with the greatest concentrations being usually observed in SF-RC. This latter diet also led to the greatest percentage of 18:2 n-6 ($P < 0.001$), although values in T-RC did not differ from this or any other treatment. On the other hand, forage legumes decreased the content of *trans*-6+7+8, *trans*-9 and *trans*-11 18:1 (vaccenic acid, VA), and *trans*-9 *cis*-12 18:2 compared with feeding ensiled T alone ($P < 0.001$). In this regard, rumen concentration of *trans*-11 18:1 was especially low in SF-RC (approx. 3.3 times less than in T; $P < 0.001$). The T-RC and SF-RC treatments also decreased the percentage of *trans*-11 *cis*-15 18:2 ($P = 0.006$). However, the effects of forage legumes on

the conjugated 18:2 isomers were relatively marginal. For example, *cis*-9 *trans*-11 (rumenic acid, RA) and *trans*-10 *cis*-12 CLA were not affected. The same lack of differences occurred for *cis*-9, *cis*-11, *cis*-13 and *trans*-10 18:1 ($P > 0.10$).

In relation to branched-chain FA, forage legumes decreased their total concentration in the rumen digesta ($P < 0.001$), this effect being less marked in T-RC (Table 3). Nevertheless, this was not observed in all cases and thus reductions in *iso* 16:0 were similar for treatments containing SF, RC or both, whereas contents of *iso* 13:0, *anteiso* 13:0, and *anteiso* 17:0 were not modified ($P > 0.10$). Total odd-chain FA proportions were neither affected by feeding forage legumes ($P > 0.10$), although there were variations in some individual compounds, such as decreases in 19:0 and increases in 17:0, 21:0 and 23:0 ($P < 0.05$), when compared with the T silage.

Finally, regarding other less abundant FA, the percentage of *cis* 14:1 and 16:1 isomers was lesser ($P < 0.10$) with treatments containing SF, which also led to greater percentages of 20:3 n-3 and 22:3 n-3 compared with the T treatment ($P < 0.05$). All forage legumes increased 22:0 and 24:0 contents and decreased those of 20:4 n-3 ($P < 0.001$).

Table 3. Fatty acid (FA) composition of the rumen digesta from lambs fed the 5 different silage mixtures

	Silage[1]						
	T	T-SF	T-RC	T-SF-RC	SF-RC	SEM	*P*-value
Total FA, g/100 g of DM	3.38^{a}	2.98^{c}	3.01^{bc}	3.26^{ab}	3.19^{abc}	0.039	0.005
FA, g/100 g of total FA							
12:0	0.13^{a}	0.11^{b}	0.11^{ab}	0.10^{b}	0.10^{b}	0.003	0.021
13:0	0.05	0.05	0.05	0.05	0.04	0.002	0.120
iso 13:0	0.07	0.06	0.08	0.07	0.07	0.003	0.294
anteiso 13:0	0.02	0.01	0.01	0.01	0.02	0.001	0.132
14:0	1.06^{a}	0.89^{b}	0.93^{b}	0.82^{b}	0.83^{b}	0.022	<0.001
iso 14:0	0.20^{a}	0.16^{b}	0.15^{b}	0.14^{bc}	0.12^{c}	0.006	<0.001
cis-9 14:1	0.025^{a}	0.016^{bc}	0.020^{ab}	0.018^{bc}	0.014^{c}	0.001	<0.001
15:0	1.03^{ab}	0.90^{b}	1.12^{a}	0.95^{b}	0.92^{b}	0.024	0.026
iso 15:0	0.41^{a}	0.28^{bc}	0.33^{b}	0.27^{c}	0.24^{c}	0.013	<0.001
anteiso 15:0	0.76^{a}	0.53^{bc}	0.61^{b}	0.52^{bc}	0.45^{c}	0.023	<0.001
16:0	16.4^{d}	17.6^{bc}	17.8^{b}	17.2^{c}	18.9^{a}	0.15	<0.001
iso 16:0	0.33^{a}	0.20^{b}	0.22^{b}	0.22^{b}	0.19^{b}	0.012	<0.001
cis-6+7 16:1	0.06^{a}	0.05^{bc}	0.05^{ab}	0.04^{c}	0.04^{c}	0.002	<0.001
cis-9 16:1	0.30^{a}	0.18^{c}	0.24^{b}	0.22^{b}	0.18^{c}	0.009	<0.001
17:0	0.65^{b}	0.69^{b}	0.80^{a}	0.64^{b}	0.66^{b}	0.012	<0.001
iso 17:0	0.29^{a}	0.16^{c}	0.23^{b}	0.20^{b}	0.13^{c}	0.010	<0.001
anteiso 17:0	0.10	0.11	0.10	0.09	0.10	0.004	0.551

18:0	41.1[a]	40.2[a]	39.1[a]	40.6[a]	34.5[b]	0.64	0.003
iso 18:0	0.13[a]	0.11[ab]	0.10[ab]	0.09[b]	0.08[b]	0.005	0.010
Σ *cis* 18:1	4.68	4.27	4.58	4.19	5.06	0.124	0.161
Σ *trans* 18:1	11.41[a]	8.42[b]	6.82[c]	8.28[b]	6.81[c]	0.330	<0.001
Σ CLA	0.52[bc]	0.64[a]	0.47[c]	0.58[ab]	0.59[ab]	0.015	0.002
Σ non-conjugated 18:2	9.11[b]	8.64[b]	10.05[ab]	9.27[b]	11.42[a]	0.288	0.012
19:0[2]	0.53[a]	0.40[b]	0.32[c]	0.39[b]	0.25[c]	0.019	<0.001
20:0	0.66[c]	0.96[a]	0.84[b]	0.84[b]	0.95[a]	0.019	<0.001
20:2 n-6	0.03[c]	0.03[bc]	0.04[ab]	0.03[bc]	0.04[a]	0.001	0.004
20:3 n-3	0.04[c]	0.06[a]	0.04[bc]	0.05[ab]	0.06[a]	0.002	0.001
20:4 n-6	0.05	0.04	0.04	0.04	0.04	0.001	0.278
20:4 n-3	0.28[a]	0.20[b]	0.19[b]	0.19[b]	0.13[c]	0.010	<0.001
20:5 n-3	0.01	0.01	<0.01	<0.01	<0.01	0.001	0.925
21:0	0.05[c]	0.09[ab]	0.08[b]	0.08[b]	0.11[a]	0.005	<0.001
22:0[3]	0.67[b]	0.81[a]	0.80[a]	0.78[a]	0.78[a]	0.009	<0.001
22:2 n-6	0.02	0.02	0.02	0.02	0.01	0.001	0.792
22:3 n-3	0.02[c]	0.03[ab]	0.03[ab]	0.02[bc]	0.03[a]	0.002	0.020
22:5 n-3	0.03	0.03	0.03	0.03	0.02	0.002	0.871
22:6 n-3	0.04	0.04	0.04	0.04	0.03	0.004	0.889
23:0	0.12[d]	0.34[b]	0.28[bc]	0.27[c]	0.43[a]	0.019	<0.001
24:0	0.56[d]	0.96[b]	0.84[c]	0.83[c]	1.06[a]	0.030	<0.001
Summary							
Σ SFA	67.2	67.1	68.3	67.4	63.2	0.59	0.055
Σ MUFA	18.0[a]	14.7[b]	13.5[b]	14.7[b]	14.3[b]	0.33	<0.001
Σ PUFA	14.8[c]	18.1[b]	18.1[b]	18.1[b]	22.4[a]	0.59	<0.001

Σ odd-chain FA	2.46	2.49	2.68	2.43	2.46	0.032	0.104
Σ branched-chain FA	3.04[a]	2.09[c]	2.52[b]	2.14[bc]	2.00[c]	0.086	<0.001

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Silages were based on timothy (T), timothy + sainfoin 1:1 (T-SF), timothy + red clover 1:1 (T-RC), timothy + sainfoin + red clover 2:1:1 (T-SF-RC), or sainfoin + red clover 1:1 (SF-RC).

[2]Coelutes with *trans*-11 *trans*-15 18:2.

[3]Contains 20:3 n-6 as a minor component.

Table 4. 18-carbon unsaturated fatty acid (FA) composition of the rumen digesta in lambs fed the 5 different silage mixtures

	Silage[1]						
	T	T-SF	T-RC	T-SF-RC	SF-RC	SEM	*P*-value
FA, g/100 g of total FA							
cis-9 18:1	3.64	3.04	3.33	2.81	3.51	0.113	0.115
cis-11 18:1	0.41	0.41	0.40	0.40	0.4	0.008	0.853
cis-12 18:1	0.20^{c}	0.27^{b}	0.26^{bc}	0.30^{ab}	0.35^{a}	0.012	<0.001
cis-13 18:1	0.11	0.12	0.12	0.13	0.13	0.004	0.497
cis-15 18:1	0.20^{d}	0.29^{c}	0.30^{bc}	0.37^{ab}	0.44^{a}	0.018	<0.001
cis-16 18:1	0.11^{c}	0.16^{b}	0.16^{b}	0.18^{b}	0.22^{a}	0.007	<0.001
trans-4 18:1	0.10^{a}	0.09^{ab}	0.09^{a}	0.09^{a}	0.08^{b}	0.002	0.032
trans-5 18:1	0.06	0.07	0.07	0.06	0.05	0.002	0.090
trans-6+7+8 18:1	0.37^{a}	0.29^{bc}	0.26^{c}	0.32^{b}	0.27^{c}	0.010	<0.001
trans-9 18:1	0.29^{a}	0.23^{bc}	0.21^{bc}	0.25^{b}	0.20^{c}	0.008	<0.001
trans-10 18:1	0.31	0.29	0.27	0.28	0.29	0.008	0.613
trans-11 18:1	8.24^{a}	4.83^{b}	3.50^{cd}	4.36^{bc}	2.49^{d}	0.361	<0.001
trans-12 18:1	0.65	0.63	0.58	0.68	0.68	0.013	0.087
trans-13+14 18:1	0.77^{d}	1.13^{bc}	1.00^{cd}	1.27^{b}	1.58^{a}	0.058	<0.001
trans-15 18:1[2]	0.71^{c}	0.89^{b}	0.91^{b}	1.01^{b}	1.24^{a}	0.036	<0.001
trans-16 18:1	0.61^{d}	0.85^{c}	0.86^{bc}	0.98^{b}	1.17^{a}	0.035	<0.001
cis-9 *cis*-12 18:2	7.63^{b}	7.37^{b}	8.89^{ab}	7.81^{b}	10.33^{a}	0.290	0.002
cis-9 *trans*-12 18:2	0.03	0.02	0.02	0.02	0.03	0.001	0.237
trans-9 *cis*-12 18:2	0.10^{a}	0.07^{b}	0.06^{b}	0.07^{b}	0.06^{b}	0.004	0.000

trans-9 *trans*-12 18:2	0.04	0.03	0.03	0.04	0.03	0.002	0.197
trans-11 *cis*-15 18:2	1.23[a]	1.05[ab]	0.96[b]	1.24[a]	0.89[b]	0.040	0.006
cis-9 *trans*-11 CLA[3]	0.24	0.27	0.20	0.24	0.23	0.010	0.404
trans-9 *cis*-11 CLA	0.06	0.10	0.06	0.08	0.08	0.005	0.089
trans-10 *cis*-12 CLA	<0.01	<0.01	<0.01	<0.01	<0.01	0.001	0.131
trans-11 *trans*-13 CLA	0.13[ab]	0.16[a]	0.10[b]	0.14[a]	0.16[a]	0.007	0.005
other *trans trans* CLA[4]	0.09	0.09	0.09	0.10	0.09	0.002	0.399
cis-6 *cis*-9 *cis*-12 18:3	0.04[c]	0.07[a]	0.05[bc]	0.06[ab]	0.07[a]	0.003	0.002
cis-9 *cis*-12 *cis*-15 18:3[5]	4.80[c]	8.82[b]	7.55[b]	8.13[b]	10.62[a]	0.406	<0.001

[a-d]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Silages were based on timothy (T), timothy + sainfoin 1:1 (T-SF), timothy + red clover 1:1 (T-RC), timothy + sainfoin + red clover 2:1:1 (T-SF-RC), or sainfoin + red clover 1:1 (SF-RC).

[2]Coelutes with *cis*-10 18:1.

[3]Contains *trans*-8 *cis*-10 CLA and *trans*-7 *cis*-9 CLA as minor components.

[4]Sum of *trans*-9 *trans*-11 CLA, *trans*-10 *trans*-12 CLA, and *trans*-8 *trans*-10 CLA.

[5]Contains *cis*-11 20:1 as a minor component.

5.2.3. Fatty Acid Composition of Intramuscular Fat

Table 5 reports the dietary effect on FA composition of i.m. fat. The proportion of total i.m. fat was not affected by the dietary treatment. The dietary treatment tended to affect the proportion of MUFA ($P = 0.081$) and of PUFA ($P = 0.079$) which were found, respectively, at the greatest and least numerical value in the T group. Within the class of PUFA, the sum of n-3 FA was lesser in T and T-SF groups compared with the mixture of legumes without grass (SF-RC; $P < 0.001$ and $P = 0.008$, respectively). The latter group also had a lesser n-6 to n-3 ratio than the T-SF ($P = 0.01$), while intermediate values were found for the other groups. The proportion of both eicosapentaenoic acid (*cis*-5 *cis*-8 *cis*-11 *cis*-14 *cis*-17 20:5; EPA) and docosahexaenoic acid (*cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:6; DHA) were almost two-fold greater in muscle from lambs in the SF-RC group compared with the T treatment ($P = 0.017$ and $P = 0.007$, respectively). In addition, the proportion of DHA was greater in the muscle from lambs in the T-SF-RC than in the T group ($P = 0.013$).

Regarding the percentages of 18-carbon FA, the *trans*-11 18:1 was greater in the i.m. fat from lambs given the T silage, in comparison with the treatment containing RC (T *vs* T-RC and T-SF-RC, $P = 0.028$ and 0.011 respectively; T *vs* SF-RC, $P < 0.001$). None of the other identified *trans*-18:1 FA were affected by the dietary treatment. The sum of the *trans*- 18:1 FA was greater in the i.m. fat from T-fed lambs as compared with the T-RC ($P = 0.02$) and SF-RC ($P < 0.001$) treatments.

No effect of the dietary treatment ($P > 0.10$) was found on the proportion of *cis*-9 18:1 and *cis*-9 *cis*-12 18:2). Rumenic acid (*cis*-9 *trans*-11 CLA was detected at greater percentage in the LM from the animals fed the T silage as compared

with the T-SF-RC treatment (P = 0.004). Contrarily, *cis*-9 *cis*-12 *cis*-15 18:3 was found at a greater concentration in the muscle from SF-RC group, compared with the other treatments (P < 0.001). Furthermore, the T-RC treatment had a greater proportion of α-linolenic acid than the T group (P = 0.03).

The experimental silages had no effect on the concentration of odd- and branched-chain FA in muscle (P > 0.10), with the exception of a decrease in *trans*-10 17:1 percentage in SF-RC group, compared with the control T silage (P = 0.017).

Table 5. Fatty acid (FA) composition of intramuscular fat (IMF) in lambs fed the 5 different silage mixtures

	Silage[1]						
	T	T-SF	T-RC	T-SF-RC	SF-RC	SEM	*P*-value
Total IMF, g/100 g of muscle	2.64	2.11	2.26	2.21	2.24	0.127	0.734
FA, g/100 g of total FA							
10:0	0.17	0.16	0.17	0.17	0.18	0.005	0.755
12:0	0.21	0.21	0.19	0.18	0.20	0.009	0.820
13:0	0.02	0.02	0.01	0.02	0.02	0.001	0.430
14:0	2.78	2.55	2.56	2.52	2.64	0.088	0.890
cis-9 14:1	0.10	0.09	0.09	0.09	0.09	0.006	0.993
15:0	0.41	0.42	0.39	0.38	0.40	0.010	0.748
iso 15:0	0.13	0.13	0.12	0.12	0.11	0.004	0.109
anteiso 15:0	0.14	0.15	0.13	0.12	0.12	0.005	0.102
16:0	25.2	24.4	25.1	24.6	25.5	0.25	0.683
cis-9 16:1	1.52	1.51	1.43	1.50	1.33	0.038	0.521
17:0	1.15	1.17	1.20	1.19	1.26	0.015	0.173
iso 17:0	0.42	0.43	0.40	0.40	0.35	0.012	0.273
anteiso 17:0	0.53	0.54	0.51	0.49	0.44	0.012	0.055
trans-9 17:1	0.19[a]	0.18[ab]	0.17[ab]	0.17[ab]	0.15[b]	0.004	0.017
18:0	16.7	17.3	16.7	16.5	17.4	0.27	0.804
cis-9 18:1	38.9	38.2	39.1	39.2	37.5	0.01	0.374

cis-11 18:1	0.97	1.04	0.95	1.01	0.92	0.009	0.314
cis-12 18:1	0.13	0.36	0.15	0.13	0.17	0.053	0.444
cis-13 18:1	0.09	0.09	0.09	0.08	0.08	0.044	0.910
cis-15 18:1	0.31	0.17	0.16	0.20	0.16	0.010	0.299
cis-16 18:1	0.20^{b}	0.21^{b}	0.24^{ab}	0.21^{ab}	0.28^{a}	0.078	0.010
Σ *cis* 18:1	40.6	40.1	40.7	40.8	39.1	0.317	0.373
trans-6+8 18:1	0.17	0.16	0.14	0.14	0.12	0.019	0.077
trans-9 18:1	0.22	0.18	0.18	0.17	0.16	0.044	0.273
trans-10 18:1	0.64	0.53	0.41	0.67	0.38	0.002	0.327
trans-11 18:1	1.30^{a}	1.20^{ab}	0.94^{bc}	0.99^{bc}	0.77^{c}	0.009	<0.001
trans-12 18:1	0.23	0.24	0.22	0.24	0.21	0.007	0.922
Σ *trans* 18:1	2.55^{a}	2.30^{ab}	1.89^{bc}	2.20^{abc}	1.64^{c}	0.306	<0.001
cis-9 *cis*-12 18:2	2.58	3.29	2.95	3.15	3.25	0.107	0.200
cis-9 *trans*-11 CLA	0.51^{a}	0.41^{ab}	0.39^{ab}	0.43^{ab}	0.34^{b}	0.016	0.009
cis-6 *cis*-9 *cis*-12 18:3	0.02	0.03	0.02	0.03	0.02	0.001	0.546
cis-9 *cis*-12 *cis*-15 18:3	0.71^{c}	1.02^{bc}	1.20^{b}	1.14^{bc}	1.74^{a}	0.072	<0.001
20:0	0.09	0.10	0.10	0.09	0.10	0.003	0.564
cis-11 20:1	0.08	0.07	0.07	0.08	0.07	0.002	0.670
20:2 n-6	0.02	0.03	0.02	0.03	0.03	0.002	0.264
20:3 n-6	0.06	0.07	0.07	0.07	0.07	0.004	0.572
20:3 n-3	0.01^{b}	0.01^{b}	0.02^{b}	0.02^{b}	0.03^{a}	0.002	<0.001
20:4 n-6	0.49	0.68	0.60	0.55	0.56	0.031	0.410
20:5 n-3	0.10^{b}	0.15^{ab}	0.19^{ab}	0.17^{ab}	0.21^{a}	0.012	0.020
22:0	0.02	0.03	0.02	0.03	0.03	0.001	0.754
22:4 n-6	0.04	0.05	0.04	0.03	0.03	0.002	0.195

22:5 n-6	0.02	0.02	0.02	0.03	0.02	0.001	0.105
22:5 n-3	0.19	0.23	0.27	0.26	0.27	0.012	0.116
22:6 n-3	0.04[b]	0.05[ab]	0.06[ab]	0.078[a]	0.08[a]	0.004	0.004
Summary							
Σ SFA	48.0	47.6	47.6	46.9	48.8	0.28	0.290
Σ MUFA	44.8	44.1	44.2	44.7	42.2	0.33	0.081
Σ PUFA	5.07	6.19	6.00	6.15	6.78	0.196	0.079
Σ n-6 FA	3.59	4.77	4.11	4.25	4.37	0.165	0.241
Σ n-3 FA	1.36[b]	1.63[b]	1.90[ab]	1.87[ab]	2.48[a]	0.094	<0.001
n-6/n-3 ratio	2.79[ab]	3.17[a]	2.18[ab]	2.32[ab]	1.78[b]	0.143	0.006
Σ odd- and branched-chain FA	2.80	2.86	2.76	2.71	2.69	0.045	0.754

[a-c]Within a row, different superscripts indicate significant differences ($P < 0.05$).

[1]Silages were based on timothy (T), timothy + sainfoin 1:1 (T-SF), timothy + red clover 1:1 (T-RC), timothy + sainfoin + red clover 2:1:1 (T-SF-RC), or sainfoin + red clover 1:1 (SF-RC).

5.4. Discussion

It has been widely demonstrated that RC, particularly if offered in the form of silage, can interfere with the ruminal lipolysis and, consequently, with the ruminal BH (Lee et al., 2006; Lee, 2014). This activity has been related to the enzyme PPO (Van Ranst et al., 2011). It is also well known that ruminal BH may be altered by dietary tannins (Vasta et al., 2009b; Vasta and Luciano, 2011; Carreño et al., 2015). Reducing the extent of ruminal BH would result in an increase of PUFA and of the beneficial intermediate VA, which would be absorbed in the small intestine and would reach the muscle (Scollan et al., 2006). Therefore, strategies to reduce BH have been studied in recent years. Both RC PPO (Lee, 2014) and tannins (Vasta and Luciano, 2011) have been indicated among the bioactive compounds that may inhibit BH. The effect of RC PPO on ruminal BH is supposed to be exerted by protecting the glycerol-based lipids from plant and microbial lipases (Lee et al., 2007), while tannins affect ruminal BH through an effect on the ruminal ecosystem (Vasta et al., 2010; Carreño et al., 2015). Here we wanted to test if the effects of PPO-containing RC and of tannin-containing SF on ruminal BH could be additive, with implications for the i.m. FA composition.

In agreement with this hypothesis, we have found that, despite similar intakes of PUFA, lambs offered SF-RC had greater proportions of these FA in the ruminal digesta, as compared with the animals fed the T silage, with intermediate values detected in the ruminal digesta from animals in which half of the T silage was replaced by either RC, SF or both. The fact that the same pattern was not found in the i.m. fat (although there was a clear tendency; P = 0.079) could be explained by the muscle metabolism that

tends to desaturate SFA and some MUFA respectively to MUFA and PUFA (Smith et al., 2006). This hypothesis is supported by the greater concentrations of *cis*-9 18:1, *cis*-9 16:1 and *cis*-9 *trans*-11 CLA in muscle than in rumen digesta, which would derive to an important extent from the activity of Δ^9-desaturase in ruminant tissues (Palmquist et al., 2004). The activity of this enzyme also appeared to explain treatment effects on muscle *cis*-9 *trans*-11 CLA proportions, which were not observed in rumen digesta and would mostly reflect the differences in substrate (*trans*-11 18:1, VA) availability.

The VA is produced in the rumen as an intermediate of the BH of 18:3 n-3 and 18:2 n-6 (Lourenço et al., 2010). Although the intake of these latter FA was similar in T- and SF-RC-fed lambs, the ruminal digesta from the lambs fed T showed a concentration of VA more than three times greater as compared with the animals fed the SF-RC mixture. This was likely dependent on an impairment of ruminal BH in the SF-RC group rather than on an increased saturation of VA to 18:0 in the rumen digesta. Furthermore, the decrease in rumen *trans*-11 18:1 concentration in this treatment (SF-RC) was probably intensified by potential shifts in the BH pathways towards an increased formation of other 18:1 intermediates, as supported by the greater percentages of *trans*-13+14, -15 and -16 18:1 and *cis*-15 and -16 18:1, which would mostly represent intermediate metabolites of 18:3 n-3 BH (Lourenço et al., 2010; Shingfield et al., 2010). On the contrary, the concentration of *trans*-9, -10 and -12 18:1 and *cis*-11 18:1, which are also intermediates of 18:2 n-6 BH (Shingfield et al., 2010), did not follow this general trend. Overall, the reasons for these inconsistent results on 18:3 n-3 and 18:2 n-6 BH are not readily apparent, which obliges to further investigate the potential mechanisms

responsible for the effects of plant secondary compounds (particularly PPO and tannins) on ruminal FA metabolism.
According to literature, PPO in RC impairs the whole onset of ruminal BH (Van Ranst et al., 2011), while it seems that tannins might exert an effect on the last step (i.e., *trans*-11 18:1 to 18:0), with an accumulation of VA in the rumen (Khiaosa-Ard et al., 2009). This consideration may help to explain why, even if lambs from the T-SF group ate less linoleic and α-linolenic acids, they had more VA in their ruminal digesta as compared with the T-RC animals: the effect of tannins from SF was probably responsible for a greater accumulation of VA because its saturation to 18:0 was impaired. This result was not confirmed in the LM, where VA was detected at comparable amounts between T-SF and T-RC groups. Again, this may be due to the confounding effect of muscle metabolism on FA.
Furthermore, 18:3 n-3 was ingested at similar amounts by the animals receiving SF-RC and those given the T silage and was found at more than double concentrations in both ruminal digesta and muscle from animals fed the legume-containing diet, compared with those given T, indicating that ruminal BH proceeded at larger extent in the rumen of the T-fed animals. In the case of α-linolenic acid, the result of ruminal digesta reflects perfectly those of LM and this was expected because 18:3 n-3 is not further metabolized in the muscle. An intermediate of the ruminal BH of this PUFA is the *trans*-11 *cis*-15 18. This FA was found at a greater concentration in the ruminal digesta from lambs given T as compared with the animals given T-RC or SF-RC. Again, considering the similar intakes of ALA, the accumulation of this FA was probably due to a larger extent of ruminal BH in the lambs offered timothy. Moreover, an effect of RC on the rumen microbiota is also feasible. Huws

et al. (2010), comparing steers fed RC, as opposed to grass silage, found that the lipolytic bacterium population *Anaerovibrio lipolytica* was greater in the rumen of grass-fed animals as compared to the RC-fed onces. This bacterium is responsible for the hydrolysis of di- and triglycerides to unesterified FA available for BH. Thus, in our case, the reduced BH in lambs receiving RC could have occurred also as a consequence of decreased bacterial lipolysis.

Greater proportions of *iso*-FA have been associated with an enrichment of cellulolytic bacteria (Fievez et al., 2012). The greater *iso* 15:0 and *iso* 17:0 in the ruminal digesta from T-fed lambs compared with all the other groups may reinforce the hypothesis that cellulolytic bacteria where present at greater concentrations in the rumen of T-fed animals. Consistently with this hypothesis, Huws et al. (2010) found a greater DNA concentration of the cellulolytic bacteria *R. flavefaciens* and *F. succinogenes* in the rumen of steers fed grass silage as compared with those receiving RC silage. In the present study, the *iso* 17:0 was present at greater concentrations in the ruminal digesta from T-RC lambs as compared to the ruminal digesta of the animals fed the T-SF diet, despite a slightly lesser NDF content of the former silage mixture (Copani et al., 2015). This is the first study in which the ruminal digesta from animals fed SF is analyzed for the FA composition, but a possible explanation of this result is that the SF tannins could have exerted an inhibitory effect on the cellulolytic bacteria similar to that exerted by tannins from other plant species (Vasta et al., 2010). Despite the observed variations in odd- and branched-chain FA in rumen digesta, experimental treatments did not affect their proportion in muscle, which may be attributed to potential

differences in the absorption, blood transport and *de novo* synthesis of these FA in body tissues (Fievez et al., 2012).
It is well known that the extent of ruminal BH is positively correlated with the NDF level of the diet because of a favorable environment to cellulolytic bacteria (Sackman et al., 2003), but also as a result of a longer retention time of the feedstuff in the rumen (Lourenço et al., 2010) and, in our experiment, the NDF content was greater ($P < 0.001$) in the T silage as compared with that of the mixtures and was the least ($P < 0.001$) in the SF-RC silage (Copani et al., 2015).
The EPA and DHA in ruminant meat derive both from the diet and from α-linolenic acid metabolism via sequential desaturation and chain-elongation steps mediated by liver enzymes (Brenner et al., 1989). In the present study, the dietary treatment did not affect the concentration of these FA in the rumen. Therefore, the greater proportion of EPA and DHA found in LM of SF-RC lambs, as compared to T silage, seems to follow the greater availability of their precursor (α-linolenic acid) in the rumen digesta of the SF-RC group.
Finally, according to Lee et al. (2006) a decline of the n-6 to n-3 ratio in the i.m. fat could be due to a greater protection of 18:3 n-3 than 18:2 n-6 related to its preferential incorporation into photosynthetic structures, such as the chloroplasts, which also contain the PPO enzyme within RC tissues. According to HMSO (1994), this ratio should not exceed the value of 4 for human nutrition. In our case, as all the animal were fed with forages, the overall value of the ratio was found to be well below the reference value, regardless of the dietary treatment.

5.5. Conclusions

From this experiment, we can conclude that the beneficial effects on ruminal BH that have been so far described for RC silage can be reported as well for SF silage. The bioactive compounds from the two plant species are chemically different but their biological effects are similar and are clearly additive. From our results, a silage composed by a mixture of RC and SF is an excellent forage for growing lambs. Furthermore, the i.m. FA composition from the animals fed the SF-RC mixture was similar to that described in previous literature from animals grazing green pasture (Aurousseau et al., 2004) with the advantage that the silage could be available during the whole year. Considering the strong effects on i.m. FA composition, further studies on the effects of legume silages on meat sensory and technological quality are advisable.

6. Experiment 2

Intramuscular fatty acids in lambs supplemented with different tannin extracts.

* Under review

6.1. Introduction

Tannins are water-soluble phenolic compounds naturally occurring in plants (Patra & Saxena, 2009) that can interact with cell wall bacteria and complex their external secreted enzymes resulting in antimicrobial activity (Scalbert et al., 1991). In the rumen, tannins can impair biohydrogenation (BH) influencing the fatty acid (FA) profile of rumen liquid (Vasta, Makkar, Mele & Priolo, 2009a) and digesta (Carreño, Hervás, Toral, Belenguer & Frutos, 2015). Thus, tannins can be exploited to favourably modify FA composition of ruminant products (for a review, see Vasta & Luciano, 2011; Morales & Ungerfeld, 2015). However, controversial results on the use of tannins for this purpose have been reported so far. *In vitro* trials showed that tannins can interfere with several steps along the BH process (Khiaosa-Ard et al., 2009; Jayanegara, Kreuzer & Leiber, 2012). In the same way, *in vivo* experiments reported varying results on the FAs accumulation in the intramuscular fat of ruminant meat in response to tannins supplementation (Vasta et al., 2009b; Willems, Kreuzer & Leiber, 2014; Brogna et al., 2014).

Such inconsistency could lie in the type of tannins which belong to a heterogeneous group of compounds. Chemically, tannins can be classified into hydrolysable (HT) and condensed (CT). The former are polymers of gallic or ellagic acid esterified to a polyhydroxylated core, while the CT can be distinguished into proanthocyanidines (polymers of antocyanidines and catechin flavan-3-ols) and leucoanthocyanidines (dimmers of flavan-3, 4-diolflavonoids; Reed, 1995). In the rumen, tannins can be differently metabolized and the capacity to interact with

feeds, bacteria and microbial molecules may depend more on their chemical characteristics than on the concentration (Waghorn & McNabb, 2003). Differences in the chemistry of dietary tannins could, therefore, exert different effects on the fatty acid composition of ruminant meat. To date, *in vitro* studies focused on the effect of a wide selection of tannin-rich plant on rumen metabolism, including fatty acid metabolism (Makkar, Blümmel & Becker, 1995; Getachew et al., 2008; Bhatta et al., 2009; Vasta et al., 2009a; Buccioni, Minieri, Rapaccini, Antongiovanni & Mele, 2011). Nevertheless, most of the *in vivo* published studies have tested the effects of the dietary administration to ruminants of one specific plant extract (Vasta et al., 2009b; Toral, Hervàs, Bichi, Belenguer & Frutos, 2013) on the fatty acid composition of meat and milk, while very few experiments have compared different extracts (Buccioni et al., 2015a). Additionally, most of the studies performed so far supplemented the diets with only quebracho (*Schinopsis lorentzii*) and/or chestnut (*Castanea sativa*) extracts as the source of condensed and hydrolyzable tannins, respectively. However, several plant extracts are commercially available and are characterized by possessing specific and different chemical groups of tannins.

Therefore, the aim of the present study was to evaluate and compare the effect of the dietary administration of different hydrolysable and condensed tannin-rich extracts on the fatty acid composition of lamb meat. To the best of our knowledge, this is the first experiment in which chestnut (*Castanea sativa*) extract as a source of HT, mimosa (*Acacia mearnsii*) and gambier (*Uncaria gambir*) extracts as a source of CT are used *in vivo* to study their effects on the fatty acid composition of lamb meat.

6.2. Materials and methods

6.2.1. Animals and diets

This study was carried out indoors in the facilities of the University of Catania (37°24'45.1"N 15°03'18.4"E) from October 2015 to January 2016. Animals were handled in accordance with the European Union Directive No.63/2010 on the protection of animals used for scientific purposes (EC, 2010). Thirty-six 2-month-old male Sarda × Comisana lambs (initial BW 19.6 ± S.D. 2.06 kg) were reared in individual pens bedded with deep straw and fresh water was always available for the duration of the trial. In a complete randomized design, lambs were divided into 4 groups (n = 9) that were balanced for initial BW. One animal from the control group died for reasons unrelated to the experimental trial. The control group (CO) received a barley-based concentrate diet containing on an "as fed" basis: barley (480 g/kg), wheat bran (230 g/kg), dehydrated alfalfa hay (150 g/kg), soybean meal (100 g/kg), molasses (20 g/kg) and mineral-vitamin premix (20 g/kg), the experimental groups received the same diet as the control lambs with the addition of 4% (as fed) of three different tannin extracts from chestnut (CH), mimosa (MI) and gambier (GA). Extracts of chestnut (commercial name: Nutri-P, minimum tannin content 75%), and mimosa (commercial name: Mimosa OP, minimum tannin content 65%) were purchased from Silvateam S.p.A. (San Michele M.vì, Cuneo, Italy) while gambier extract (commercial name: Retan FGC, minimum tannin content 48%) was purchased from Figli di Guido Lapi S.pA. (Castelfranco di Sotto, Pisa, Italy); information about the tannin percentage in each extract were provided by the manufacturers. All the concentrates were in the form of a

pellet and the tannins extracts were added before pelleting at the temperature of 40°C. Table 1 reports the chemical and fatty acid composition of the experimental diets.

Table 1 – Chemical and fatty acid composition of the experimental diets

	CO	CH	MI	GA
Chemical composition				
Dry matter (%)	90.2	89.4	89.4	89.8
Crude Protein	13.12	14.5	14.8	15.3
Ether Extract	2.59	2.60	2.53	2.59
NDF	28.3	26.6	26.2	25.9
ADF	15.4	14.1	13.4	14.2
ADL	3.21	3.22	2.69	3.16
Ash	5.85	6.33	6.09	6.40
Fatty acid composition				
14:0	0,03	0,03	0,07	0,04
16:0	4,36	3,87	4,61	4,32
cis-9 16:1	0,04	0,03	0,07	0,04
18:0	0,45	0,40	0,42	0,48
18:1 *n*-9	3,86	3,37	3,94	3,95
cis-9 *cis*-12 18:2	12,2	11,2	12,9	12,8
cis-9 *cis*-12 *cis*-1518:3	1,26	1,16	1,28	1,32
20:0	0,09	0,08	0,15	0,07
22:0	0,09	0,10	0,15	0,09

CO: Control, CH: Chestnut, MI: Mimosa, GA: Gambier.
NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: neutral detergent lignin.

The experiment lasted a total of 84 days. During a 9-day-adaptation period, the animals received the same commercial starter concentrate used for weaning, with gradual increases in the proportion of the experimental concentrates. During the experimental period (75 days), the lambs were fed *ad libitum* with their respective diets. Daily, before morning feeding, individual intakes were measured

according to the refusal left by each animal. Weekly, lambs were weighed before morning feeding and samples of each experimental diets were collected. At the end of the experimental period, all the lambs were slaughtered on the same day at a commercial abattoir according to the European Union welfare guidelines. All the animals had free access to their respective diet and water until approximately 3 hours before slaughter. Each carcass was immediately weighted to record the hot carcass weight. After 24-h-storage at 4 °C, carcasses were halved and the entire *longissimus thoracis et lumborum muscle* (LTL) was removed from the right side, packed under vacuum and stored at -80 °C until analysis.

6.2.2. Analyses of feedstuffs

Feed samples of each diet, collected during the trial, were pooled together and analysed for neutral detergent fibre (NDF) according to Van Soest, Robertson, & Lewis (1991). Furthermore, crude protein, crude fat (ether extract) and ash, were analysed according to AOAC (1995). Lipid from the control and experimental diets was extracted and converted to fatty acid methyl esters (FAME) with a 1-step procedure using chloroform (Sukhija & Palmquist, 1988) and 2% (v/v) sulfuric acid in methanol (Shingfield et al. 2003). Gas chromatographic analysis was carried out as later described for muscle. Fatty acids were expressed as g/100g of dry matter.

6.2.3.Muscle fatty acid analysis

Intramuscular fat was extracted in double from 10 g of finely minced LTL samples with a mixture of chloroform and methanol (2:1, v/v) as described by Folch, Lees, & Stanley (1957) and 30 mg of lipids were converted to FAME by base-catalyzed transesterification (Christie, 1982), using 0.5 mL of sodium methoxide in methanol 0.5 *N* and 1 mL of hexane. Nonadecanoic acid was used as an internal standard. Gas chromatographic analysis was carried out with a GC 8000 Top ThermoQuest (Milan, Italy) equipped with a flame ionization detector (FID; ThermoQuest, Milan, Italy) and 100-m high-polar fused silica capillary column (WCOT-fused silica CP-Select CB for FAME Varian, Middelburg, the Netherlands; 100m×0.25mm i.d.; film thickness 0.25 μm). Helium was the carrier gas at a constant flow of 1 mL/min. Total FAME profile in a 1 μL sample volume (2 μL for the feed lipid) at a split ratio of 1:80 was determined using the following GC conditions: the oven temperature was programmed at 40 °C and held for 4 min, then increased to 120 °C at 10 °C/min, held for 1 min, then increased up to 180 °C at 5 °C/min, held for 18 min, then increased up to 200 °C at 2 °C/min, held for 15 min, and then increased up to 230 °C at 2 °C/min, held for 19 min. The injector and detector temperatures were at 270 °C and 300 °C, respectively. FAME identification was based on a standard mixture of 52 Component FAME Mix (Nu-Chek Prep Inc., Elysian, MN, USA) and 77 individual FAME standards (Larodan Fine Chemicals, Malmo, Sweden). The identification of 18:1 and 18:2 isomers was based on commercial standard mixtures (Larodan Fine Chemicals) and on chromatograms published by Kramer, Hernandez, Hernandez, Kraft, & Dugan (2008) and Alves & Bessa (2007). For each FA, response factors to

FID and inter- and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Bureau of Reference, Brussels, Belgium). Fatty acids were expressed as g /100g of total methylated fatty acids.

6.2.4. Statistical analysis

Data of animal performance and intramuscular FA composition were analyzed by means of one-way analysis of variance using the statistical software Minitab, version 16 (Minitab Inc, State College, PA). The differences between means were separated using the Tukey's adjustment for pairwise comparisons. Differences were declared significant at $P \leq 0.05$. Furthermore, the FA composition of intramuscular fat was subjected to a multivariate discriminant analysis (SPSS, ver. 18.0.0, 2009), using a stepwise selection of those variables (FAs) that better discriminated between the experimental groups. Variables with $F > 0.10$ were retained at the end of the stepwise procedure and submitted to a canonical discriminant analysis. This procedure derives the canonical functions (a linear combination of the quantitative variables) that best summarize the variance of the dataset, with the aim to maximize the distance between the groups. The efficiency in group discrimination was evaluated through the Wilks's lambda test of significance. The discriminating efficacy of the model was expressed as the percent correct assignment of each individual to the respective group using the *leave-one-out* cross-validation. In this procedure, each individual is treated as an unknown subject and the canonical functions, previously developed with the entire dataset, are used to assign it to a group. The higher the discriminating

capacity of the model, the higher the percentage of cases correctly assigned to their respective group.

6.3. Results

6.3.1. Animal performance, intramuscular fat and fatty acid composition

Table 2 reports data on the main animal performance parameters. Dietary tannins significantly affected dry matter intake (DMI, $P < 0.001$), final body weight (BW, $P = 0.005$), carcass weight ($P = 0.009$), and average daily gain (ADG, $P < 0.001$), which were lower in the CH group as compared to all the other groups. The feed efficiency (FE), calculated as the ratio between ADG and DMI during the 75-day-experimental period, was lower in the CH group in comparison with GA ($P = 0.015$).

Table 3 reports the effect of the dietary treatment on each individual FA in the intramuscular fat. Total saturated (SFA), monounsaturated (MUFA), polyunsaturated fatty acids (PUFA), as well as the sum of *n*-3 and *n*-6 FAs and their ratio were not affected by treatments. Dietary tannins tended to affect ($P = 0.056$) odd- and branched-chain fatty acids (OBCFA) that were higher in the CO group as compared to CH ($P = 0.038$). Within the class of OBFCA 17:0 and *cis*-9 17:1 were higher in the i.m. fat of the CO lambs as compared to CH ($P = 0.023$ and $P = 0.024$, respectively) while 15:0 only tended ($P = 0.068$) to be higher; *anteiso* 18:0 was lower in CO in comparison with CH and MI ($P = 0.041$ and $P = 0.047$, respectively). Moreover *iso* 14:0 ($P = 0.002$), *iso* 15:0 in tendency ($P = 0.059$), *iso* 16:0 ($P = 0.026$) and *iso* 17:0 ($P = 0.020$) were higher in the CH lambs as compared to GA; *iso* 14:0 was higher in the CH in comparison with MI ($P = 0.029$).

Table 2 – Effect of dietary tannins on feed intake and growth performance

	CO	CH	MI	GA	SEM	*P*-value
N. of lambs	8	9	9	9		
Dry matter intake (g/d)	1167[a]	919[b]	1174[a]	1093[a]	0.0241	<0.001
Final BW (kg)	35.5[a]	30.3[b]	34.1[a]	35.1[a]	0.6280	0.005
ADG (g/d)	256[a]	180[c]	205[b,c]	249[a,b]	0.0082	0.001
FE	0.221[a]	0,194[a,b]	0,173[b]	0.227[a]	0,0062	0,003
Carcass weight (kg)	17.1[a]	14.7[b]	16.7[a]	17.2[a]	0.3180	0.009

CO: Control, CH: Chestnut, MI: Mimosa, GA: Gambier.
BW: body weight; ADG: average daily gain.
FE: feed efficiency (gain : intake ratio)
[a-c] within row, different superscripts indicate significant different.

Table 3 – Effect of dietary tannins on the fatty acid composition of intramuscular fat (g/100g total FA).

	CO	CH	MI	GA	SEM	*P*-value
IMF(g/100g of muscle)	2.074	1.853	1.982	1.770	0.077	0.535
10:0	0.120	0.124	0.136	0.114	0.004	0.270
12:0	0.128	0.144	0.130	0.110	0.005	0.145
14:0	2.896	3.013	2.755	2.697	0.072	0.410
iso 14:0	$0.018^{a.b}$	0.022^{a}	0.016^{b}	0.014^{b}	0.001	0.002
cis-9 14:1	0.111	0.118	0.101	0.103	0.004	0.382
15:0	0.357^{a}	0.294^{b}	$0.309^{a.b}$	$0.304^{a.b}$	0.009	0.073
iso 15:0	$0.068^{a.b}$	0.070^{a}	$0.057^{a.b}$	0.054^{b}	0.002	0.026
anteiso 15:0	0.108	0.107	0.096	0.095	0.003	0.183
16:0	23.598	23.285	23.095	23.045	0.201	0.789
iso 16:0	$0.121^{a.b}$	0.127^{a}	$0.114^{a.b}$	0.104^{b}	0.003	0.033
cis-7 16:1	0.310^{a}	0.309^{a}	0.268^{b}	$0.275^{a.b}$	0.006	0.023
cis-9 16:1	1.772	1.666	1.658	1.694	0.031	0.601
trans-5 16:1	0.056^{a}	0.035^{b}	$0.051^{a.b}$	$0.048^{a.b}$	0.003	0.029
trans-9 16:1	0.041^{a}	0.044^{a}	0.030^{b}	0.029^{b}	0.002	0.003
17:0	1.152^{a}	0.877^{b}	$1.057^{a.b}$	$1.017^{a.b}$	0.034	0.036
iso 17:0	$0.366^{a.b}$	0.391^{a}	$0.352^{a.b}$	0.328^{b}	0.008	0.031
anteiso 17:0	0.486	0.431	0.449	0.442	0.008	0.120
cis-9 17:1	0.708^{a}	0.510^{b}	$0.621^{a.b}$	$0.637^{a.b}$	0.025	0.037
18:0	12.129	12.961	13.006	12.796	0.168	0.254
iso 18:0	0.096	0.127	0.119	0.118	0.006	0.330
anteiso 18:0	0.019^{b}	0.036^{a}	0.036^{a}	$0.033^{a.b}$	0.002	0.027
cis-9 18:1	38.781	37.684	38.477	38.469	0.357	0.749

cis-11 18:1	1.575	1.428	1.407	1.655	0.046	0.162
cis-12 18:1	0.435[a]	0.416[a.b]	0.329[c]	0.344[b.c]	0.014	0.008
cis-13 18:1	0.108	0.098	0.121	0.121	0.004	0.086
cis-14 18:1	0.049	0.044	0.042	0.043	0.001	0.217
cis-15 18:1	0.087	0.088	0.107	0.112	0.005	0.131
trans-5 18:1	0.015	0.014	0.016	0.013	0.001	0.511
trans-6+8 18:1	0.283[a.b]	0.212[c]	0.302[a]	0.243[b.c]	0.009	<0.001
trans-9 18:1	0.264[a.b]	0.222[b.c]	0.296[a]	0.209[c]	0.009	<0.001
trans-10 18:1	1.579[a.b]	0.79[c]	1.759[a]	1.121[b.c]	0.097	<0.001
trans-11 18:1	0.753[a]	0.669[a]	0.612[a.b]	0.458[b]	0.031	0.005
trans-16 18:1	0.148[a.b]	0.173[a]	0.124[a.b]	0.138[b]	0.006	0.007
cis-9 *trans*-11 18:2	0.434[a]	0.379[a.b]	0.355[a.b]	0.304[b]	0.015	0.016
trans-8 *cis*-10 18:2	0.008	0.011	0.008	0.014	0.001	0.193
cis-11 *trans*-13 18:2	0.017[a.b]	0.008[b]	0.024[a]	0.013[b]	0.001	< 0.001
cis-10 *cis*-12 18:2	0.024	0.033	0.029	0.025	0.002	0.232
cis-9 *cis*-12 18:2	6.846	8.110	7.354	7.667	0.283	0.480
trans-8 *cis*-13 18:2	0.065[b]	0.196[a]	0.140[a]	0.155[a]	0.012	<0.001
trans-9 *cis*-12 18:2	0.065[a]	0.066[a]	0.044[b]	0.043[b]	0.003	<0.001
trans-9 *cis*-13 18:2	0.267	0.362	0.294	0.293	0.132	0.061
trans-11 *cis*-15 18:2	0.138[a.b]	0.103[b]	0.152[a]	0.112[a.b]	0.007	0.027
cis-6 *cis*-9 *cis*-12 18:3	0.055	0.063	0.058	0.066	0.008	0.700
cis-9 *cis*-12 *cis*-15 18:3	0.525	0.570	0.542	0.534	0.003	0.259
20:0	0.093	0.100	0.097	0.097	0.002	0.687
20:3 *n*-3	0.020	0.023	0.022	0.019	0.001	0.595
20:3 *n*-6	0.153	0.190	0.142	0.186	0.010	0.234

20:4 *n*-6	1.404	1.800	1.476	1.988	0.124	0.312
20:5 *n*-3	0.082	0.091	0.064	0.097	0.007	0.377
22:0	0.037	0.031	0.031	0.044	0.002	0.223
22:4 *n*-6	0.131	0.177	0.163	0.202	0.014	0.332
22:5 *n*-3	0.221	0.286	0.230	0.334	0.022	0.215
22:5 *n*-6	0.036	0.046	0.038	0.059	0.005	0.243
22:6 *n*-3	0.050^{b}	$0.079^{a.b}$	0.054^{b}	0.097^{a}	0.007	0.025
Σ SFA	38.984	39.658	39.254	38.901	0.323	0.851
Σ MUFA	47.075	44.520	46.321	45.710	0.391	0.127
Σ PUFA	10.538	12.593	11.187	12.210	0.445	0.365
Σ OBCFA	$2,791^{a}$	$2,480^{b}$	$2,605^{a.b}$	$2,507^{a.b}$	0.067	0.047
Σ PUFA *n*-6	8.439	10.144	9.010	9.899	0.406	0.443
Σ PUFA *n*-3	0.898	1.049	0.910	1.082	0.039	0.224
n-6 / *n*-3	9.329	9.692	9.900	9.247	0.237	0.751
Σ *trans*- 18:1	3.043^{a}	2.081^{b}	3.110^{a}	2.182^{b}	0.112	<0.001
VA / total *trans*-18:1	$0.343^{a.b}$	0.499^{a}	0.262^{b}	0.297^{b}	0.027	0.005

CO: Control; CH: Chestnut; MI: Mimosa; GA: Gambier.

SEM: standard error of mean.

IMF: intramuscular fat; Σ: sum of; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; OBCFA: odd and branched chain fatty acids; VA: *trans*-11 18:1.

[a-c] within row, different superscripts indicate significant different ($P \leq 0.05$).

Regarding the 18-carbon fatty acids, no effect of the dietary treatment ($P > 0.05$) was found for stearic (18:0), oleic, (*cis*-9 18:1), linoleic (*cis*-9 *cis*-12 18:2) and α-linolenic acid (*cis*-9 *cis*-12 *cis*-15 18:3). Dietary tannins significantly affected total *trans*-18:1 fatty acids ($P < 0.001$), with higher percentage in the CO lambs in comparison with GA and CH ($P = 0.005$, $P = 0.001$, respectively); total *trans*-18:1 were also higher in the i.m. fat of MI lambs as compared to GA and CH ($P = 0.002$, $P < 0.001$, respectively). Within the identified *trans*-18:1 isomers, *trans*-6+8 18:1 and *trans*-10 18:1 were lower in the CH group as compared to CO ($P = 0.004$ and $P = 0.005$, respectively) and MI ($P < 0.001$ and $P < 0.001$, respectively), and in the GA group in comparison with MI ($P = 0.019$ and $P = 0.023$, respectively). *Trans*-9 18:1 was lower in the GA animal as compared to CO and MI ($P = 0.040$ and $P < 0.001$, respectively), and in the CH group in comparison with MI ($P = 0.003$). A lower percentage of vaccenic acid (*trans*-11 18:1, VA) was found in the i.m. fat of GA lambs in comparison with CO ($P =0.003$) and CH ($P = 0.039$). *Trans*-16 18:1 was significantly higher ($P = 0.043$) in the CH lambs than in MI. Rumenic acid (*cis*-9 *trans*-11 18:2, RA) was significantly higher in the CO group as compared to GA ($P = 0.001$); among the other identified conjugated linoleic acid (CLA), *cis*-11 *trans*-13 CLA was found at a higher percentage in the i.m. fat of MI lambs as compared to GA ($P = 0.013$) and CH ($P < 0.001$). Regarding other PUFA, *trans*-8 *cis*-13 18:2 was significantly affected by experimental diets resulting in lower proportion ($P < 0.001$) in the CO group as compared to the other groups. *Trans*-9 *cis*-12 18:2 was higher in CO group in comparison with GA and MI ($P = 0.001$ and $P = 0.002$, respectively) as well as in the CH group as compared to GA and MI ($P < 0.001$ and $P < 0.001$, respectively).

Trans-11 *cis*-15 18:2 was lower in the CH group as compared to MI (P = 0.034). Docosahexaenoic acid (cis-4 *cis*-7 *cis*-10 *cis*-13 *cis*-16 *cis*-19 22:6; DHA) was affected by dietary tannins, with a higher percentage in GA as compared to CO and MI.

6.3.2. Multivariate approach to intramuscular fatty acids profile

From the entire dataset, eleven FAs were retained at the end of the stepwise selection and were linearly combined to obtain three canonical discriminant functions (CAN), which together explained the total variance of the dataset. The multivariate structure could be appropriately described by the first two CAN, explaining 88.8% and 8.8% of the variance, respectively.

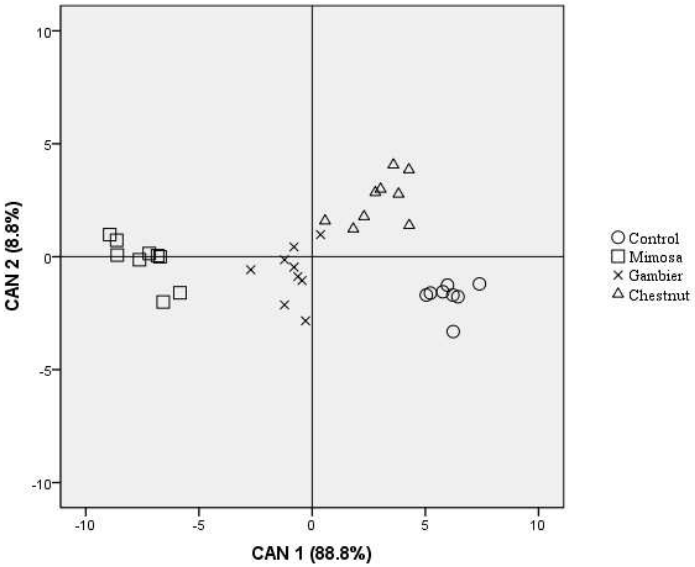

Figure 1 Discrimination of the dietary treatments achieved by plotting the first two canonical functions (cumulative variance explained: 97.6%)

Therefore, the scatterplot (Figure 1) represents the distribution of the lambs in the multivariate space, with CAN 1 as the X axis and the CAN 2 as the Y axis; the distance between the groups was always significant ($P \leq 0.05$).

The CAN 1 alone allowed the complete discrimination between the four groups with only one subject overlapping between the CH and MI groups. The CAN 2 tends to separate the CH lambs from all the other groups with some overlaps with the MI or GA groups. All the animals from CO, GA and MI were correctly assigned to their original groups, while two animals from the CH group were wrongly assigned to MI treatment after the cross-validation. Table 4 reports the standardized coefficients of the variables describing the total canonical structure. The magnitude (absolute value) of the standardized coefficients assigned to each variable is a measure of the relative contribution of the variable itself to the discriminating capacity of the whole multivariate function: the greater the value of the coefficient, the higher the contribution of the variable to the discrimination. The CAN 1 was mainly influenced by iso 14:0, *cis*-14 18:1, *trans*-16 18:1, RA, *cis*-11 *trans*-13 18:2 and *trans*-9 *cis*-12 18-2; while *cis*-14 18:1 accounted most in the CAN 2.

Table 4 – Standardized coefficients of each canonical function and variance explained by each canonical function

	CAN 1	CAN 2	CAN 3
iso 14:0	2.190	0.302	0.378
cis-14 18:1	-2.172	-1.507	-0.315
trans-10 18:1	1.889	-0.133	-0.302
trans-16 18:1	-2.679	0.085	-0.634
cis-9 *trans*-11 18:2	2.410	0.096	-0.112
cis-11 *trans*-13 18:2	-2.050	-0.296	0.789
trans-8 *cis*-13 18:2	-1.022	0.390	0.339
trans-9 *cis*-12 18:2	2.631	0.323	0.321
trans-9 *cis*-13 18:2	1.037	0.880	0.040
C20:3 *n*-3	0.797	0.047	0.356
C22:6 *n*-3	-1.510	0.216	0.519
Explained variance (%)	88.8	8.8	2.4

6.4. Discussion

The CH lambs showed a reduced DMI in comparison with all the other groups, resulting also in a lower ADG, final BW and carcass weight. It is known that tannins in the diet of ruminants can negatively affect the voluntary feed intake, which may be related to both their chemical nature and to their concentration in the diet (Min, Barry, Attwood & McNabb, 2003). The reduction of DMI has been commonly associated with high concentrations of tannins in the diet (over 50 g/kg DM), while limited or no negative effects have been observed at a concentration lower than 20 g/kg DM (Patra & Saxena 2011). Several possible mechanisms have been considered, alone or in association, to explain how tannins influence DMI (Frutos, Hervás, Giráldez & Mantecón, 2004). Among them, the reduction of the palatability of the feed is due to the astringent sensation

induced by a direct reaction of tannins with the taste receptors or through the interaction with the tannin-binding salivary proteins. However, the occurrence of tannin-binding salivary proteins in sheep is controversial (Jeronimo et al., 2016) and taking into account that the level of tannins in our experimental diets varied between 20 and 30 g/kg DM, a noticeable impairment of the feed intake was not expected. Also, the available literature on the use of HT from *castanea sativa* does not report detrimental effects on DMI when similar concentrations have been included in sheep diet (Toral, Hervás, Bichi, Belenguer & Frutos, 2011; Liu, Li, Mingbin, Zhao & Xiong, 2016). Nonetheless, the response of the animals in terms of feed intake cannot be linearly correlated to the quantity of tannins in the diet. Indeed, no reduction of ingestion has been reported when 80 g/kg of tannins were included in concentrate feed (Buccioni et al., 2015b). In addition, it is possible that different tannins have varying degree of affinity with salivary proteins in sheep (Vargas et al., 2013). Another plausible mechanism for the reduction in DMI could be related to the effects of dietary tannins to reduce the rate of ruminal feed digestion which could result in decreased feed intake without hampering the efficiency of feed utilization (Makkar et al., 1995). This factor could explain why CH have comparable FE with other groups (with the only exception of GA) despite the lower DMI.

The main objective of this study was to evaluate the intramuscular fatty acids in lambs supplemented with the three different tannin-rich extracts. No information on the effect of the here-used extracts on the FA profile of lamb meat is available in literature, while limited and contrasting results were provided by studies in which the effect of dietary HT form CH on the fatty acid composition of ewe

milk (Buccioni et al., 2015b, Carreño et al., 2015, Toral et al., 2013) was investigated.

The use of tannins in ruminant feeding is based on the hypothesis that they can affect the ruminal BH of the ingested PUFA, impairing their progressive saturation to 18:0 and, therefore, increasing the outflow of health-promoting intermediates such as rumenic and vaccenic acids that can be absorbed, further metabolized and/or accumulated in the tissues (Shingfield, Bernard, Leroux & Chilliard, 2010; Piperova et al., 2002). An increase of PUFA would be observed when the first steps of the BH are inhibited (Priolo et al., 2005; Willems et al., 2014; Campidonico et al., 2016). On the contrary, the inhibition of the terminal step of the BH (the conversion of VA to 18:0) might result in the reduced formation of SA and in the accumulation of the BH intermediates rumenic and vaccenic acids (Vasta et al., 2009b; Whitney, Lupton & Smith, 2011.) and of other *trans*- 18:1 FAs (Whitney & Smith, 2015). In our experimental conditions only *cis*-9 *cis*-12 18:2, the main substrate of BH (Chilliard et al., 2007), and as a consequence total PUFA and PUFA *n*-6, were numerically lower in the meat of CO lambs, suggesting a higher BH activity in this group. However, RA and VA were found at similar proportions between the control and the tannin-supplemented groups, with the exception of GA. The lower proportion of RA, VA as well the reduced percentage of total *trans*- 18:1 in the meat from GA lambs could indicate that GA extract reduced BH to a higher extent. This hypothesis seems to be confirmed also by the lower percentage of *trans*-9 *cis*-11 18:2 and *cis*-11 18:1 arising from an alternative pathway of linoleic acid BH (Shingfield et al., 2010). A similar trend was observed by Vasta et al. (2007), who reported a lower level of RA and VA in lamb

fed carob pulp as compared to lambs consuming the same diet with the addition of polyethylene glycol, a binding agent that inactivate the action of tannins in the rumen. Later, it was found that the supplementation of quebracho extract in the diet at the concentration of 60 g CT/kg increased the accumulation of total *trans*- 18:1 in the rumen liquid and in lamb muscle (Vasta et al., 2009b;c). We also observed a significant reduction of the total *trans*- 18:1 in the CH group in comparison with the CO. Taking into account the ratio between VA and total *trans*- 18:1, it is evident how in the case of GA this reduction mainly depends on the lower percentage of VA, while for the CH lambs it depends on *trans*- 18:1 isomers other than VA. In contrast with our results, Buccioni et al. (2015b) found increased levels of *cis*-9 *cis*-12 18:2 and *cis*-9 *cis*-12 *cis*-15 18:3, lower percentages of VA and RA and no effect on other *trans*- 18:1 FAs in milk of grazing ewes supplemented with a concentrate containing linseed and HT from CH. Nevertheless, it is worth mentioning that, in the study of Buccioni et al., (2015b), ewes consumed a diet higher in fat (3.5 fold) and *α*-linolenic acid (4 fold) compared to our conditions, while the amount of consumed tannins was similar; it is plausible to suppose that a higher availability of PUFA in the rumen could enhance the effect of tannins on BH, with this making not straightforward the comparison between the studies. Indeed, Whitney et al. (2011) observed that increasing amounts of dietary crude fat and of *α*-linolenic acid resulted in a linear increase of RA and reduction of 18:0 in the fat of lambs receiving dry juniper leaves. In another study, Toral et al. (2011) did not observe effects on rumen fermentation or on milk FA profile when a commercial mixture of condensed and hydrolysable tannin extracts was given to lactating ewes fed a TMR containing

sunflower oil, probably due to the low level of tannin supplementation (10g/kg DM).
In the present study, both MI and GA lambs showed a reduced VA / total *trans*- 18:1 ratio as compared to CH. As stated before, this depends on the lower percentage of VA for GA, while it is mainly due to a higher percentage of other *trans*- 18:1 for MI. Tannins present in GA and MI extracts, catechins and profisetinidins respectively, could have differently affected the BH pathway. It has been reported that the chemistry of the tannins can influence their ability to interact with feed and bacterial protein or other molecules (Makkar et al., 2003). In an *in vitro* trial, Khiaosa-Ard et al. (2009) reported that condensed tannins from *Acacia mearnsii*, the same used here, inhibited the conversion of VA to 18:0, while the same amount of tannins from *Onobrychis viciifolia* did not affect the last step of BH but reduced the disappearance of linoleic and linolenic acids.
Trans-10 18:1 was predominant among the *trans*- fatty acids in all the groups. It should be mentioned that the characteristics of the basal diet greatly impact on BH in the rumen. According to Sackman et al. (2003), BH activity is positively related to the level of NDF in the diet. This is due both to a more favourable environment for the development of cellulolytic bacteria involved in the BH, and to a higher retention time in the rumen as compared to starch-rich feed (Van Soest, 1991). Vaccenic acid accounts up to 70% of *trans*- 18:1 fatty acid in forage-fed ruminants (Bessa et al., 2007), but the progressive replacement of forages with concentrates increases *trans*-10 18:1 due to a changed BH pathway (Aldai, de Renobales, Barron & Kramer, 2013). In agreement with the literature, we found that the percentage of *trans*-10 18:1 was doubled in comparison with VA in all

the groups. As reviewed by Bessa, Alves & Santos- Silva (2015), *trans*-10 18:1 negatively correlates with RA. This depends on the fact that RA in the fat of ruminants mainly arises endogenously from the action of the Δ^9- *desaturase (*Griinari et al., 2000*)*. In the muscle, this enzyme converts VA to RA, but cannot use *trans*-10 18:1 as a substrate for the production of RA. In our conditions, the ratio between *trans*-10 18:1 and VA was slightly lower in the CH than the other treatments, but this did not cause a proportional increase of the RA level.

Regarding the OBCFA, their presence in both milk and intramuscular fat largely arises from ruminal bacteria; thus these fatty acids represent a potential diagnostic tool for rumen function (Vlaemink et al., 2005). In this experimental trial, dietary tannins tended to affect total OBCFA, which were lower in the CH as compared to CO lambs, probably because of a general reduction of microbial activity. This is consistent with the literature, reporting that chestnut HT and quebracho CT lowered branched chain fatty acids in sheep milk (Buccioni et al. 2015a;b; Toral et al., 2013). Moreover, dietary HT seem to play a role on the proportion of *iso* FAs, which are positively related to the presence of cellulolytic bacteria in the rumen (Fievez, Colman, Montoya, Stefanov & Vlaeminck 2012). In our study, except for *iso* 18:0, all the other identified *iso* FAs were higher in the CH lambs as compared to MI, GA or both. As *iso* FAs arise from isovalerate and isobutyrate (Vlaeminck, Fievez, Cabrita, Fonseca & Dewhurst, 2006), a different availability of volatile fatty acids in the rumen of HT- and CT-supplemented lambs is supposable as well as a different composition of the rumen population (Vasta et al., 2010; Buccioni et al., 2015a).

Multivariate statistical approaches are often used to understand the combined contribution of the individual variables in the identification of overall patterns within the dataset. Several approaches can serve to this objective and, among the more commonly used methods, the canonical discriminant analysis is specifically aimed at verifying if the selected variables, taken together, possess a discriminant capacity able to differentiate subjects belonging to different groups. Based on this, canonical discriminant analysis has been previously applied to datasets related to the fatty acid composition of muscle with the aim to, for instance, discriminate meat from different geographic origin and/or from animals belonging to different breeds or raised under different production systems (Dias et al., 2008; Garcia et al., 2008). Moreover, in the case of complex datasets with a high number of variables (such as the fatty acid composition of meat), the use of the stepwise procedure allows identifying those variables with the highest discriminating ability, thus driving the attention on those which are more relevant to the specific objective. In the present study, the canonical discriminant analysis, together with the stepwise selection of the variables, was used to: *i*) assess whether the multivariate structure of the whole FA profile was able to define overall patterns able to discriminate among the tested treatments, *ii*) to evaluate the discriminant power of each fatty acid and *iii*) to identify those with a higher discriminating capacity. As stated previously, eleven fatty acids were retained and linearly combined into three canonical discriminant functions, two of which alone explained 97.6% of the total variance and allowed to correctly assign each animal to its original feeding group with only a 5% error after the cross-validation. All the fatty acids with high standardized coefficients in the CAN 1 and

CAN 2 were significantly affected by the dietary treatments with the exception of *cis*-14 18:1 and are FAs arising from rumen metabolism or synthesized during the BH. Moreover, it is noteworthy that among the intermediates of the principal pathway of BH, only RA was retained after the stepwise discriminant analysis. A similar finding was reported by Morales et al. (2015) after a regression analysis conducted on 12 different experiments to investigate the response of fatty acids in meat fat to tannins supplementation. In that case, tannins were associated only with the reduction of 18:0. Therefore, although the results of this study cannot lead to identifying specific patterns describing the effects of dietary tannins upon the fatty acid metabolism, taking together the results of the univariate ANOVA and of the multivariate analysis shows that dietary tannins exert general modifications along the whole BH process.

6.5. Conclusion

The dietary supplementation of three different tannin extracts affected the fatty acid composition of lamb meat. However, our findings do not allow to describe specific mechanisms of action of the tannins on precise steps of the fatty acid metabolism but, rather, suggest a general action on the whole BH process. This supports findings recently provided by *in vitro* and *in vivo* studies that highlight how the effect of tannins on the fatty acid metabolism in ruminants cannot be explained in a straightforward manner, as previously supposed. Moreover, it should be taken into account the difficulty in comparing results of different studies conducted under different conditions, such as the source of tannins, their dose in the diet and the interaction

with the basal diet. Nevertheless, in our study, a multivariate approach to the data made it possible to clearly discriminate the dietary treatments by means of the intramuscular fatty acid profile. The fatty acids with a high discriminant capacity arise from microbial metabolism and from the principal or alternative BH pathways. This confirms the impact that dietary tannins exert on the rumen BH and suggests that they can have a generalized influence on BH rather than effects on one or more specific steps of the process.

7. Conclusions

Literature has shown that the content of functional fatty acids (CLA, EPA, DHA) in red meat is strongly affected by animal diet. An adequate ingestion of these fatty acids is recommended for the normal physiological functioning and health of humans (Palmquist, 2009). Nevertheless, the consumption of these compounds is less than recommended in an optimal diet. Therefore, increasing the content of these fatty acids in meat is an important objective in the meat industry and this goal can be almost exclusively achieved by the opportune manipulation of the animal diet. Such strategies need to be particularly effective in ruminants, for the well know lipid metabolism in these species, leading to a generally low content of polyunsaturated fatty acids in muscle. Effective strategies to increase the content of healthy compounds in muscle could also confer on the meat an added value, representing a commercial opportunity, especially for the small farmers addressing a niche market.

It is well known that pasture feeding increase omega-3 and CLA content in food. However, alternative solutions are needed in several areas worldwide, where the availability of good quality pastures is limiting. The use of plants, or plant-extracts, containing bioactive compounds represents an achievable prospect; however, literature shows that the effect of dietary plant secondary compounds and of other plant bioactive components on meat fatty acid composition is strongly affected by the diet supplied to the ruminant.

The experiments planned in this PhD represent a contribution to this issue.

The experiment in which lambs were fed the different silages showed an effect of both red clover (RC) and sainfoin (SF) silages on the ruminal BH of fatty acids. A clear additive effect was observed when the two plants were mixed together in the silage mixture. Lambs fed with silage mixture RC-SF had a clear greater content of PUFA in rumen digesta. In the muscle this mechanism was not totally confirmed; indeed PUFA proportion in RC-SF group just partially confirmed the results observed in the rumen digesta, which may be partially explained considering the post-ruminal metabolism of fatty acids in the anial organism.

Polyphenol oxidase (PPO) in RC is believed to impair the whole onset of ruminal BH, while it is often reported that tannins might exert an effect on the last step of the BH, leading to the *trans*-11 18:1 to 18:0 conversion, with an accumulation of VA in the rumen. In this study, a contribution of tannins to this process might have been responsible for the higher proportion of VA in ruminal digesta from lambs fed the mixture T-SF, which was higher compared to T-RC even with a lower intake of LA and ALA. The effect of tannins from SF was probably responsible for a greater accumulation of VA because its saturation to 18:0 was impaired. This result was not confirmed in the LM, where VA was detected at comparable amounts between T-SF and T-RC groups. This inconsistency between ruminal digesta and muscle might be explained by the muscle metabolism that tends to desaturate SFA and some MUFA respectively to MUFA and PUFA (Smith et al., 2006). This hypothesis is further supported by the greater concentrations of *cis*-9 18:1, *cis*-9 16:1 and *cis*-9 *trans*-11 CLA in muscle than in rumen digesta, which would

derive to an important extent from the activity of Δ^9-desaturase in ruminant tissues.
In the second experiment, including 4% of tannin extract from chestnut or mimosa or gambier in lambs diet did not produce the expected beneficial effects on lipid fatty acid profile. In our experimental conditions, tannins generally did not show any advantage in favor of VA or PUFA, which indicates that probably they only slightly affected the BH process and did not exert effects on precise steps of the BH. The slight differences found in the muscle fatty acids in the control group of lambs, as compared to the tannin fed lambs, show the partial impairment of the BH of LA, which suggests a possible involvement of the tannins in the fatty acid metabolism. A distinctive effect of the three extract was observed using a multivariate approach. From the initial number of fatty acids, the system retained a pool of 12 that better discriminate among dietary treatments. It is noteworthy that all of them are related to microbial metabolism in the rumen. This confirms the potential of tannins to affect rumen BH and fatty acid profile of ruminant meat and highlight that, in different experimental conditions, the effects of tannins may not be univocal and straightforward.

Both experiments report an effect of plant bioactive compounds on BH. However, in the silage experiment the hypothesis about impairing of BH and improvement of FA profile was verified, while in the second experiment the response did not clearly explain how tannins interact with metabolism.

The results from these studies allow to confirm the potential benefit of ensiling legumes for ruminant feeding, because,

unlike the pasture, silage is available during the whole year and because their use have improved meat nutritional quality.

Regarding the addition of tannin containing plant extracts, the chemically and physically characteristic of the basal diet appear to be a discriminant factor driving their potential activity. As previously reported, tannins activity is strongly affected by basal conditions, such as fibre/starch ratio and fat content and composition of the diet, which can modify important factors for ruminal bacteria populations.

Literature shows that tannins can affect and modify meat quality, although the results provided are often controversial. Therefore more investigation is necessary to unravel their exact mechanisms of action in order to be able to calibrate their use as feed supplements and to optimize their efficacy.

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