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Effect of Different Kind of Polyphenols on Nutraceutical Milk Quality, Ruminal Lipids Metabolism and Rumen Microbiota

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DECLARATION of AUTHORSHIP

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

ABSTRACT

Nowadays, European and World policies encourage the reduction of feed inputs in livestock productions. Moreover, in the last decade, there was a steady and inexorable increase of livestock feed costs, due to the increase of row matrixes price. Many farmers, particularly in marginal lands, are in crisis due to these problems; however, these difficulties are much more evident in large intensive farms, because large ruminants have high requirements.

In order to overcome these economic problems, it seems evident that the pastoral resource represent a low-cost solution; nevertheless, it does not meet the large-scale needs of intensive farming. Another winning solution could be represented by the use of high biological value agro-industrial by-products. In this way it would be possible to turn a waste, that is disposed with considerable costs, into a food resource for the animals.

Most by-products contain notably quantities of secondary compounds, such as polyphenols, which act on digestion and animal performances, but also on the quality of animal derived products. The composition of milk and meat fatty acids can be manipulated by polyphenols naturally present in several plants, modifying the biohydrogenation of polyunsaturated fatty acids, as consequence of changes in ruminal ecology.

Small ruminants, even more than large ones, have a remarkable ability to convert feed into products with high nutritional value (meat and milk); they are also endowed with a marked frugality and adaptability to unconventional feed matrices, since they have lower energy requirements. Therefore, a diet formulated with low-input content and with alternative feeds, such as by-products, instead of more expensive products, is an innovative but functional choice. Recently, the increased interest in secondary plant metabolites has led to studies that have shown interesting results regarding animal health and product quality. Plant polyphenols fall into this category but their vastness and chemical diversity imply constant research in the fields of animal nutrition. Furthermore, the use of by-products rich in functional molecules, such as polyphenols, represents a sustainable strategy and meets consumer demand, which increasingly seek natural, healthy and environmentally sustainable products.

The rumen and its microbiota represent the key of these issues, because all pathways that convert feed in bioactive compounds occur in a preponderant way in the pre-stomach.

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Introduction abbreviations

BH, biohydrogenation; CH_4, methane; CT, condensed tannins; GAL, gallic acid; HT, hydrolysable tannins; OOP, olive oil pomace; PEG, polyethylene-glycole; PP, polyphenols; VFA, volatile fatty acid.

INTRODUCTION

1. Polyphenolic compounds

Plants synthesize very numerous organic compounds, traditionally classified as primary and secondary metabolites. Primary metabolites are molecules that have essential roles in plant growth and development (i.e. in photosynthesis and respiration). In contrast, the other phytochemicals, commonly referred as secondary metabolites (Crozier et al., 2007), are related to plant defence responses (Lin et al., 2016). Indeed, these compounds provide protection against pathogens and predators (Balasundram et al., 2006), since they have antibacterial or antifungal actions (Lin et al., 2016) and plant synthesizes them in the occasion of infections or external damages. They have also structural function and contributing to colour and sensory characteristics of fruits and vegetables (Balasundram et al., 2006).

From chemical standpoint, secondary metabolites are aromatic polymers, called polyphenols (PP), that contain benzene rings with one or more hydroxyl substituents (Lin et al., 2016). Among PP, there are simple molecules, such as phenolic acids, or complex structures, such as tannins, that are highly polymerized molecules (Lin et al., 2016). The majority of these phenols exist in nature as glycosides (Tsao, 2010), conjugated with mono- and polysaccharides, linked to one or more phenolic groups (Balasundram et al., 2006); the different sugar units can be acylated at different position on the polyphenol skeleton (Tsao, 2010). PP may also occur in nature as functional derivatives, such as esters and methyl esters (Balasundram et al., 2006).

Plant synthesize PP in two main pathways, that can carry out independently or together. One produce activate acetate, binding two carbon units, which undergo the subsequent cyclisation. The other way is the shikimic acid way, through which the most part of PP is produced. In this way, carbohydrates, derived from pentose phosphate pathway, are converted to the aromatic aminoacids fenilalanin, tyrosine and tryptophan, before to go to the glycolysis. Then, an ammonia molecule is removed by the phenil alanine lyase, forming cinnamic acid, that could be converted into the p-OH-cinnamic acid, to form, by the sequential addition of hydroxyl and methoxy groups, caffeic acid and ferulic acid, which are the precursors of flavonoids, the largest subclass of PP (Cutrim and Cortez, 2018) (fig. 1).

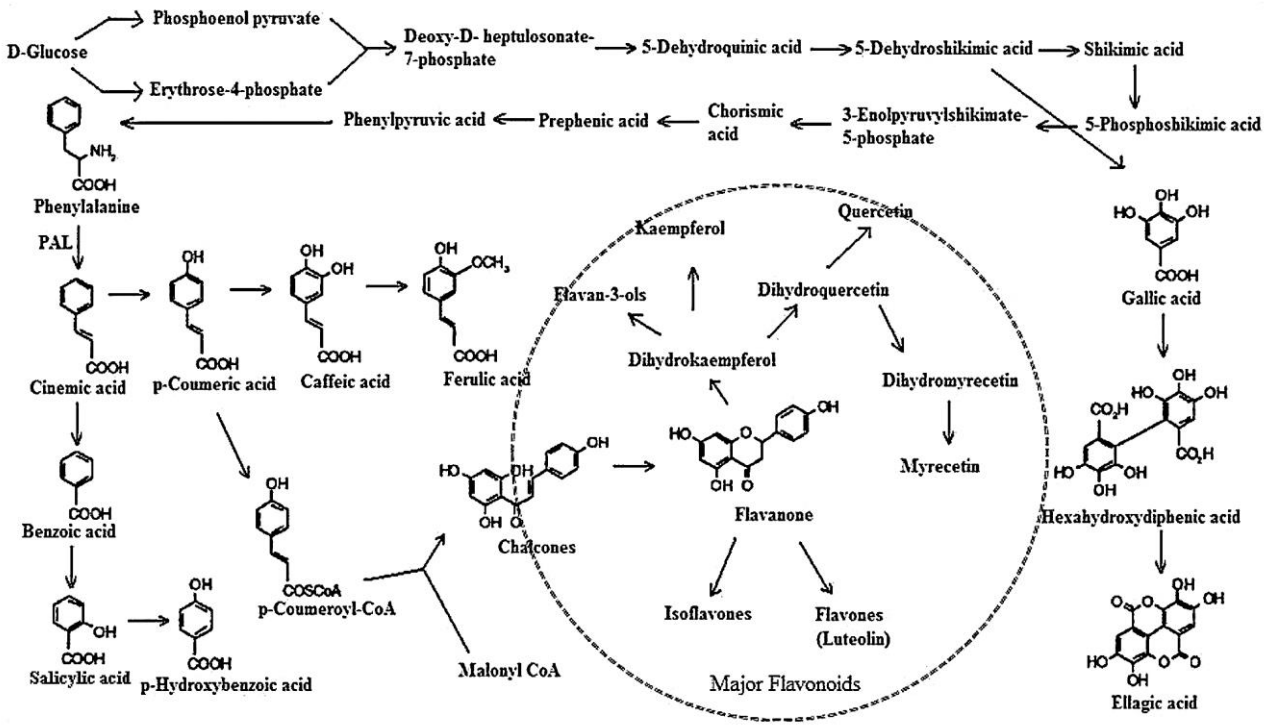

Fig. 1. Phenil propanoid biosynthetic pathway of phenolic acids and flavonoids (Mandal et al., 2010, accessed on September 2018).

1.1 Classification

Plant PP constitute one of the most numerous and widely distribute group of natural products. Actually, more than 8000 phenolic compounds have been discovered and partially studied (Tsao, 2010). The classification of PP was carried out through years and they can be categorized in several ways (Cutrim and Cortez, 2018): by vegetal origin, use, functions and chemical structure and properties. However, the most accredited way of classification is the one that consider their chemical structure and properties (Tsao, 2010) (fig. 2).

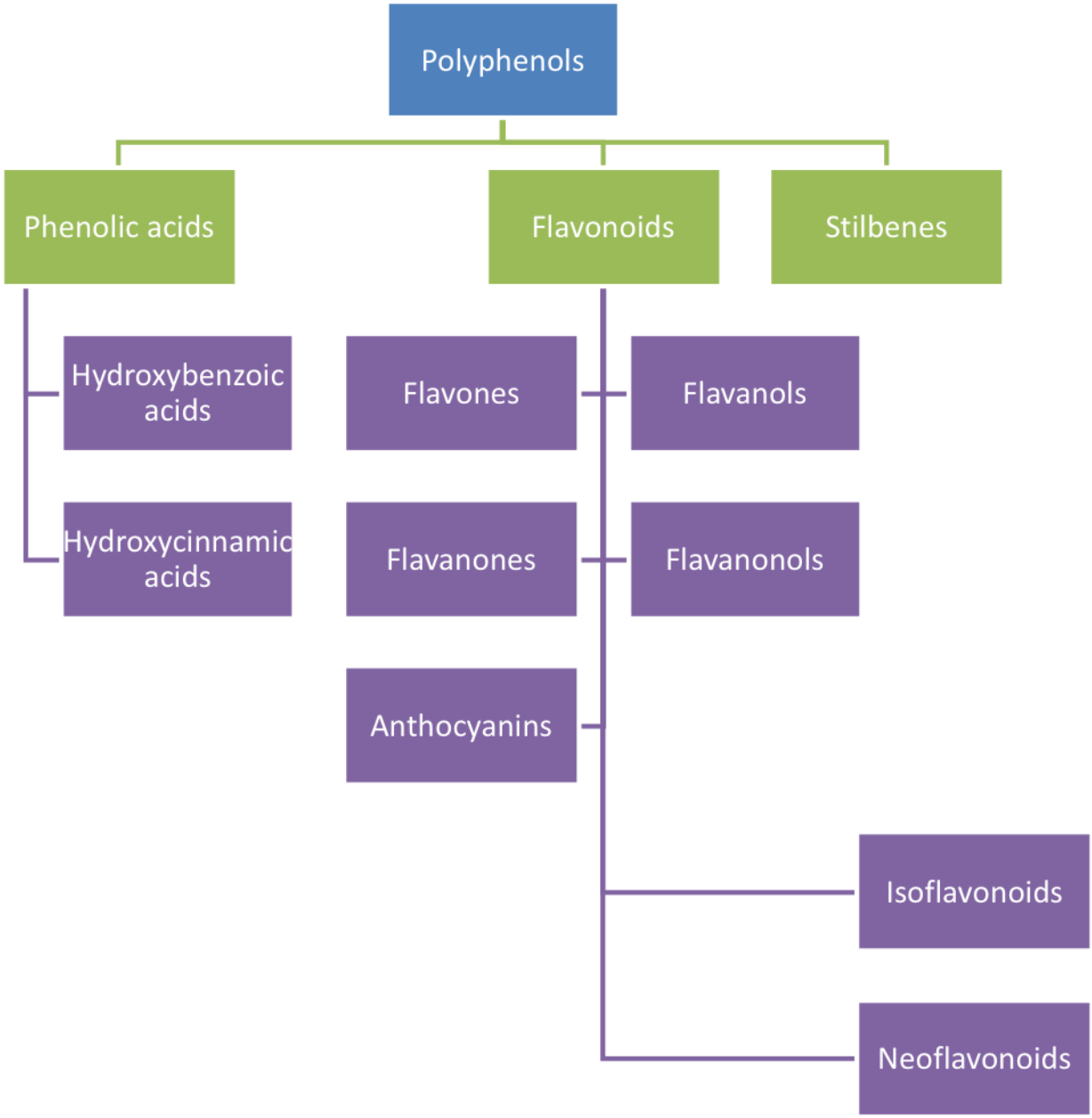

Fig. 2. Polyphenols classification.

1.1.1 Phenolic acids

Phenolic acids are the smallest plant phenolic products, formed by only one benzene ring. Some phenolic acids are found in free form, but structural tissues contain them in bound form (Basheer and Kerem, 2015), for instance in plant cell walls and lignin (Mandal et al., 2010). These molecules are subdivided in two subgroups: the *hydroxybenzoic acids*, with a skeleton of C_6-C_1 and the *hydroxycinnamic acids*, with a skeleton of C_6-C_3 (Balasundram et al., 2006).

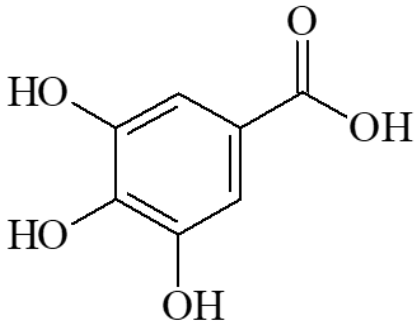

Fig. 3. An example of phenolic acid, gallic acid.

They have a very stable structure and only few bacteria can use them as Carbon source (Mandal et al., 2010). The most important hydroxybenzoic acid is gallic acid (GAL) (fig. 3), which is the base unit of gallotannins and with hesahydroxyphenol forms ellagitannins. Both ellagitannins and gallotannins are commonly referred as hydrolysable tannins (HT) because are easily hydrolyzed, freeing GAL (Crozier et al., 2007). As hydroxybenzoic acids, the hydroxycinnamic ones are incorporated in cell walls with covalent bound, but are also present in soluble form in the cytoplasm even though, this form occurs very rarely in nature (Setti et al., 2001). Caffeic acid is one of the most common hydroxycinnamic acid and it is found in coffee (Basheer and Kerem, 2015), tea leaves, fruits (in particular in red ones)(Teixeira et al., 2013) and in olive oil (Basheer and Kerem, 2015).

1.1.2 Flavonoids

Flavonoids constitute the largest group of plant phenolics, accounting over 5000 different compounds (Balasundram et al., 2006). They are present in the outer surfaces of leaves and flowers, but also in the cell walls, in the cytoplasm and in the vacuoles (Andersen and Markham, 2006). High concentration of flavonoids are found in young leaves and in fruits skin. They are involved in several processes as protection from UV (Crozier et al., 2007), from oxidation and free radical, insect attraction or repulsion, protection against viral, fungal and bacterial infections, pollen germination, etc... (Andersen and Markham, 2006). They are common in free form, but, in the case of catechin, epicatechin and gallocatechin, are also the monomeric constituents of the condensed tannins (CT) (Bravo, 1998).

Chemically, flavonoids are low molecular weight compounds, with a simple C_6-C_3-C_6 configuration. Essentially, the structure consists of two aromatic rings, A and B, joined by a 3-carbon bridge, usually, in the form of etherocyclic ring, named C (fig. 4). The A ring

derived from the acetate/malonate pathway, while the B ring is derived from shikimate pathway (Balasundram et al., 2006).

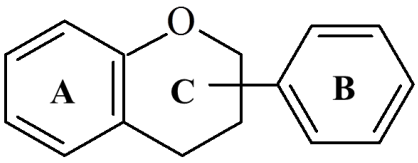

Fig. 4. Flavonoid generic structure.

The basic flavonoid skeleton can have numerous substituents, like sugars, that are very common substituents with the majority of flavonoids, that exist naturally as glycosides. The nature of the substituent may modify the chemical behaviour of flavonoids; hydroxyl groups and sugars increase the water solubility of them, while other substituents, such as methyl groups, make flavonoids lipophilic (Crozier et al., 2007).

Flavonoids can be subdivided into different subgroups depending on the carbon binding site on which the B ring is linked to the C one and on the degree of unsaturation and oxidation of the C ring. Thus, flavonoids in which the B ring is linked in position 3 are called *isoflavonoids* (Panche et al., 2016) (fig. 5).

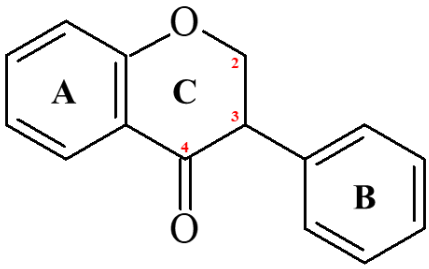

Fig. 5. Isoflavonoid generic structure.

Isoflavonoids are also called phytoestrogenes, since they have an estrogen-like behaviour (Andersen and Markham, 2006). They are found almost exclusively in leguminous plants, above all in soybeans (Crozier et al., 2007).

Those flavonoids in which B ring is linked to the C one in position 4, without any hydroxyl group in position 2, are called *neoflavonoids* (Panche et al., 2016) (fig. 6).

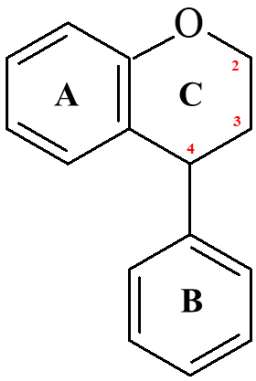

Fig. 6. Neoflavonoid generic structure.

These compounds are found mostly in tropical plant, for instance in *Guttifereae*, *Rubiaceae* and *Passifloraceae* (Donnelly and Sheridan, 1988).

Flavonoids in which the B ring is linked in position 2 can be further subdivided on the basis of the structural features of the C ring. These subgroups are: flavones, flavonols, flavanones, flavanonols, flavanols or catechins and anthocyanins.

Flavones have a double bond between position 2 and 3 and a ketone on position 4 of the C ring (fig. 7).

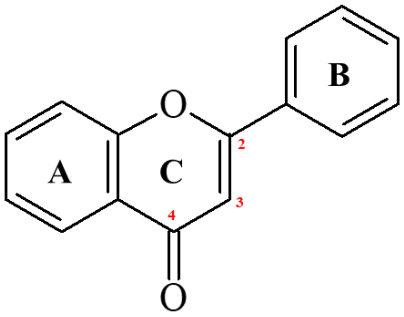

Fig. 7. Flavone generic structure.

Most flavones of vegetables and fruits have a hydroxyl group in position 5 of the A ring, while hydroxylation in other position may vary according to species (Panche et al., 2016). A wide range of substitutions, like methylation and glycosylation is possible too (Crozier et al., 2007). Celery, parsley, red peppers, chamomile, mint and ginkgo biloba are the major sources of flavones (Panche et al., 2016). In addition, polymethoxylated flavones have been found in citrus species, especially in peels (Crozier et al., 2007).

Flavonols, compared to flavones, have a hydroxyl group in position 3 of the C ring, which may also be glycosylated (fig. 8).

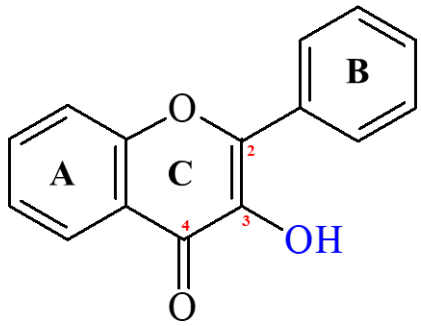

Fig. 8. Flavonol generic structure.

Like flavones, they are very diverse in methylation and hydroxylation patterns (Panche et al., 2016) and the distribution and structural variation of these compounds are extensive (Crozier et al., 2007). In fact, they are the most widespread subclass of flavonoids, being dispersed throughout the plant kingdom, with the only exception of the algae (Crozier et al., 2007). Flavonols occur abundantly in a large variety of fruits and vegetables; in particular onions, cabbage, lettuce, tomatoes, apples, grapes and berries are rich sources of these compounds, such as red wine and tea (Panche et al., 2016). However, sizeable differences are found in the amounts present in similar sources, possibly due to seasonal changes and varietal dissimilarities (Crozier et al., 2007). Flavonols are also building blocks of proanthocyanidins. The most studied flavonols are kempferol, quercetin and myricetin (Panche et al., 2016).

Flavanones are characterized by the absence of the 2-3 double bond and by the presence of a chiral centre on the C2 of the C ring (Crozier et al., 2007) (fig. 9). So, they have the C ring completely saturated and this is the only difference between flavanones and flavones (Panche et al., 2016).

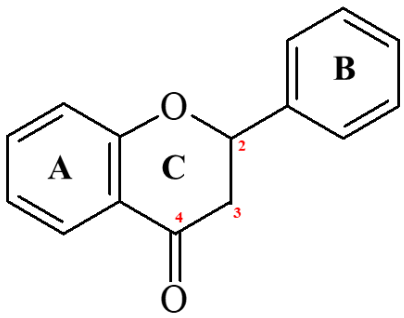

Fig. 9. Flavanone generic structure.

This structure is highly reactive and undergo hydroxylation, glycosylation and O-methylation reactions (Crozier et al., 2007). Flavanones are present in especially high concentration in citrus fruits (Crozier et al., 2007) and are responsible for the bitter taste of peel and juice

(Panche et al., 2016), even though exist several flavanones without any taste. The most common flavanone is the hesperidin, which is found in citrus peel (Crozier et al., 2007).

Flavanonols can be considered as flavanones with a hydroxyl group on position 3 (de la Rosa et al., 2010) and with no double bond between positions 2 and 3 of the C ring (Panche et al., 2016) (fig. 10).

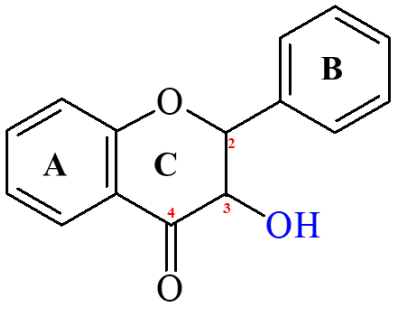

Fig. 10. Flavanonol generic structure.

Flavanols are the most complex subclass of flavonoids and are often referred to as flavan-3-ols, as the hydroxyl group is almost always attached to the position 3 of the C ring.

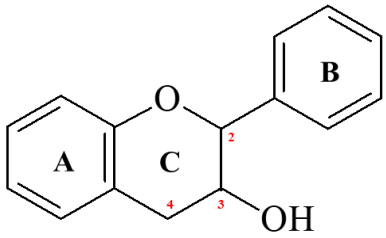

Fig. 11. Flavanol generic structure.

The term “flavanols” is also interchangeable with the term “catechins” (de la Rosa et al., 2010), that is used more often in reference to the monomers of oligomeric and polymeric proanthocyanidins, also known as CT. Unlike flavones, flavonols, anthocyanins, iso- and neoflavonoids, which are planar molecules, flavanols, but also flavanones and flavanonols, have a saturated element in position 3 of the heterocyclic C ring, and are, thus, non-planar (Crozier et al., 2007). Catechins have two epimers depending on the stereo-configuration of the bond between ring B and the C2 of the C ring, and other two depending on the hydroxyl group orientation on position 3 of the C ring (de la Rosa et al., 2010). Of these four isomers ((+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin), two of them ((+)-catechin and (-)-epicatechin) are widespread in nature, whereas the other two are comparatively rare.

Proanthocyanidins can occur as polymers of up to 50 units and have an additional chiral centre at C4. Moreover, flavanols can be hydroxylated, undergo esterification and form

gallocatechins with GAL. Red wine and chocolate are rich of these compounds; instead, green tea contains high level of epigallocatechins, the epimers of gallocatechins (Crozier et al., 2007). Flavanols are also found in bananas, apples, blue berries, peaches and pears (Panche et al., 2016).

Anthocyanins are pigments responsible for most of the red, blue and purple colours of fruits, vegetables, flowers and other plant tissues (D'Archivio et al., 2007). Colour variations among these compounds reflect structural differences in the number of hydroxyl groups, the presence or absence of methylations and glycosylations, as well as the distribution of positive and negative charge on the chroman ring system (Swanson, 2003). Anthocyanins occur primarily as glycosides of their respective aglycone form, called anthocyanidins (D'Archivio et al., 2007) plus one or more mono- and oligosaccharides linked with a glycosidic bond (Swanson, 2003). The sugar moiety is attached on the C ring, at position 3, or on the A ring, at position 5 or 6 (D'Archivio et al., 2007). The aglycone form is highly unstable (Manach, 2004) and anthocyanidins undergo structural alterations, depending on the pH and the ionic strength of the aqueous environment (Swanson, 2003). In plants, the degradation of these molecules is prevented by glycosylation, generally with a glucose, and by the esterification with various organic and phenolic acids. In addition, anthocyanins are stabilized by complexation with flavonoids (Manach, 2004).

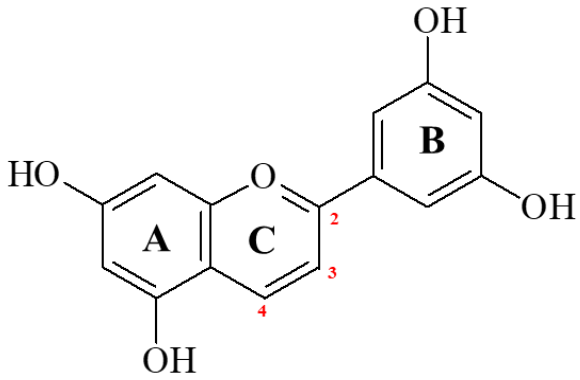

Fig. 12. Anthocyanin generic structure.

1.1.3 Stilbenes

Stilbenes are a minor class of PP, produced as defence, especially against fungal pathogens (Grotewold, 2006) but also in response to stress and bacterial infections. Stilbenes occur in many plant species, including grapevine, peanuts and several berry and tree species (Reinisalo et al., 2015). These molecules are commonly present in roots, barks, rhizomes and leaves (Cassidy et al., 2000). All higher plants are able to synthesize malonyl-CoA and CoA-esters, which are stilbenes precursors, but only few plants seem to be able to produce stilbenes

(Chong et al., 2009). Stilbenes are considered as phytoalexins (Crozier et al., 2007), derived from the phenil propanoid pathway.

Members of stilbene family have a C_6-C_3-C_6 structure, without the heterocycle C, and occurs in both *cis* and *trans* isomers (Crozier et al., 2007) (fig. 13).

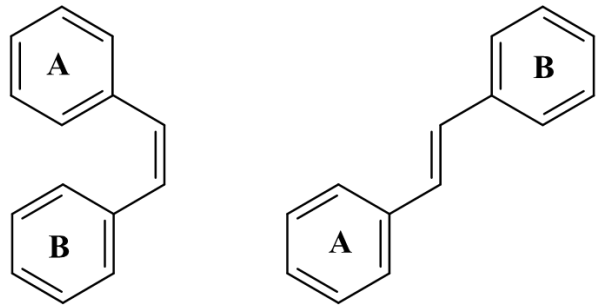

Fig. 13. Stilbene generic structures in *cis* and *trans* form.

Usually, ring A carries two hydroxyl groups in *meta*-position, while ring B have hydroxyl and methoxy substituents in *orto*-, *meta*- and *para*-position (Cassidy et al., 2000). Glycosylation is a common modification of plant secondary metabolites that interest also stilbenes and can alter their bioactivity. In plants these molecules are generally accumulated in the glycosylated form. Glycosylation of stilbenes could be involved in their storage, transport and protection against peroxidative degradation. Also methylation can occur on stilbene skeleton and a large number of natural stilbenes is found as oligomers (Chong et al., 2009). The fresh skin of red grapes is particularly rich in resveratrol (D'Archivio et al., 2007), the most common stilbene (Crozier et al., 2007), which contributes to a relatively high concentration of it in red wine (D'Archivio et al., 2007).

1.1.4 Tannins

Unlike the previously described groups of plant PP, the classification of some of it as tannins must be carefully considered, because this term is used in reference to phenolic compounds belonging to different classes (Aron and Kennedy, 2008). The term "tannin" come from the French word *tanin* (Khanbabaee and van Ree, 2001), in reference to the capacity of certain substances to transform animal skin into leather (Bravo, 1998). However, only during the twentieth century, thanks to modern analytical techniques, it was possible to understand what happens to the skin during the tanning process (Khanbabaee and van Ree, 2001).

Tannins are highly hydroxylated molecules, able to form complexes with carbohydrates, proteins and mineral ions. In fact, polyphenolic substances can react with skin proteins, preserving them from degradation. They can be found in especially high concentration in barks, seeds and lignificate fruits, such as achenes, and in grapevine seeds.

Plant tannins are subdivided in HT and CT. *Hydrolysable tannins* consist of an esterification between GAL (and its dimers) and oligosaccharides, mainly of glucose. These molecules can be also further condensed to form high-molecular-weight polymers. As their name indicates, these tannins are easily hydrolysed in presence of acids, alkalis and simple hot water, but also by enzymatic action. *Condensed tannins*, or proanthocyanidins, are high-molecular-weight compounds. In this case, the monomeric unit is a flavan-3-ol with a flavan-3,4-diol. Oxidative condensation occurs between the C4 of the C ring and the C6 or the C8 of the A ring of the second unit. CT and HT with high molecular weight are insoluble. Moreover, when tannins form complexes with proteins or the cell wall polysaccharides, they remain insoluble. Unfortunately, this insolubility is responsible for significant errors during the quantification analysis of the phenolic content in plants, because PP are usually analysed in extract form (Bravo, 1998).

1.2 Properties and chemical actions

All phenolic compounds, according to their –OH groups, are able to make several reactions. They are good H-donating antioxidants, and act as terminators of free radicals and chelators of metal ions (Bravo, 1998). Phenolic acids antioxidant activity is strictly related to the number and the position of hydroxyl groups, while the activity of flavonoids is generally more complicated and depends on the features and the nature of substituents on rings B and C (Balasundram et al., 2006).

Furthermore, PP, especially flavanonols, are able to bind divalent transition metal, which are catalyst in the Haber-Weiss reactions (Bravo, 1998). These reactions produce hydroxyl radicals starting from hydrogen peroxide and superoxide, and can occur inside cells, representing a potential source of oxidative stress. The overall reaction, subdivided in two sub reactions, is catalysed by iron ions, that can be stolen by flavan-3-ols (Aron and Kennedy, 2008).

PP can also bind with proteins, forming pH-dependent complexes with low solubility in water solutions. Highly polymerized tannins, like CT, are the most effective in protein precipitation, interfering with digestion and bacterial enzymes and with diet proteins (Bravo, 1998). Due to these properties, they can play important roles in several metabolic pathways,

like protein and lipid ones and, as antioxidants, they can scavenge reactive oxygen species and breaks the new-radicals-generation cycle. They act as antioxidant also by inhibiting enzymes involved in radical development (Leopoldini et al., 2004; El Gharras, 2009).

After all, phenolic compounds can interfere with microorganism growth and nutrition, in different manner, expressing antimicrobial effects. However, for the most part, these mechanisms of action still unknown and literature can't be comprehensive of that. Due to their chemical properties, PP can surely interact with microbial enzymes, inactivating them or changing their course to other pathways and they are also able to stole metallic ions, necessary to microbial growth (Smith et al., 2005; Patra and Saxena, 2011).

PP, due to their properties, play important roles in human health and nowadays are used in medicine as complementary in several therapies. Current findings have highlighted the contribute of these molecules in cardiovascular diseases, cancers and osteoporosis prevention and have suggested a role also in neurodegenerative diseases and diabetes mellitus prevention. Finally, PP present antimutagenic, antiviral, antibacterial (bactericidal, bacteriostatic), algicidal, antifungal, insecticidal, estrogenic and keratolytic activities, that may serve to protect plant from competing other organism in their biological environment. These properties can be used to take advantage in human and animal welfare, through nutrition, and in foods from animal production (Buccioni et al., 2015a; b).

2. Polyphenols in animal feeding

Plant secondary metabolites, and especially PP are largely being used through centuries, in the leather and in the naval industries. While, in food industries, these molecules have been used for a long time and widespread in the technology of wine production, as antioxidants, clarifying, chelating, bacteriostatic additive and colour stabilizers. On the contrary, the application of PP in the biomedical field is more recent, where them have been introduced as food supplements and, as therapeutic, in the treatment of certain diseases; GAL, for instance, is used as anti-haemorrhagic and antiseptic. Also, the use of polyphenolic compounds in the animal field is quite recent and mainly concerns CT. There are many studies on the use of PP in the diet of small ruminants (Vasta and Luciano, 2011) and fish (Sicuro et al., 2010) that have reported positive outcomes: improvement of production performances and of the diet energy/protein balance, through the reduction of nitrogen excretion; reduction of the production methane (CH_4) in the rumen, through the selection of digestive microorganisms; antiparasitic action at the intestinal level (Getachew et al., 2008; Sicuro et al., 2010; Vasta and Luciano, 2011).

2.1 Polyphenols and ruminants

Thanks to the microflora present in the rumen, ruminants are able to transform the dietary nutrients into nutraceutical and bioactive molecules, which will eventually be transferred in products of animal origin (meat, milk and also cheese), thanks to its peculiar digestive metabolism. The polyphenolic substances, if taken by the animals through the diet, have the ability to interact with the ruminal metabolism, modifying the interactions among different microbial strains and modulating the protein degradation (Waghorn and McNabb, 2003) or the CH_4 production (Waghorn et al., 2002). Dietary supplementation with feeds naturally rich in PP may steer rumen degradation towards more favourable metabolic pathways, preserving animal welfare and including nutraceutical component in animal derived food (Aerts et al., 1999; Buccioni et al., 2017b; Cappucci et al., 2018).

Literature shows a list of positive results when PP are used in dairy ruminants feeding: lower content of urea in the blood flow, by the modulation of ruminal protein degradation (Patra and Saxena, 2011); prevention of intestinal infections and the risk of bloat (Aerts et al., 1999); reduction of oxidative stress, by antioxidant action (Vasta and Luciano, 2011); reduction of incidence and severity of mastitis, according to a modulation of the inflammatory response. Moreover, at the ruminal level, changes in the metabolic pathways have been found, in particular, regarding: the degradation processes of the dietary fibrous components, strongly linked to the CH_4 genesis; the biohydrogenation (BH) of unsaturated fatty acids (Toral et al., 2011, 2018); the nitrogen excretion, related to the balancing of the energy/protein ratio (Patra and Saxena, 2011).

Nevertheless, it is necessary to pay attention at the dose of these substances (which varies according to the kind of PP) because detrimental phenomena can occur, such as the inhibition of some digestive processes, compromising quantity and quality of production (Hervás et al., 2003). For instance, CT and HT are different in molecular weight and structure, and have, consequently, different behaviour. Generally, CT are more resistant to microbial metabolisation, than HT, but metabolites of HT are more toxic than CT ones (Scalbert, 1991). PP toxicity is expressed through the same interaction mechanisms through which they exert their positive effects, and paying attention to the inclusion level used is essential. This explains the conflicting results found in the literature.

2.2 Temperate forages as polyphenols source

Animals naturally fed PP, by grazing pasture and feeding forages. PP abundance and their profile depends on botanical composition and plant quality. These characteristics are

influenced by environment and soil fertility, intensity of utilisation and human footprint (seeding and cultivation). Pasture PP are mostly CT, that can bind proteins, affecting voluntary feed intake, proteins and carbohydrate digestion. This matches with the natural aim of tannin synthesis, in order to protect plant against animal consumption or pathogen attack.

The principal sources of PP in temperate areas are plant belonging to *Lotus* species, as *L. pedunculatus* and *L. corniculatus* (Waghorn et al., 1987; Barry and McNabb, 1999). These essences have a different concentration of PP and consequently affect animal metabolism in a different way. CT concentration of *L. pedunculatus* is higher respect to *L. corniculatus* and this is reflected on the animal voluntary feed intake, when they grazing *Lotus* species plant. Animal grazing *L. pedunculatus* showed a low voluntary feed intake respect to the ones fed *L. corniculatus*. While, considering N intake, CT of *L. corniculatus* increased the abomasal flow and the net absorption of essential aminoacids in the small intestine, binding diet proteins (Waghorn et al., 1987; Barry and McNabb, 1999). Similarly, sulla (*Hedysarium coronarium*) have a considerable concentration of CT (about 12.5% of DM in leaves)(Cabiddu et al., 2009; Molle et al., 2009). Stienezen et al. (1996) reported an increase digestibility in animal fed with sulla and supplemented with PEG, respect to other fed the same diet without it.

Moreover, literature reported positive effects in animals grazing forage containing CT. Plant containing CT can exert positive effects on intestine parasites, as nematodes (Niezen et al., 1995; Piluzza et al., 2014).

2.3 Agro-industrial by-products as polyphenols sources

In the Mediterranean area there are several agro-industrial sectors which produce large quantities of wastes. Most of them, for their chemical composition, represent a great problem for manage and disposal, barding industries with additional costs. The current European legislation allows the re-entry into kind of these by-products, through spreading on agricultural land, but to a very limited extent, as they can cause the alteration of the soil structure and ecosystem. The most important for abundance are: skins, stalks and grape seeds, deriving from the pressing of the grapes and lees from must fermentation; pomace and vegetation waters produced during olives milling process; the so-called *pastazzo*, fruit peels deriving from the squeezing of citrus fruits; tomato peels deriving from the extraction of the pulp; chipboard and sawdust resulting from the reduction of wood in timber.

These by-products represent an unused PP source, which can be used for animal feeding, especially for ruminants.

3. Tannin extracts

Tannin extracts are derived from discard of wood and lignificate wastes, by physical or chemical extraction. They are commercialized in fluid form (with added water) or in powder and are very simple to manage, omogenized with concentrate and pelleted for animal nutrtion. When used as supplements, especially in ruminant feeding, these extracts are ingested with the diet and, in the rumen, they interact with the microbiota and the diet components. There, they may modulate protein degradation and modify the metabolic pathways of nitrogen compounds, acting on both feed and microbial proteins. In the first case the formation of insoluble tannin-protein complexes, which occurs already at the time of chewing (Jones and Mangan, 1977), prevents the enzymes from recognizing the substrate to be degraded; while, in the second case, the tannins bind directly to the proteolytic bacterial enzymes, which are therefore unable to perform their function, or to the polymers of the bacterial walls (Jones et al., 1994), inducing changes in the morphology of different species of ruminal bacteria (Chiquette et al., 1988; Jones et al., 1994). In general, the affinity of tannin molecules appears to be greater for bacterial proteins than for dietary ones (Molan et al., 1999; Min et al., 2005), although this affinity may differ if tannins are condensed or hydrolysable (Mcallister et al., 2005). Tannins used in animal feeding may have other positive effects, as: the reduction of CH_4 emission (Liu et al., 2014; Aboagye et al., 2018; Jafari et al., 2018), of the incidence of gastrointestinal parasites (Molan et al., 1999; Min and Hart, 2003) and the modulation of microbial BH (Buccioni et al., 2017a).

However, the effects of tannins on animal biology depend on numerous factors, such as the kind of extract and tannin, their concentration, animal requirements, diet composition, etc. Min et al. (2003) have shown that high concentrations of CT in fodder (> 55g / kg of DM) generally reduce the voluntary feed intake and its digestibility, and depress the rates of body growth and wool production. Therefore, it is critical to evaluate the possible side effects related to the administration of tannin extracts in animal diet.

3.1 Effects on rumen metabolism

Tannins bind to proteins forming tannin-protein complexes. It is well known that these complexes are insoluble and are formed at pH values close to neutrality (Jones and Mangan, 1977). Moreover, they are poorly degraded under anaerobic conditions (McSweeney et al., 1999). The formation of these complexes reduces the degradability of dietary proteins fed by animals (Aerts et al., 1999). This decreases the concentration of ruminal ammonia and, consequently, the synthesis of urea in the liver, the concentration of blood urea nitrogen

(Carulla et al., 2005) and the subsequent excretion of urea through the urine (Grainger et al., 2009); on other hand, after the dissociation of the tannin-protein complexes at the low pH of the abomasum (Waghorn et al., 1987), the share of digestible protein reaching the intestine, increases (Min et al., 2003).

Consequently, it is clear that the use of tannin extracts as diet supplement can reduce the accumulation of urea in blood and milk, especially in animals reared on young and fresh pasture, in which the energy/protein ratio is unbalanced in favour of the protein component (Buccioni et al., 2015b). In this case, the excess of proteins is not used to derive amino acids but degraded to ammonia, which through the portal circulation reaches the liver where it is metabolized in the urea cycle, accumulating in both milk and urine. If the production of urea becomes excessive, damage to animal health may occur, as well as an increase in the contribution of nitrogenous excretions to the environment. The tannin-protein complexation subtracts from the digestive microflora the excess share of the nitrogenous matter necessary for its metabolism (biosynthesis), modifying the energy/protein ratio of the diet; in this way, the share of not-digested protein, reaching the intestinal tract is greater with a consequent reduction in urea production.

Another important advantage of tannins in ruminant feeding is represented by the modulation of methanogenesis (Waghorn et al., 2002; Piñeiro-Vázquez et al., 2015). Furthermore, over the past 20 years, non-chemical anti-parasitic strategies, based on the use of fodder containing tannins, have been developed (Niezen et al., 1995), reducing both the incidence of gastrointestinal parasites and the impact they have on animal health. Nowadays, literature reported that the use of PP and tannin extracts or plant naturally rich in PP may affect methanogen bacteria and, consequently, CH_4 excretions. Both CT and HT extracts had shown an action on total methanogens bacteria population *in vitro* with a significative reduction of CH_4 emission (HT from chestnut and sumach, ↓ 7.7-14.4% and CT from mimosa and quebracho, ↓ 6.3-8.9%; Jayanegara et al., 2015). This effect seems to be independent from the kind of PP and from its dose, because also other PP source have shown *in vitro* the same effects (pure flavonoids, ↓ 8.1-38%; Oskoueian et al., 2013).

The action of tannins on methanogens and protozoa rumen communities is responsible also of changes on volatile fatty acid (VFA) production. However, in this case, the effects are dose and kind dependent. CT seems to be able to reduce VFA production with an increase of acetate acid and a decrease of propionate and butyrate acids (action on *Methanobrevibacter ruminantium*; Tavendale et al., 2005 in an *in vitro* study). While, HT evidence, with a reduction of total VFA, a low concentration of acetate and a higer concentration of propionate

and butyrate acids (action on total methanogen population, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*; Jayanegara et al., 2015 in an *in vitro* study).

3.2 Antinutritional effects

The ability of tannins to complex proteins is responsible for many effects. Tannins may have an antinutritional effect, since they are able to bind salivary enzymes, causing astringency (Murdiati et al., 1992; Mueller-Harvey, 2006), and to digestive enzymes (Makkar, 2003; Gemede and Ratta, 2014). Moreover, if ingested in large quantities and for long time, they can be hepatotoxic, cause of the inactivation of liver enzymes, favouring the consequent inhibition of the metabolic pathways in this district (Mueller-Harvey, 2006). In the rumen, tannins are partially degraded and HT metabolites are much more toxic than those of CT. For istance, GALis decomposed in the rumen to pyrogallol, becoming a source of Carbon for the ruminal flora and turning into resorcinol, a toxic molecule; moreover, the ellagic acid (which can be formed in the rumen from the GAL) is much more toxic than resorcinol. Some rumen bacteria, such as the *Eubacterium oxidoriducens*, are able to completely degrade the GAL, as source of Carbon; however, they are in little percentage and, therefore, are not able to set off the dietary intake of this substance (Murdiati et al., 1992). For this reason, dietary inclusion level represents a key point in nutritional integration with tannins, because an excessive quota could cause problems to animal physiology (Piluzza et al., 2014). In contrast, tannins at moderate concentrations may have beneficial effects on animal health, such as the prevention of meteorism, tympanism, lameness and intestinal parasites, especially for ruminants rared on young pasture (Min et al., 2003).

4. Olive oil pomace

Pomaces are a by-product derived from olive milling processes and are available in large quantities in whole the Mediterranean area. They represent a low-cost source of bioactive compounds, since most of the PP present in the olive fruit are lost during the oil extraction and are found in the processing waste, such as the pomace. However, the quantity and the kind of PP present in milling biowaste depend on the cultivar, the fruit maturity, the processing technique, the climate and storage conditions. The major phenolic compounds found in olive oil pomace (OOP) are hydroxytyrosol, oleuropein, tyrosol, caffeic, gallic, p-coumaric and vanillic acids and rutin (Cioffi et al., 2010; Morsi et al., 2016). These PP are responsible for the antioxidant capacity of virgin olive oil, even though only 1% of them remains after the milling process; the other 99% is lost in vegetation water and pomace (Cioffi

et al., 2010). Especially hydroxytyrosol seems exert a high antioxidant capacity (Casalino et al., 2002).

The recycling of this by-product in animal feeding represent a great advantage, since it is a low-cost resource with high nutritional highs.

4.1 Effects of milling by-products on rumen metabolism

OOP and the other olive milling by-products may affect rumen metabolism and modulate microbial community profile. However, there is a lack of knowledge about how these matrixes act on rumen ecology. There are several studies on the supplementation with OOP that highlighted positive effects on animal growth, milk and meat quality, hypothesizing that pomace PP affect rumen BH with an increase of PUFA in food of animal origin (Chiofalo et al., 2004; Sadeghi et al., 2009; Terramoccia et al., 2013; Pallara et al., 2014).

4.2 Technical problems, contraindications and antinutritional effects

Olive oil bio-wastes used in animal feeding present some technical problems and contraindications. Firstly, these matrixes have a variable composition, that depends on cultivar, climate and milling process. Vegetation waters have a low DM (<10%), but are rich in soluble PP. In contrast, pomaces are more complex matrixes that contain less water (30-75%), may contain a high percentage of fiber, and their concentation of PP may vary according to water presence (Mulinacci et al., 2001). Thus, it is fundamental to analyse the proximate composition of these by-products before utilizing them in animal nutrition and formulating a balanced diet; it is also important to evaluate PP profile and concentration. Secondly, especially OOP, have a low nutritive value, since it has a high percentage of acid detergent fibre (ADF) and lignin, because contains olive stones (Yansari et al., 2007).

Moreover, since olive milling wastes (wet OOP and vegetation water) have a high content of water, they need of a special management to be included in animal diet. Phenolic extracts are originated from vegetation waters which have a high PP concentration, but they are not so commercially common. In contrast, pomaces, could be used after easy treatment. They are dryed, grinded and then pelleted with the other diet ingredients. Finally, for wetter pomaces, they are absorbed on powdered concentrate as dryed alphalpha hay. Moreover, in some cases, new milling technologies permitted to destoned pomaces (Amirante et al., 2006).

Other perplexities on the use of milling by-products concern their seasonal presence on the market and the palatability for the animal, since they may alter concentrate flavour.

OBJECTIVE

The increasing demand of livestock food products, due to human population growth, had led to more intensive animal managements. This approach involves high protein feed, in order to push animal growth, perturbing rumen fermentation and rising the gas excretion. It is not so simple to manage the rumen environment, that act like a big fermenter with over 200 microbial species, which are in mutual relationship and in competition for nutritional substrates.

In addition to a better balance of the diet, the use of natural supplements could improve animal health and ruminal metabolism. PP are a valuable tool to modulate feed digestion and microbiota metabolism. Thus, the main objective of the present PhD thesis is to improve the knowledge about the effect exerted by PP substances on rumen microbial community, responsible of nutrient fermentation. This knowledge will be useful to setting up animal nutrition strategies, aimed to improve environmental sustainability of animal livestock production, also through the recycling of bio-wastes from agri-food industries, such as chestnut tannin and OOP.

Eventually, an unconventional approach to animal livestock production that include feed strategies with by-products supplementation match with FAO spotlight on environment and with European Community policy about the recycling of wastes from agriculture.

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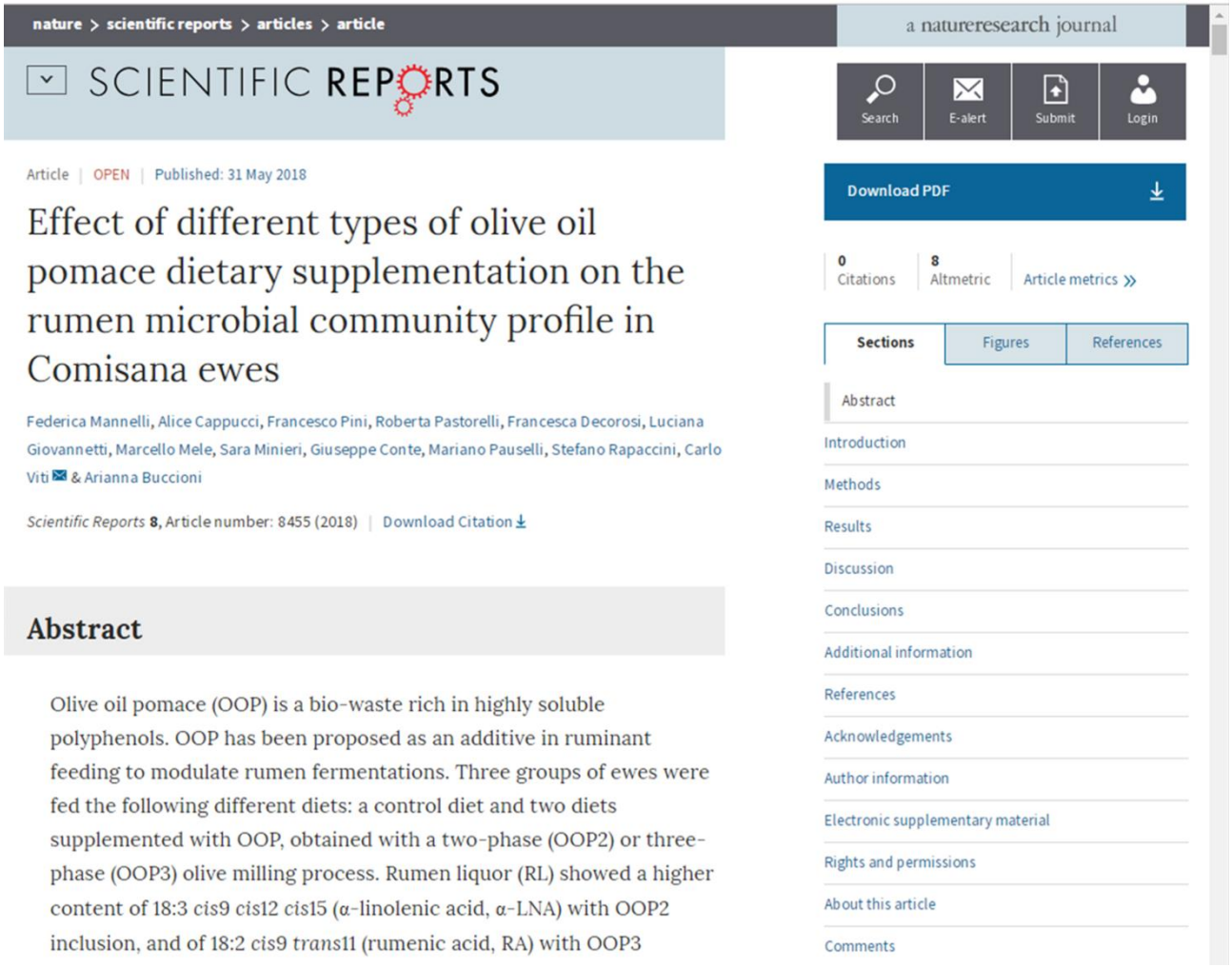
TRIAL 1

Effect of different types of olive oil pomace dietary supplementation on the rumen microbial community profile in Comisana ewes. (Federica Mannelli, Alice Cappucci, Francesco Pini, Roberta Pastorelli, Francesca Decorosi, Luciana Giovannetti, Marcello Mele, Sara Minieri, Giuseppe Conte, Mariano Pauselli, Stefano Rapaccini, Carlo Viti and Arianna Buccioni) Received: 30 November 2017; Accepted: 9 May 2018 on Scientific reports (www.nature.com/scientificreports); DOI:10.1038/s41598-018-26713-w



Author Contributions

Conceived the experiments: A.B., M.M., L.G., S.R. and C.V.; Animals care and sampling: F.M., A.C., S.M. and M.P.; Metataxonomic analysis: F.P., F.D. and R.P.; Fatty acid analysis: F.M., A.B. and A.C.; Statistical analysis: F.M., A.C., F.P. and G.C.; Wrote the manuscript: F.M., F.P., A.B. and C.V.

Abstract

Olive oil pomace (OOP) is a bio-waste rich in highly soluble polyphenols. OOP has been proposed as an additive in ruminant feeding to modulate rumen fermentations. Three groups of ewes were fed
the following different diets: a control diet and two diets supplemented with OOP, obtained with a two-phase (OOP2) or three-phase (OOP3) olive milling process. Rumen liquor (RL) showed a higher content of 18:3 *cis*9 *cis*12 *cis*15 (α-linolenic acid, α-LNA) with OOP2 inclusion, and of 18:2 *cis*9 *trans*11 (rumenic acid, RA) with OOP3 inclusion. The overall composition of the RL microbiota did not differ among treatments. Significant differences, between control and treated groups, were found for six bacterial taxa. In particular, RL microbiota from animals fed OOPS showed a reduction in *Anaerovibrio,* a lipase-producing bacterium. The decrease in the *Anaerovibrio* genus may lead to a reduction in lipolysis, thus lowering the amount of polyunsaturated fatty acids available for biohydrogenation. Milk from animals fed OOPS showed a higher content of 18:1 cis9 (oleic acid, OA) but the α-LNA concentration was increased in milk from animals treated with OOP2 only. Therefore, inclusion of OOP in ruminant diets may be a tool to ameliorate the nutritional characteristics of milk.

Increasing the content of polyunsaturated fatty acids (PUFAs) in ruminant derived products is particularly important for human health (i.e., decrease in plasma cholesterol and low-density lipoprotein-cholesterol). Different approaches are used to achieve this result, including the use of diet supplements, such as vegetable oils and other natural compounds, to alter the rumen microbiota[1]. Adding extruded linseed to ewe diets approximately doubles the contents of 18:2 *cis*9 *trans*11 (rumenic acid, RA), 18:1 *trans*11 (vaccenic acid, VA) and 18:3 *cis*9 *cis*12 *cis*15 (α-linolenic acid, α-LNA) acids in milk[2]. The combination of extruded linseed with natural bioactive compounds may further boost milk quality[3,4]. Olive oil pomace (OOP) is the main by-product of olive oil manufacturing and constitutes an important source of nutraceutical molecules with antioxidant and antimicrobial activities, including polyphenols (flavonoids, anthocyans, cyanidins and phenolic acids), tyrosol, hydroxytyrosol and oleuropein[5–8]. OOP requires specific management and storage because it cannot be directly disposed of in the environment[9]. The chemical characteristic of this bio-waste could be valorized by ruminant metabolism[10]. Indeed, several studies have demonstrated that dietary supplementation with OOP at low concentrations in small ruminant diets increases the yield and nutritional quality of milk without having a negative influence on animal welfare, as ewes have a higher sensitivity to polyphenols than goats[11,12]. In an *in vitro* trial, Pallara et al.[13] demonstrated that OOP affects rumen biohydrogenation (BH) of PUFAs, especially linoleic acid (18:2 *cis*9

*cis*12; LA). Other matrices rich in polyphenols, such as tannins from wine peels, chestnut wood or quebracho seeds, are already used in ruminant feeding strategies to improve milk and meat quality with significant results[3,4,14,15]. OOP is usually produced with a three-phase (OOP3) or two-phase (OOP2) decanter. These two bio-wastes differ in their chemical and physical properties, and OOP2 is richer in polyphenols because phenol washing is limited[16]. It is hypothesized that inclusion of the two OOPs in a diet may have similar but not identical effects on the rumen microbiota and the BH process. Hence, this study aimed to investigate the effects of these two different OOPs added to ewe diets on rumen liquor (RL) microbiota, RL fatty acid (FA) and the milk FA profile.

Methods

Experimental design. Twenty-four multiparous Comisana ewes at 97 ± 12 days in milking (kept with the experimental flock of the Department of Agriculture, Food and Environmental Sciences at University of Perugia) were allotted into 3 experimental groups (8 animals per pen), with similar body weight (65 ± 8 kg) and milk yield (735 ± 15 g/day). The trial lasted 28 days, after 15 days of adaptation to the new diets.

OOP characterization. The OOPs used as supplementation in this trial were obtained from local virgin olive oil producers, processed according to Servili et al.[17] and pitted. OOP2 was derived from a two-phase milling process, resulting in a considerable percentage of water (approximately 75%). To make this matrix more technologically suitable, the pomace was adsorbed on ground dried alfalfa to be pelleted with the other ingredients of the concentrate (CMS-IEM – Colognola ai Colli, Verona, Italy). OOP3 was derived from a three-phase process resulting in a low content of water (approximately 55%). The total amount and characterization of OOP polyphenols in the experimental diets were determined by HPLC analysis according to Mele et al.[18] (Supplementary Tables S1 and S2).

Table S1. Ingredients (g/kg of DM[1]) of the experimental diets used.

Ingredients (g / kg of DM[1])	**Experimental concentrates**		
	C[1]	**COOP2[1]**	**COOP3[1]**
Wheat bran	201.9	20.2	201.9
Corn	102.3	30.7	102.1
Broad bean	30.6	30.7	20.4
Sunflower meal	10.2	25.6	81.7
Corn gluten	10.2	20.4	10.2

Dehydrated alfalfa	201.9	272.9	-
Barley	126.2	50.5	80.8
Molasses	50.1	50.2	50.1
Stoned olive pomace	-	272.9	227.1
Extruded linseed	199.6	199.8	199.6
Olive oil	40.8	-	-
$CaCO_3$	10.4	10.4	10.4
Sodium bicarbonate	5.2	5.2	5.2
Di-calcium phosphate	5.2	5.2	5.2
Sodium chloride	5.2	5.2	5.2

[1]DM = Dry matter; C = control diet; COOP2 = control diet added with OOP extracted with a two-phase procedure; COOP3 = control diet added with OOP extracted.

Diet composition. Diets were composed of chopped alfalfa hay (particle size >3 cm in length) administered ad libitum with 800 g/head/day of a concentrate formulated to contain the same amount of OOPs as follows: 10 g/100 g of dry matter (DM) of extruded linseed as an α-LNA source (control, diet C), 10 g/100 g of DM of extruded linseed and 13.5 g/100 g of DM of OOP2 (diet COOP2), or 10 g/100 g on DM of extruded linseed and 11.25 g/100 g of DM of OOP3 (diet COOP3) with 100 g/head/day of rolled barley. All concentrates were obtained by pelleting the ingredients (diameter was 5 mm) and offered in two equal doses with rolled barley, during each milking at 7:30 a.m. and 5:30 p.m. (Supplementary Tables S1 and S2). The experimental diets were formulated to be isoproteic and isoenergetic according to the nutrient requirements of an ewe weighing 68 kg and producing 1 kg of milk at 6.5% fat[19]. Animals had free access to water. Dry matter intake (DMI) of concentrates and hay was registered daily and individually on the basis of residuals.

Table S2. Chemical composition (g/kg of DM1) of the experimental diets used in this trial.

	Alfalfa hay	**Rolled barley**	**Experimental concentrates**		
			C[1]	**COOP2[1]**	**COOP3[1]**
Chemical composition (g / kg of DM[1])					
CP	139.9	118.6	148.9	153.8	149.1
EE	11.7	34.4	129.6	121.4	127.4
NDF	499.8	269.3	290	318.4	300.5
ADF	349.7	102.5	149	195.2	151.7
ADL	110.6	24.4	37.7	78.8	97.8
Ash	77.4	26.8	68.1	91.8	64.7
FA (% of total FAME)					
C16:0	24.5	23.6	9.0	7.8	8.6
C16:1	2.8	1.2	0.4	0.2	0.3
C18:0	5.1	1.8	2.7	2.8	2.6

C18:1 *cis*-9	5.4	18.8	34.1	36.6	33.9
C18:2 *cis*-9,*cis*-12	21.0	47.4	19.4	16.4	19.9
C18:3 n-3	39.6	4.4	31.5	33.6	31.8
SFA	31.2	27.5	12.0	10.9	11.4
MUFA	8.2	20.0	36.3	38.0	36.0
PUFA	60.6	51.8	51.6	51.0	52.2
Polyphenol profile (g/kg of DM[1])					
3,4-DHPEA	-	-	-	1.2	1.0
p-PEA	-	-	-	0.3	0.2
Verbascoside	-	-	-	1.1	0.3
3,4-DHPEA-EDA	-	-	-	2.1	1.0
Rutin	-	-	-	0.2	0.1
Total polyphenols	-	-	-	4.9	2.7

[1]DM = Dry matter; C = control diet; COOP2 = control diet added with OOP extracted with a bi-phasic procedure; COOP3 = control diet added with OOP extracted with a three-phasic procedure; FA = Fatty acid; FAME = Fatty acid methyl ester.

Rumen liquor (RL) sampling and fatty acid (FA) and dimethyl acetal (DMA) determination. At day 28, RL samples were individually collected by a stomach tube, connected to a manual pump, after overnight fasting and before morning feeding[20,21]. Animals were fasted before rumen sampling to facilitate the introduction of the stomach tube in the rumen as common in the veterinary practice. Five samples from each animal were collected and examined visually and tactilely to check the presence of saliva contamination. Samples from each animal were then combined, strained through a cheesecloth and allotted (20 ml)[20]. Two ewes (one belonging to the C group and one belonging to the COOP3 group) were not considered at sampling time due to diseases. Immediately after collection, each sample was measured for pH, divided into 2 aliquots and stored at −80 °C until analysis. One aliquot was freeze-dried and used for FA and DMA identification according to Alves et al.[22]. DMAs are secondary artifacts formed during the methylation of microbial fatty acid methyl esters (FAMEs) derived from bacterial plasmalogen lipids contained in the external membrane and, hence, strictly related to microbial species[22]. First, FAs were trans-esterified[22,23] using 5:0 and 19:0 (1 mg/ml) as internal standards. FA and DMA fractions were then separated by thin-layer chromatography (TLC). The DMAs were identified by GC/MS22, while the FAME profile was determined using a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD), equipped with a flame-ionization detector and a high-polarity fused-silica capillary column (Chrompack CP-Sil 88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness of 0.20 μm). The programming used has been previously described by Buccioni et al.[3] (specifications are also available in the supplementary information).

DNA extraction, PCR amplification, illumina MiSeq sequencing and sequencing data processing. DNA was extracted from 1 ml of RL using a Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH) with the following modifications: 1 ml of RL was thawed and vortexed for 30 s; 185 µl of RL was then mixed lysis buffer, and the mixture was then added to a tube containing the lysis matrix and homogenized with a Retsch MM300 disrupter (90 s at 30 cycles/s). Samples were incubated for 20 min at 70 °C and centrifuged at 14,000 × g at 4 °C. The supernatant was recovered and processed according to the manufacturer's specification. DNA integrity was verified by agarose gel electrophoresis. DNA purity and quantity were measured using a ND-1000 Spectrophotometer (NanoDrop Technologies, Labtech, Ringmer, UK) and standardized to a concentration of 10 ng/µl. For each sample, the V3-V4 region of the 16S rRNA gene was amplified with Pro341f and Pro805R primers[24], and barcodes were added to the forward primer (Supplementary Table S3). Amplicons for each library were purified and mixed in equal proportions. Illumina MiSeq v3 chemistry 300 base paired-end (PE) sequencing was performed at BMR Genomics (Padova, Italy). MiSeq 300 PE sequencing produced a total of 3,983,079 reads. Reads were merged with FLASh v1.2.11[25] with the following parameters: -m 20, -M 280, and Phred score default of 33, resulting in 3,362,386 reads correctly aligned reads. The sequences were then trimmed to discard primers with Prinseq-lite[26], and sequences shorter than 200 bp were filtered out. Chimeras were removed with USEARCH 6.1[27]. Open reference OTU picking was performed with SUMACLUST within QIIME 1.9.1[28] using a similarity threshold of 0.97 and Greengenes 13.8[29] as a reference database. OTUs representing less than 0.005% of the total read abundance were discarded[30]. Sequences identified as chloroplasts, mitochondria and unassigned sequences (approximately 5% of sequences in each library) were removed from further analysis. A total of 1,003,318 high-quality sequences were obtained with an average of 45,605 ± 12,742 sequences per sample, and libraries were then rarefied to 30,000 sequences per sample. QIIME tables at different taxonomic levels are available in Supplementary Tables S4–S8 (at the end of the paper).

Table S3. Primer and barcode sequences used in this work.

Sample ID	BarcodeSequence	LinkerPrimerSequence	Treatment	Reverseprimer	Description
CD-01	AAAAAAAAAAAACCCC	TCCTACGGGAGGCAGCAGT	C[1]	TCCTACGGGAGGCAGCAGT	MicrobIT10
CD-02	AAAAAAAAAAAAGGGG	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT16
PSD-01	AAAAAAAAAAAAAACC	TCCTACGGGAGGCAGCAGT	COOP3[1]	TCCTACGGGAGGCAGCAGT	MicrobIT6
CD-03	TAAAAAAAAAAAAACC	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT22
CD-04	AAAAAAAAAAAAAAAA	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT21
CSD-01	AAAAAAAAAAAAAATT	TCCTACGGGAGGCAGCAGT	COOP2[1]	TCCTACGGGAGGCAGCAGT	MicrobIT3
CD-05	AAAAAAAAAAAAAAGG	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT20
CD-06	TTAAAAAAAAAAAAAA	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT5
PSD-	GGCCAAAAAAAAAAAA	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT1

02					
CSD-02	AAAAAAAAAAAATTAA	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT13
CD-07	AAAAAAAAAAAAGGTT	TCCTACGGGAGGCAGCAGT	C	TCCTACGGGAGGCAGCAGT	MicrobIT19
PSD-03	AAAAAAAAAAAAGGCC	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT18
PSD-04	AAAAAAAAAAAACCAA	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT9
CSD-03	AAAAAAAAAAAATTCC	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT14
PSD-05	AAAAAAAAAAAATTGG	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT12
CSD-04	AAAAAAAAAAAACCTT	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT11
CSD-05	TTCCAAAAAAAAAATT	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT7
PSD-06	GGAAAAAAAAAAAACC	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT2
CSD-06	TTAAAAAAAAAAAAGG	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT4
CSD-07	AAAAAAAAAAAATTTT	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT15
CSD-08	AAAAAAAAAAAACCGG	TCCTACGGGAGGCAGCAGT	COOP2	TCCTACGGGAGGCAGCAGT	MicrobIT8
PSD-07	AAAAAAAAAAAAGGAA	TCCTACGGGAGGCAGCAGT	COOP3	TCCTACGGGAGGCAGCAGT	MicrobIT17

[1]C = control diet; COOP2 = control diet added with OOP extracted with a bi-phasic procedure; COOP3 = control diet added with OOP extracted with a three-phasic procedure.

Milk sampling and analysis. Individual milk samples were collected weekly, during the morning and evening milking. Milk samples were gathered in a single sample according to the morning and afternoon yield and subsequently split into two aliquots for analysis. The first aliquot was processed to evaluate fat, lactose, protein and urea contents using a Milkoscan 6000 FT (Foss Electric, Hillerød, Denmark) and to determine the somatic cell count (SCC) according to ISO 13366-2/IDF 148-2 (ISO-IDF, 2006) using a Fossmatic 5000 (Foss Electric). Somatic cell count data were expressed as a linear score (LS) according to Shook et al.[31] as follows: $LS = \log_2 (SCC/12{,}500)$. Milk production was standardized as fat-corrected milk (FCM) at 6.5% fat according to Pulina and Nudda[32]. The second milk sample aliquots were analyzed for FA composition. Milk fat was extracted as reported by Buccioni et al.[3], methylated according to Christie[33] with nonanoic (C9:0) and nonadecanoic (C19:0) acid methyl ester (Sigma Chemical Co., St. Louis, MO) as the internal standards and analyzed by gas chromatography using the same program as described for RL samples.

Statistical analysis. Data on RL FA and DMA were analyzed by the following general linear model:

$$y_i = \mu + diet_i + e_{ij}$$

where y is the observation, μ is the overall mean, diet is the fixed effect of i^{th} diet (i = 1 to 3), and e_{ij} is the residual error (SAS 9.2, 2013)[34].

Data related to animal performances, milk composition and yield recorded over the course of the trial were processed as a completely randomized design with repeated measures using the following linear mixed model (SAS 9.2, 2013)[34]:

$$y_{ijk} = \mu + D_i + P_j + (D \times P)_{ij} + A_k[D_i] + e_{ijk}$$

where y is the observation, μ is the overall mean, D_i is the fixed effect of diet (i = 1 to 3), P_j is the fixed effect of sampling time (j = 1 to 4), $(D \times P)_{ij}$ is the interaction between diet and sampling time, A_k is the random effect of the animal nested within the diet (k = 1 to 8), and e_{ijk} is the residual error. The covariance structure was compound symmetry, which was selected based on Akaike's information criterion of the mixed model of SAS[34]. Statistical significance of the diet effect was tested against variance of ewe nested within diet according to repeated measures design theory[35]. Multiple comparisons among means were performed using the Tukey test[34].

Rarefaction analysis was performed using observed OTUs with 10 iterations at each sampling depth. α-diversity was estimated using observed OTUs, Chao1 value and Shannon index within QIIME. Effects of different diets on FA concentrations, and the relative abundances of different taxa were analysed using a one-way ANOVA with Tukey's HSD post hoc comparison procedure available within the agricolae package in R[36]. A non-metric multi-dimensional scaling (nMDS) plot was constructed using the OTU table with the Bray-Curtis index within PAST[37]. Microbial community profiles were further evaluated with multivariate statistical tests within PAST[37]: One-way analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were performed using Bray-Curtis and Dice indexes (9,999 permutation test).

Pairwise correlation among bacterial taxa and FA or DMA composition was performed out by multivariate analysis (SAS, 9.2, 2013).

Use of experimental animals. All experiments in this study were performed in accordance with the

approved guidelines from the European directive 2010/63/UE and DL 4/03/2014 n 26 comma g. All experimental protocols requiring animal handling and the collection of samples were approved by the Institutional Animal Care and Use Committee of University of Perugia.

Accession codes. The 16S rRNA gene amplicon sequence data supporting the conclusions of this article are available at the National Centre for Biotechnology Information Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) bioproject number PRJNA397032, under the following SRA experiment accession numbers: SRR5895869 – SRR5895890.

Results

Animal performances and influence of polyphenol-enriched diets on rumen liquor FA and DMA. Three experimental groups of ewes were fed different diets based on alfalfa and extruded linseed as the α-LNA source. All diets were balanced and formulated according to the nutrient requirements of lactating ewes. A control diet (C) without any polyphenol supplementation and two treated diets including OOP obtained by a two-phase or a three-phase milling process (COOP2 and COOP3, respectively, Supplementary Table S1) were generated. During the trial, the administered concentrate was completely consumed by the animals regardless of the treatment (800 g/head/day). The average DMI was 2.26, 2.10 and 2.27 kg/head/day (SEM = ±0.19; P = 0.47) for the C, COOP2 and COOP3 groups, respectively.

The FA profile of RL from all groups was obtained. Significant differences ($P < 0.05$) between the control and treated groups were related to RA and α-LNA (Table 1). In particular, RA was significantly higher only in the RL of the COOP3 group, and α-LNA was significantly higher in ewes fed with COOP2 (45% increase respect to C group for both FAs). Moreover, the C16:1 *cis*7 content in the COOP3 group was lower than that in the other groups (Table 1).

Table 1. Effect of olive oil pomaces on FA[1] production in rumen liquor.

	Diet			
	g/100 g of FAs[1] ± SEM[1]			
FA[1]	C[1]	COOP2[1]	COOP3[1]	P[2]
12:0	0.131 ± 0.019	0.158 ± 0.0178	0.114 ± 0.019	0.261
13 iso	0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.001	0.583
13:0	0.028 ± 0.012	0.060 ± 0.011	0.032 ± 0.012	0.140
14 iso	0.084 ± 0.005	0.092 ± 0.005	0.089 ± 0.005	0.485
14:0	0.420 ± 0.081	0.641 ± 0.076	0.396 ± 0.081	0.074
15 iso	0.527 ± 0.042	0.525 ± 0.040	0.546 ± 0.042	0.930

15 ante	0.381 ± 0.022	0.388 ± 0.020	0.393 ± 0.022	0.928
15:0	0.462 ± 0.029	0.532 ± 0.027	0.460 ± 0.029	0.148
16 iso	0.211 ± 0.020	0.223 ± 0.019	0.226 ± 0.020	0.852
16:0	9.386 ± 0.739	8.034 ± 0.691	9.068 ± 0.739	0.388
17 iso	0.189 ± 0.014	0.189 ± 0.013	0.184 ± 0.014	0.963
16:1 cis7	0.191 ± 0.040 a	0.237 ± 0.037 a	0.090 ± 0.040 b	0.043
16:1 cis9	0.032 ± 0.004	0.039 ± 0.004	0.032 ± 0.004	0.386
17 ante	0.264 ± 0.028	0.296 ± 0.026	0.297 ± 0.028	0.628
17:0	0.256 ± 0.016	0.245 ± 0.015	0.252 ± 0.016	0.885
18:0	25.494 ± 2.363	20.677 ± 2.210	25.502 ± 2.363	0.245
OA (18:1 cis9)[1]	4.598 ± 0.470	5.022 ± 0.440	5.183 ± 0.470	0.666
18:1 cis11	0.333 ± 0.087	0.188 ± 0.081	0.220 ± 0.087	0.464
18:1 cis12	0.157 ± 0.025	0.153 ± 0.023	0.144 ± 0.025	0.937
18:1 cis13	0.033 ± 0.004	0.030 ± 0.004	0.034 ± 0.004	0.763
18:1 cis15	0.167 ± 0.028	0.266 ± 0.026	0.211 ± 0.028	0.053
18:1 trans5	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.001	0.989
18:1 trans6-8	0.095 ± 0.011	0.080 ± 0.011	0.086 ± 0.011	0.660
18:1 trans9	0.050 ± 0.005	0.041 ± 0.005	0.046 ± 0.005	0.514
18:1 trans10	0.075 ± 0.012	0.047 ± 0.012	0.052 ± 0.012	0.264
VA (18:1 trans11)[1]	0.382 ± 0.103	0.239 ± 0.097	0.167 ± 0.103	0.344
18:1 trans12	0.065 ± 0.008	0.058 ± 0.007	0.069 ± 0.008	0.602
18:1 trans15	0.066 ± 0.009	0.061 ± 0.008	0.071 ± 0.009	0.686
18:1 trans16	0.631 ± 0.72	0.464 ± 0.067	0.623 ± 0.072	0.183
18:2 trans9 trans12	0.106 ± 0.011	0.116 ± 0.010	0.113 ± 0.011	0.825
LA (18:2 cis9 cis12)[1]	0.582 ± 0.054	0.695 ± 0.051	0.712 ± 0.054	0.208
18:3 trans9 trans12 trans15	0.048 ± 0.016	0.021 ± 0.015	0.049 ± 0.016	0.349
20:0	0.320 ± 0.026	0.293 ± 0.025	0.302 ± 0.026	0.755
α-LNA (18:2 cis9 cis12 cis15)[1]	0.343 ± 0.029 b	0.496 ± 0.023 a	0.441 ± 0.029 ab	0.004
RA (18:2 cis9 trans11)[1]	0.211 ± 0.021 b	0.216 ± 0.020 b	0.307 ± 0.021 a	0.007
21:0	0.083 ± 0.011	0.084 ± 0.010	0.079 ± 0.011	0.944
22:0	0.188 ± 0.014	0.196 ± 0.013	0.194 ± 0.014	0.906
23:0	0.103 ± 0.005	0.094 ± 0.005	0.087 ± 0.005	0.162
24:0	0.203 ± 0.014	0.217 ± 0.013	0.217 ± 0.014	0.721

[1]Acronyms used in this table: FA (fatty acid), C (control diet), COOP2 (control diet added with olive oil pomace extracted with a two-phase procedure), COOP3 (control diet added with olive oil pomace extracted with a three-phase procedure), OA (oleic acid), VA (vaccenic acid), LA (linoleic acid), α-LNA (α linolenic acid), RA (rumenic acid) and SEM (Standard Error Mean). [2]Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

Total DMA concentration did not vary among groups, and DMA16:0 was the most abundant DMA for all three diets (Table 2). Significant differences were found among the DMA profiles (3 out of 18 DMAs analyzed). DMA13:0 was higher in the RL of the C group

than in the COOP2 and COOP3 groups. DMA18:0 reached the highest value when OOPs were included in the concentrates. DMA17:0 increased with OOP diet inclusion, with the highest value found in the COOP3 group (Table 2).

Table 2. Total DMA1 (mg/g dry matter ± SEM1) and DMA composition (g/100 g of total DMA ± SEM1) of rumen liquor.

	Diet			
DMA	C[1]	COOP2[1]	COOP3[1]	P[2]
Total DMA	1.507 ± 0.118	1.775 ± 0.111	1.607 ± 0.119	0.267
DMA12:0	0.798 ± 0.218	1.244 ± 0.204	1.027 ± 0.218	0.348
DMAi13:0	2.434 ± 0.674	2.405 ± 0.630	1.931 ± 0.674	0.837
DMA13:0	1.149 ± 0.123 a	0.729 ± 0.115 b	0.587 ± 0.123 b	0.012
DMAi14:0	6.039 ± 0.328	5.602 ± 0.307	5.202 ± 0.328	0.223
DMA14:0	8.044 ± 0.394	7.296 ± 0.369	6.785 ± 0.394	0.102
DMAi15:0	1.157 ± 0.204	1.391 ± 0.191	1.237 ± 0.204	0.697
DMAa15:0	5.639 ± 0.456	4.976 ± 0.435	4.712 ± 0.465	0.366
DMA15:0	6.168 ± 0.161	6.381 ± 0.151	6.010 ± 0.161	0.262
DMAi16:0	1.756 ± 0.233	1.655 ± 0.218	2.024 ± 0.233	0.507
DMA16:0	46.422 ± 1.754	46.625 ± 1.641	49.001 ± 1.754	0.518
DMA16:1	6.364 ± 0.763	6.209 ± 0.714	5.237 ± 0.763	0.534
DMAa17:0	2.617 ± 0.206	2.120 ± 0.192	2.419 ± 0.206	0.230
DMA17:0	0.482 ± 0.331 c	1.564 ± 0.310 b	2.989 ± 0.331 a	<0.001
DMA18:0	2.481 ± 0.144 b	3.127 ± 0.135 a	3.051 ± 0.144 a	0.009
DMA18:1t11	0.637 ± 0.070	0.693 ± 0.065	0.667 ± 0.070	0.844
DMA18:1c9	3.115 ± 0.380	3.901 ± 0.356	3.349 ± 0.380	0.317
DMA18:1c11	1.914 ± 0.244	1.836 ± 0.229	1.924 ± 0.244	0.958
DMA18:1c12	0.146 ± 0.074	0.334 ± 0.069	0.183 ± 0.074	0.166
DMA17:1	2.637 ± 0.540	1.910 ± 0.505	1.665 ± 0.540	0.430

[1]Acronyms used in this table: DMA (dimethyl acetal), C (control diet), COOP2 (control diet added with olive oil pomace extracted with a two-phase procedure), COOP3 (control diet added with olive oil pomace extracted with a three-phase procedure) and SEM (Standard Error Mean). [2]Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

Metataxonomy of rumen liquor (RL). The results of RL microbiota sequencing produced a total of 1,813 OTUs (based on 97% nucleotide sequence identity). In each sample, a similar number of OTUs was observed, with an average of 1,452 OTUs (ranging from 1,190 to 1,566). Rarefaction curves showed high sequencing coverage for all the samples (Supplementary Fig. S1).

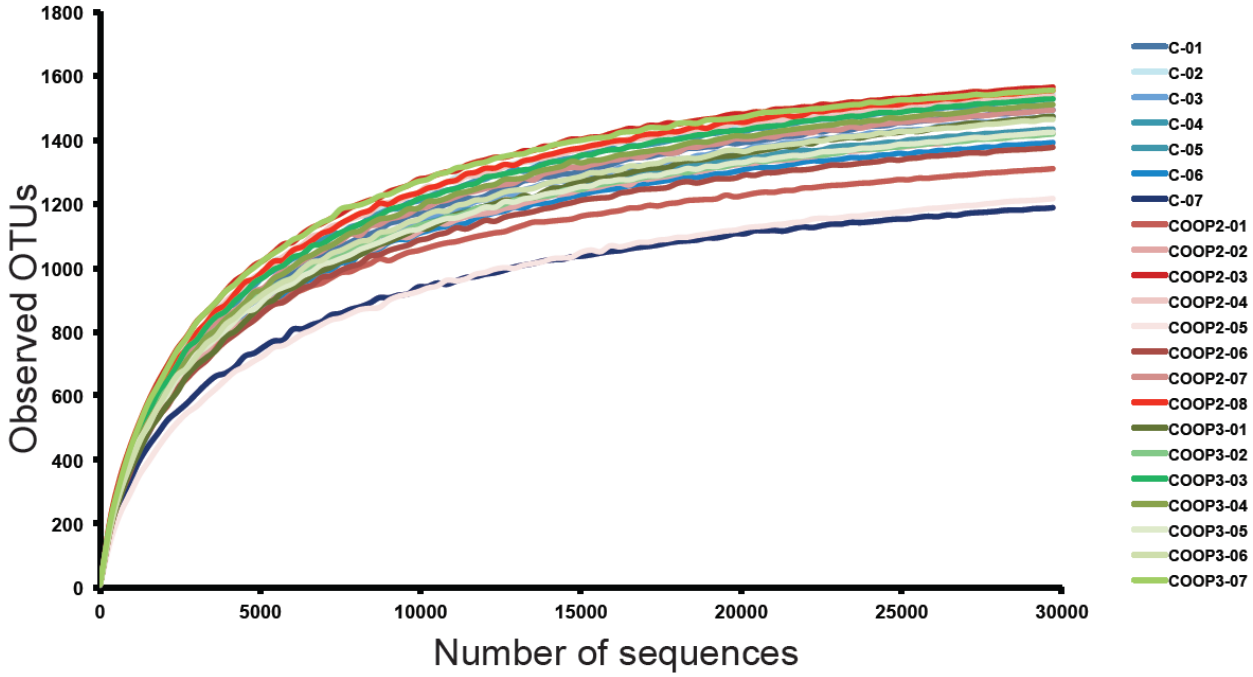


Figure S1. Sample-based rarefaction curves representing the number of observed OTUs at different sequencing depths (each point is the average of 10 iterations). RL microbiota are labeled respect to the ewe diet regimen (C = control diet; COOP2 = control diet added with OOP extracted with a two-phase procedure; COOP3 = control diet added with OOP extracted with a three-phase procedure).

The α-diversity was calculated from the number of OTUs observed, and the Chao 1 value and Shannon diversity index did not differ significantly among the three groups (Supplementary Fig. S2).

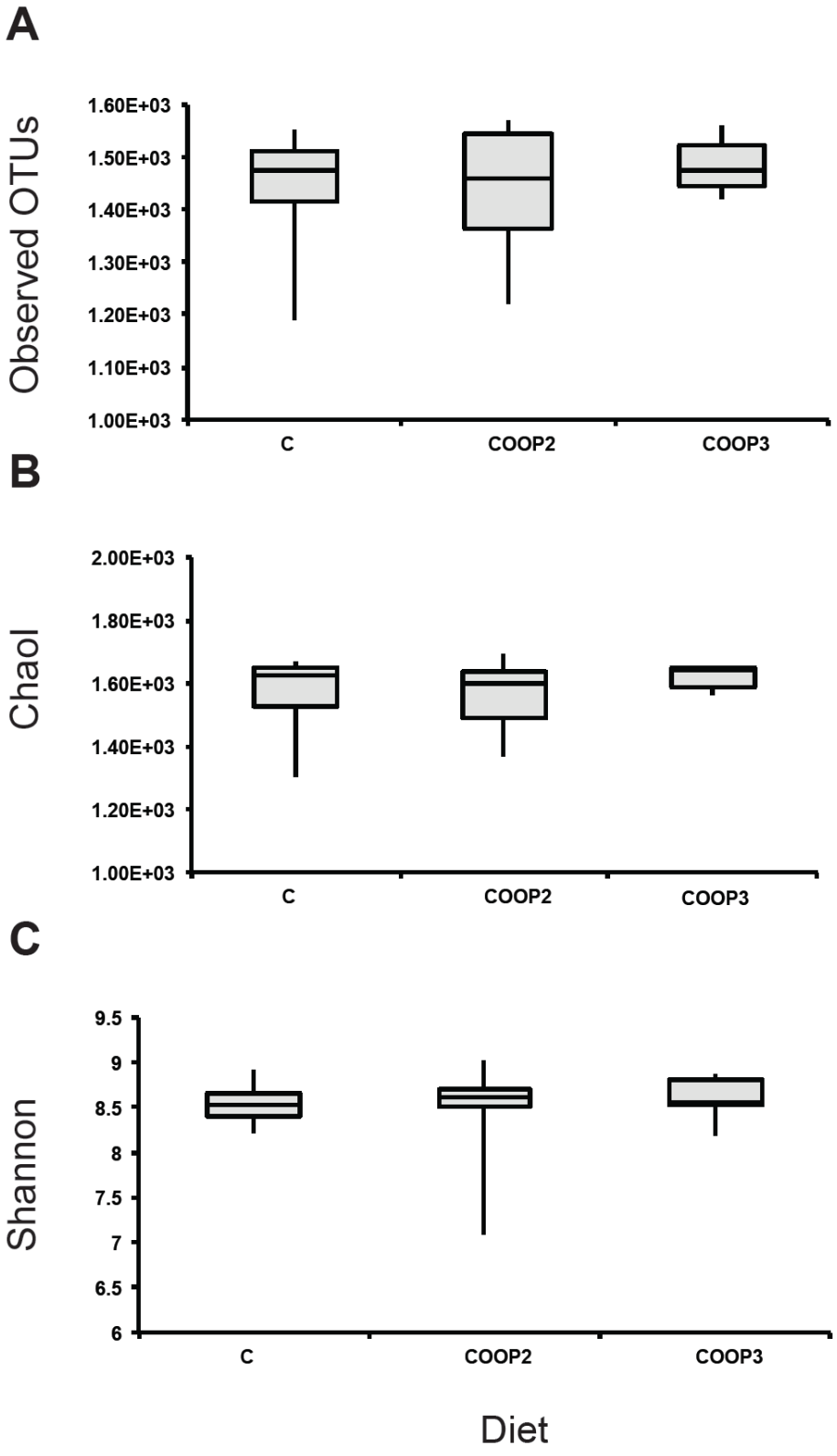


Figure S2. Box-plots of bacterial α-Diversity based on: A) Observed OTUs, B) Chao 1 value and C) Shannon index. Each box is labeled respect to the ewe diet regimen (C = control diet; COOP2 = control diet added with OOP extracted with a two-phase procedure; COOP3 = control diet added with OOP extracted with a three-phase procedure).

Addition of OOPs did not alter the overall microbiota composition, as indicated by the non-metric multidimensional scaling plot (nMDS, Fig. 1A), where all samples were evenly

scattered. Sample COOP2-5 formed an out-group, and it was also one of the two specimens with the lowest number of OTUs observed (Supplementary Fig. S1). No significant differences related to diet were found with one-way ANOSIM and PERMANOVA (data not shown).

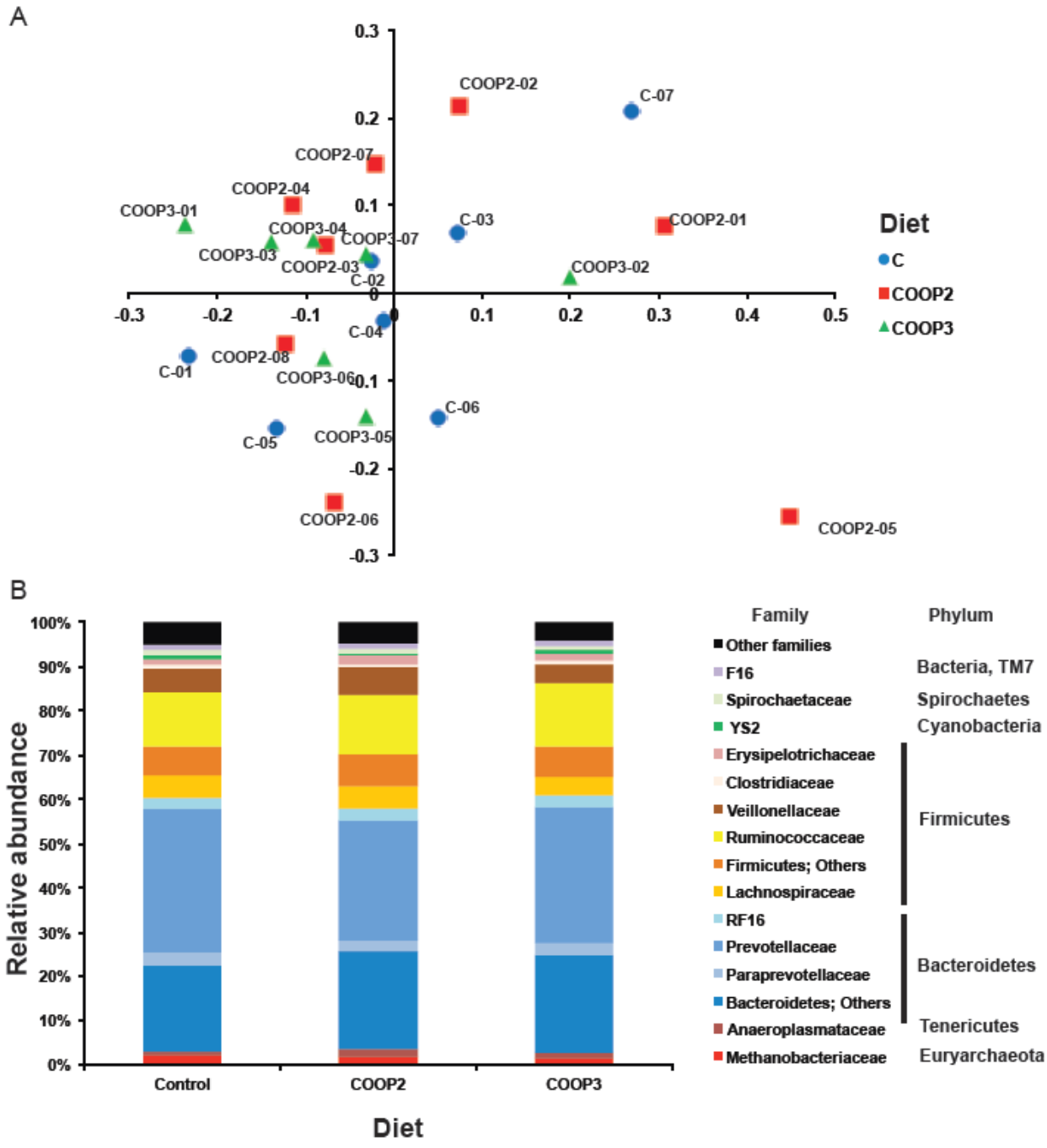


Figure 1. Rumen microbiota of ewes. Rumen microbiota are labelled respective to the ewe diet regimen (C = control diet; COOP2 = control diet added with olive oil pomace extracted with a two-phase procedure; COOP3 = control diet added with olive oil pomace extracted with a three-phase procedure). (A) ß-diversity, non-metric MDS plot. (B) Prokaryotic community composition of ewe RL at family level. Only families whose relative abundance was higher than 0.8% are shown.

The microbiota composition of the three groups was analyzed at different taxonomic levels. At the *phylum* level, the microbiota was dominated by *Bacteroidetes* and *Firmicutes* (approximately 56% and 32%, respectively) (Fig. 1B). Together, these two *phyla* accounted for 89 ± 0.7% of the total microbiota (Fig. 1B), but their relative abundance was highly variable (Supplementary Fig. S3), ranging from 0.9 to 2.9 (ratio *Bacteroidetes*/*Firmicutes*). *Prevotellaceae* was the most represented family (30%), followed by *Ruminococcaceae* (13.4%), *Veillonellaceae* (5.2%) and *Lachnospiraceae* (4.7%) (Fig. 1B).

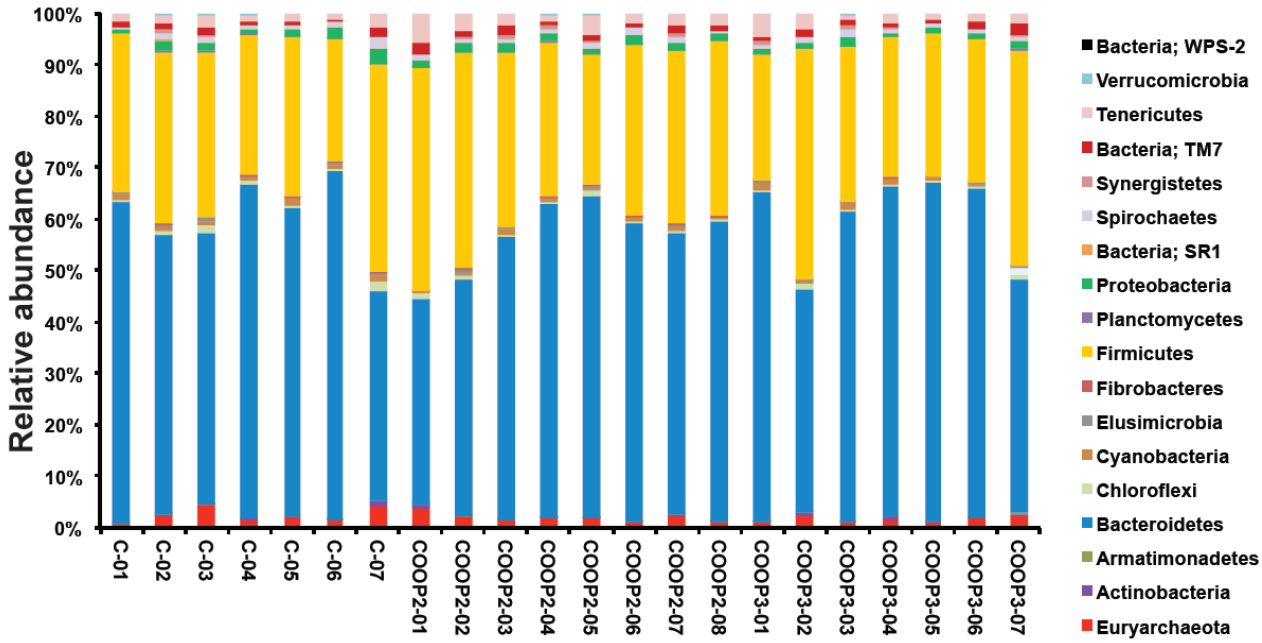


Figure S3. Prokaryotic microbiota composition (expressed as relative abundance) for each sample at *phylum* level. Bars are labeled respect to the ewe diet regimen (C = control diet; COOP2 = control diet added with OOP extracted with a two-phase procedure; COOP3 = control diet added with OOP extracted with a three-phase procedure).

Anaerostipes, *Anaerovibrio*, RFN20, *Anaeroplasma*, *Desulfobulbus* and *Sphaerochaeta* genera were influenced by OOP diet inclusion (Fig. 2). Excluding *Anaerostipes* and *Anaerovibrio*, the other four genera were the only representatives (in the present dataset) of their respective families and orders, so these differences were also reflected at higher taxonomic levels. Among the six different genera influenced by OOPs, the genus *Desulfobulbus* was the only taxon that had opposite behavior depending on the OOP used. The abundance of the genus *Desulfobulbus* was significantly higher in the COOP2 group than in the COOP3 group, but neither was significantly different from the C group. An increase in the relative abundance of RFN20 and *Anaeroplasma* was significant for the RL microbiota of ewes fed COOP2 (Fig. 2C and F). In particular, a three-fold increase in the *Anaeroplasma*

genus was observed (Fig. 2F). This trend was opposite for *Anaerovibrio* and *Sphaerochaeta*, which resulted significantly lower ($P < 0.01$) in both OOP supplemented diets (Fig. 2B and E) respect to control diet. For the *Anaerovibrio* genus, a tenfold reduction was found in the RL microbiota of ewes fed COOP2, while in COOP3 was 80% less than that in the C group (Fig. 2B). A similar trend was observed for the *Sphaerochaeta* genus, which was the 65% and 72% less in COOP2 and COOP3 groups, respectively (Fig. 2E). The reduction in *Anaerostipes* abundance was significant only for the COOP2 group ($P < 0.05$, Fig. 2A).

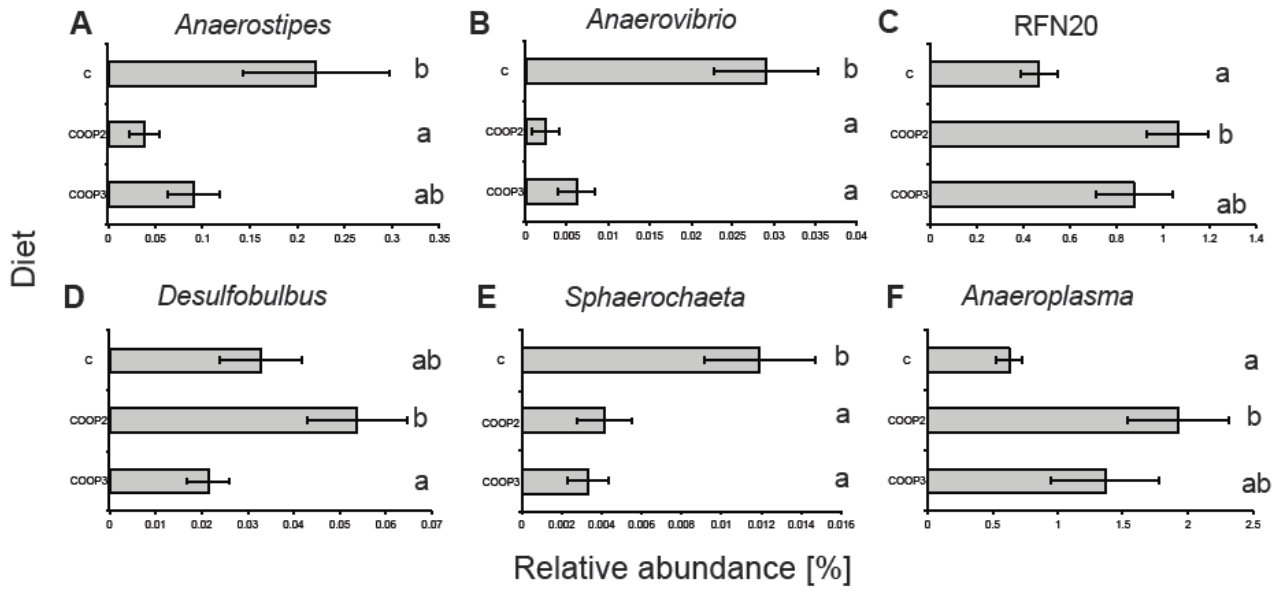


Figure 2. Effect of OOPs at genus level. Each bar is labeled respective to the ewe dietary regimen (C = control diet; COOP2 = control diet added with olive oil pomace extracted with a two-phase procedure; COOP3 = control diet added with olive oil pomace extracted with a three-phase procedure). Bacterial genera influenced by diet (ANOVA, $p < 0,05$): (A) *Anaerostipes*, (B) *Anaerovibrio*, (C) RFN20 genus, (D) *Desulfobulbus*, (E) *Sphaerochaeta* and (F) *Anaeroplasma*. Means sharing the same letter are not significantly different (post hoc Tukey's HSD test).

Pair-wise correlation. Pair-wise correlation showed that DMA13:0 was positively related to *Anaerostipes* (corr. coeff. +0.4661; $P = 0.0287$) and that DMA17:0 was positively related to *Sphaerochaeta* (corr. coeff. +0.4909; $P = 0.0203$), while DMA18:0 was positively correlated to RFN20 (corr. coeff. +0.5411; $P = 0.0009$) and to *Anaeroplasma* (corr. coeff. +0.4290; $P = 0.0046$) but negatively correlated to *Anaerovibrio* (corr. coeff. −0.5836; $P = 0.0043$).

Desulfobulbus was positively related to DMAi13:0 (corr. coeff. +0.4702; $P = 0.02723$). Considering the FAs involved in BH processes, *Anaerovibrio* was negatively correlated with α-LNA (corr. coeff. −0.6096; $P = 0.0025$) and LA (corr. coeff. −0.4795; $P = 0.0239$), while RFN20 genus was positively related to α-LNA (corr. coeff. +0.5789; $P = 0.0047$) only. RA

showed a negative correlation with *Veillonellaceae* (corr. coeff. −0.4363; P = 0.0423) and a positive correlation with *Coriobacteriaceae* (corr. coeff. +0.5425; P = 0.0090).

Milk composition and FA profile. Dietary treatments did not significantly affect milk yield and composition (Table 3).

Table 3. Effect of olive oil pomaces on milk yield and composition.

	Diet				
	C[1]	COOP2[1]	COOP3[1]	SEM[1]	P[2]
Milk yield (g/days)	575.27	603.58	617.21	80.92	0.72
FCM[1] (g/days)	698.06	699.31	744.56	75.41	0.69
Milk fat (g/100 g)	8.65	8.19	8.62	0.28	0.52
Milk protein (g/100 g)	6.52	6.07	6.38	0.17	0.89
Milk lactose (g/100 g)	4.41	4.54	4.38	0.07	0.53
Milk urea (mg/dL)	49.00	37.97	46.85	4.24	0.54
Linear Score[3]	3.66	2.95	3.93	0.44	0.65

[1]Acronyms used in this table: C (control diet), COOP2 (control diet added with olive oil pomace extracted with a two-phase procedure), COOP3 (control diet added with olive oil pomace extracted with a three-phase procedure), SEM (Standard Error Mean) and FCM (Fat-Corrected Milk); [2]Probability of significant effect due to experimental diets; means within a row with different letters differ (P < 0.05); [3]Linear score: log2 (Somatic cell count/12,500).

The FA composition of milk from ewes fed COOP2 was significantly different from that in the C group (Table 4). In contrast, the effect of the COOP3 diet was intermediate between the COOP2 and C diets (Table 4). In particular, the content of several short- and medium-chain fatty acids (SMCFAs, 6:0, 8:0, 10:0, 10:1 *cis*9, 12:0 and 14:0) was lower in COOP2 milk samples than in the other samples. Moreover, milk from ewes fed COOP2 was higher in unsaturated long-chain fatty acids (ULCFAs), such as oleic acid (18:1 *cis*9, OA) and α-LNA.

Table 4. Effect of olive oil pomaces on FAs1 production in milk.

	Diet				
	g/100 g of FAs[1]				
FA[1]	C[1]	COOP2[1]	COOP3[1]	SEM[1]	P[2]
4:0	2.65	2.81	2.67	0.08	0.28
6:0	1.73 a	1.33 b	1.63 ab	0.10	0.02
8:0	1.49 a	0.98 b	1.33 ab	0.11	0.01
10:0	4.22 a	2.62 b	3.69 ab	0.35	0.01
10:1 *cis*9	0.14 a	0.08 b	0.13 ab	0.02	0.03
11:0	0.04	0.03	0.03	<0.01	0.08
12:0	2.42 a	1.67 b	2.15 ab	0.17	0.01
13:0 *iso*	0.01	0.02	0.01	<0.01	0.18

13:0 *anteiso*	0.01	0.01	0.01	<0.01	0.06
12:1 cis11	0.02	0.02	0.02	<0.01	0.77
13:0	0.04	0.04	0.04	<0.01	0.60
14:0 *iso*	0.07	0.08	0.07	<0.01	0.15
14:0	6.95 a	5.64 b	6.61 ab	0.33	0.02
15:0 *iso*	0.16	0.18	0.15	0.01	0.10
15:0 *anteiso*	0.27	0.29	0.26	0.01	0.35
14:1 *cis*9	0.11	0.08	0.12	0.01	0.08
15:0	0.69	0.77	0.69	0.03	0.05
16:0 *iso*	0.15	0.15	0.14	0.01	0.72
16:0	16.72	15.62	16.86	0.42	0.09
16:1 *trans*9	0.28	0.34	0.28	0.02	0.14
17:0 *iso*	0.26	0.28	0.26	0.01	0.35
16:1 *cis*7	0.24 b	0.28 a	0.24 b	0.01	0.02
16:1 *cis*9	0.54	0.46	0.57	0.03	0.06
17:0 *anteiso*	0.23	0.25	0.23	0.01	0.20
17:0	0.42	0.49	0.43	0.02	0.07
17:1 *cis*9	0.12	0.13	0.12	0.01	0.23
18:0	9.22	9.72	9.72	0.55	0.75
18:1*trans*6-8	0.91	1.01	0.89	0.04	0.13
18:1 *trans*9	0.69	0.75	0.73	0.05	0.66
18:1 *trans*10	0.90	0.96	0.95	0.08	0.84
VA (18:1 *trans*11)[1]	4.24	4.64	4.29	0.33	0.63
18:1 *trans*12	0.85	0.85	0.84	0.02	0.96
OA (18:1 *cis*9)[1]	19.53 b	22.26 a	21.06 a	0.69	0.03
18:1 *trans*15	0.45 b	0.52 a	0.48 ab	0.02	0.02
18:1 *cis*11	0.43	0.41	0.45	0.02	0.29
18:1 *cis*12	0.34	0.31	0.31	0.02	0.33
18:1 *trans*16	0.51	0.54	0.53	0.02	0.57
18:1 *cis*14	0.07	0.08	0.08	0.01	0.27
LA (18:2 *cis*9 *cis*12)[1]	1.86	1.98	1.95	0.11	0.68
α-LNA (18:2 *cis*9 *cis*12 *cis*15)[1]	1.90 b	2.38 a	1.90 b	0.13	0.02
RA (18:2 *cis*9 *trans*11)[1]	2.03	2.25	2.19	0.14	0.53
18:3 *cis*9 *trans*11 *cis*15	0.05 b	0.06 a	0.04 b	<0.01	0.02
20:0	0.13 b	0.17 a	0.14 b	0.01	0.01
20:3n6	0.02	0.02	0.02	<0.01	0.63
20:3n3	0.03	0.03	0.03	<0.01	0.20
20:4n6	0.10	0.11	0.11	0.01	0.32
20:5n3	0.05	0.06	0.06	<0.01	0.23
22:5n3	0.10	0.12	0.10	0.01	0.13
22:6n3	0.04	0.04	0.04	<0.01	0.51

[1]Acronyms used in this table: FA (fatty acid), C (control diet), COOP2 (control diet added with olive oil pomace extracted with a two-phase procedure), COOP3 (control diet added with olive oil pomace extracted with a three-phase procedure), OA (oleic acid), VA (vaccenic acid), LA (linoleic acid), α-LNA (α linolenic acid), RA (rumenic acid) and SEM (Standard Error Mean); [2]Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

Discussion

RL microorganisms are highly sensitive to dietary composition and, in particular, to supplements with antimicrobial activity, such as polyphenols[38]. Much of our knowledge related to rumen metabolism of feeds and additives has been gained by *in vitro* studies[13]. Thus, it is necessary to perform *in vivo* trials for better understanding the effects of diet quality. Pomace is a by-product of the olive oil extraction process and is rich in polyphenols, the amount of which is variable depending on the production technique used (two-phase *vs* three-phase method). The addition of polyphenols to animal diets may alter rumen microorganism activities[4,13,39]. The lipid content of milk and meat is influenced by rumen metabolism. Thus, modulation of RL microbiota to increase the amount of nutraceutical PUFAs may be exploitable to ameliorate food production.

In this trial results related to DMI did not show significant differences among experimental groups. Therefore, the effects of dietary supplementation with OOPs on RL and milk composition were due to the different chemical profiles of the experimental diets. OOP addition led to an increase in PUFAs. In particular, a gain of α-LNA was obtained with OOP2, while the RA concentration was enhanced with OOP3. The different changes observed in FAs among ewe groups may be due to the different contents of polyphenols into the two extracts used as supplements in the treated diets. COOP2 showed a higher content of verbascoside, 3,4 DHPEA-EDA, and rutin than COOP3, as a consequence of the different extraction processes. Verbascoside is a molecule with antioxidant, anti-inflammatory and antimicrobial activities[40], suggesting that it may play a role in the modulation of rumen microbiota.

Since the 1980s, chemotaxonomic techniques have been considered of important value to identify and classify bacterial strains in rumen microbial ecosystems[41]. Several authors have found that DMAs are associated with specific bacterial taxa[41–44], indicating that plasmalogen lipid profiles may be considered a tool for microbial community characterization. These molecules are present in bacterial membrane, especially of anaerobic species. However, their function is not completely known. DMAs play a key role in the regulation of membrane fluidity, and their profile changes when environmental conditions vary. The DMA profile

reflects the FAME composition, and a characteristic DMA profile could be associated with a specific microbial strain[41–44]. In this study, DMA 16:0 was the most abundant in all groups, which was in agreement with previous results of Alves et al.[22]. Significant variations were observed for DMA13:0, DMA17:0 and DMA18:0, even if the total DMA concentration did not vary among groups.

Metataxonomic analysis of RL microbiota showed that overall composition was unaffected by OOP addition. Although unrelated to diet composition high variability was observed, especially in the relative abundance of *Bacteroidetes* and *Firmicutes*. Moreover, in this trial, a low abundance of the *Lachnospiraceae* family (<5%) was found, and it has been reported to be generally higher (>10%) in ewe rumen microbiota[45]. The low abundance of *Lachnospiraceae* may be linked to the addition of a high content of concentrate (rich in starch and fat, and poor in fiber and polysaccharide xylan, which are the main substrates for cellulolytic bacteria growth) in all the ewe dietary regimens. However, the method used for rumen fluid collection may also affect solid-associated bacteria content because the esophageal pump may be selective in feed particle extraction, although it is a general veterinary practice. The *Lachnospiraceae* family is mainly represented by the genus *Butyrivibrio*, accounting for 1% of the total microbiota, and it has been known for its role in BH since the 1960s[46]. An increase in α-LNA and RA contents was observed upon OOP2 or OOP3 addition to the diet, which may be due to a decrease in BH or to lower availability of substrates for BH. Although the *Butyrivibrio* relative presence did not vary among groups, other microorganisms are known to be involved in the BH process (i.e., *Megasphaera elsdenii* and *Propionibacterium acnes*) and have been characterized in recent decades[1]. Furthermore, a putative new role has recently been assigned to several known microorganisms, which were considered until now to be involved in other processes[47,48].

In this study, variations linked to diet were observed for *Anaerostipes*, *Anaerovibrio*, RFN20, *Anaeroplasma*, *Desulfobulbus* and *Sphaerochaeta*. Within *Firmicutes*, the genus *Anaerovibrio* was less represented in animals fed with OOP. For *Anaerostipes*, the decrease was significant only for COOP2. *Anaerostipes* is a butyrate-producing bacterium whose activity is strongly linked to fermentation of dietary carbohydrates, and its lower relative abundance in RL from ewes fed COOP2 may be related to the lower quality of fiber contained in this diet as lignin is indigestible[49]. The opposite behavior was observed for bacteria belonging to RFN20 genus, which was significantly higher in the COOP2 group, but the role of this taxon within the rumen microbial community remains unclear[50]. Similarly, *Anaeroplasma* was higher in the COOP2 diet. These microorganisms are anaerobic

mycoplasmas, which in some cases have bacteriolytic capabilities affecting nutrient cycling and protein turnover. Indeed, their activity may reduce Gram-negative bacteria, thus interfering with rumen processes[51]. *Mycoplasmas* may also parasite ruminal fungi and protozoa modifying their activity[51]. Pairwise analysis showed that a variation of DMA13:0 may be related to a variation of *Anaerostipes*, and that changes in DMA17:0 may be related to changes in *Sphaerochaeta*, whereas DMA18:0 variations may be related to a variation of *Anaeroplasma*, RFN20 and *Anaerovibrio*.

Increase in PUFAs may be linked to the lower abundance of the *Anaerovibrio* in the RL microbiota. This relation was confirmed by the pairwise correlation, which showed a significant and negative correlation between α-LNA and *Anaerovibrio* content (corr. coeff. = −0.6096, P = 0.0026). *Anaerovibrio lipolyticus* is the only species described within this genus, and it is a key player in the lipolysis process[52]. Lipolysis is a fundamental requirement for the next step of lipid metabolism in RL, bacterial membrane structure formation, cell replication and PUFA-BH. *A. lipolyticus* growth is enhanced with diets having a high content of concentrate respect to the diet based on hay[53]. Hence, according to the high level of concentrate used in this trial, an increase in *A. lipolyticus* was expected in all ewe groups. In contrast, the decrease of *A. lipolyticus* in the COOP2 and COOP3 groups respect to the C group, showed the negative action of OOP on *A. lipolyticus* growth. This bacterium uses glycerol as a nutrient[54,55]. Thus, it could be hypothesized that the presence of polyphenols in the diets may have complexed lipase enzymes, avoiding the triglyceride hydrolysis[56,57] and ultimately resulting in less free glycerol available to *A. lipolyticus* for its growth[58,59]. Reduction of *A. lipolyticus* led to a decrease in lipolytic activity, and hence, to a low availability of PUFAs for BH, which agreed with the increase of α-LNA and RA. It has been reported that polyphenols, such as those from chestnut or quebracho, interfere with the last step of the BH process, inhibiting the activity of microorganism, such as *B. proteoclasticum*[3,39]. This study showed that polyphenols from OOP act in a different manner by affecting *A. lipolyticus* abundance and consequently lipolysis, which is the step before BH. The lower abundance of *A. lipolyticus* may explain the differences observed for PUFA concentrations in the RL of animals fed with OOP diets. Nevertheless, considering the putative role of *Butyrivibrio* group in the BH, it is possible that within the *Butyrivibrio* genus, the relative abundance of different *Butyrivibrio* species (i.e., *B. fibrisolvens*) may affect the BH process.

Dietary treatment did not significantly affect milk yield and composition. Inconsistent results have been reported in previous studies, and these differences are probably due to the

inclusion levels of OOP and/or to their associative effects with specific diets[12,60,61]. Regarding milk urea content, the results suggested that olive phenols do not interact with the dietary protein metabolism unlike to other phenolic substances, such as tannins[62]. The lower content of several SMCFAs in milk fat from ewes fed COOP2 should be related to a lowering of mammary gland *de novo* fat synthesis[63]. Considering long-chain fatty acids, OA content was higher in milk fat from ewes fed OOP diets than with control, although the OA supply was similar across diets. OA is largely generated by mammary Δ^9-desaturation of 18:0[64]. Further studies are required to better understand the role of OOPs on mammary gland metabolism. An increased content in α-LNA was found only in milk fat from ewes fed COOP2, as observed in the RL of the same animals. In contrast, no significant differences in RA concentrations in milk fat were observed, which may have been due to mammary gland activity for the Δ^9-desaturation of VA[64].

Conclusions

The use of different types of OOP in dairy ewe diets did not negatively affect milk yield or composition. COOP2 and COOP3 diets led to an enrichment of the milk fat with α-LNA and OA. The changes in microbiota profile due to OOPs are limited and do not alter rumen functionality, preserving animal welfare. The data of this trial highlighted that *A. lipolyticus* is particularly sensitive to OOPs. Hence, depending on the types of polyphenol added to diet, it might be possible to modulate rumen metabolism at different levels as they affect relative abundance of different microorganisms related to BH. In conclusion, this study suggested that OOPs may be used in ruminant feeding because they induce a decrease in lipolysis, favoring the accumulation of healthy FAs in milk.

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Additional Information

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TRIAL 2

2.1 Chestnut or quebracho tannins in the diet of grazing ewes supplemented with soybean oil: effects on animal performances, blood parameters and fatty acid composition of plasma and milk lipids. (Arianna Buccioni, Mariano Pauselli, Sara Minieri, Valentina Roscini, Federica Mannelli, Stefano Rapaccini, Paola Lupi, Giuseppe Conte, Andrea Serra, Alice Cappucci, L. Brufani, Francesca Ciucci, Marcello Mele) Received 24 November 2016; Received in revised form 21 April 2017; Accepted 13 May 2017 Available online 15 May 2017 on Small Ruminant Research (www.elsevier.com/locate/smallrumres); DOI: http://dx.doi.org/10.1016/j.smallrumres.2017.05.006

Author Contributions

Conceived the experiments: A.B., M.P., M.M., A.S. and S.R.; Animals care and sampling: F.M., A.C., F.C., V.R. and L.B.; Proximate analysis: A.C. and V.R.; Fatty acid analysis: F.M. and S.M.; Blood analysis: P.L. and S.M.; Statistical analysis: G.C. and F.M.; Wrote the manuscript: A.B., A.S. and F.M.

A B S T R A C T
The aim of the present study was to evaluate the effect of the inclusion of chestnut or quebracho tannin extracts in the diet of grazing ewes supplemented with soybean oil, on the blood plasma and milk fatty acid profile, milk quality traits and animal metabolic profile. Eighteen Comisana ewes at 172 ± 6 days in milking were allotted into 3 experimental groups. Diets were characterized by pasture ad libitum administered and by 800 g/head and day of 3 experimental concentrates containing 84.5 g of soybean oil/kg of DM and 52.8 g/kg DM of bentonite (Control diet) or 52.8 g/kg DM of chestnut tannin extract (hydrolysable tannins, CHE diet) or 52.8 g/kg DM of quebracho tannin extract (condensed tannins, QUE diet). The trial lasted 4 weeks after 15 days of adaptation to the feeding regimen. Milk yield was daily recorded while milk composition and blood parameters were weekly analysed. CHE and QUE did not affect the milk yield and composition. Casein Index was affected by diet and it was significant higher in milk from animals fed QUE ($P < 0.0259$). The clotting parameters with the exception of a_{30} were affected by tannins: r was higher for QUE milk while k_{20} increased regardless the kind of tannin. Blood parameters were not affected by tannins and the oxidative status of ewes, determined using MDA as indicator, did not present significant differences among groups, regardless the concentrates fed to animals. Fatty acid profile of blood plasma demonstrated that tannin extract, regardless the source, favored the accumulation of vaccenic acid (trans-11 18:1) reducing the hematic concentration of stearic acid (18:0). Only few significant differences in milk fatty acid profile were found. In particular, rumenic acid (cis-9, trans-11 18:2) increased when the concentrates contained polyphenols and the stronger effect is reached with QUE ($P< 0.0002$).

Introduction

Chestnut (*Castanea sativa* Miller) trees, widespread in the Mediterranean region, and Quebracho (*Schinopsis lorentzii*) trees, widespread in South America, have their wood or fruits rich in hydrolysable and condensed tannins, respectively. Several authors demonstrated the efficacy of these two extracts in modulating biohydrogenation (BH) of polyunsaturated fatty acids (PUFA) in dairy ewes fed diets based on hay and concentrates supplemented with oils or full fat seeds (Buccioni et al., 2015 a; Hervàs et al., 2003; Toral et al., 2011; Toral et al., 2013). Recently, Buccioni et al. (2015a) and Correddu et al. (2015) reported that the interaction between dietary polyphenols such as chestnut (CHE), quebracho (QUE) or grape seeds (GS) and lipid supplements (soybean oil or extruded linseed) resulted in an increase of the concentration of linoleic (LA; *cis*-9, *cis*-12 18:2), vaccenic (VA, *trans*-11 18:1), rumenic (RA, *cis*-9, *trans*-11 18:2) acids and in a decrease of total saturated fatty acids (SFA) in sheep milk. These effects were probably due to the ability of tannins to interfere with rumen microbial metabolism, as indirectly confirmed by changes in the concentration of volatile

fatty acids (VFA) and by changes in rumen microbial communities (Buccioni et al., 2015a; Carreño et al., 2015; Minieri et al., 2014; Pallara et al., 2014; Vasta et al., 2010).

In a previous experiment, Buccioni et al. (2015a) demonstrated that QUE tannins were more efficient than CHE tannins in perturbing rumen BH when diets were based on preserved forages and supplemented with soybean oil as main dietary source of PUFA. However, little information is available on the interaction between different kind of tannin extracts and rumen BH when PUFA contemporary originate from oil and pasture. Since in oil PUFA are mainly present as triglycerides whereas in forage are constituents of structural lipids such as phospholipids (Buccioni et al., 2012), some differences might occur in the BH process due to the timing of lipolysis or to the kind of bacteria strains involved. Consequently, also the interaction between different kinds of tannins and the individual step of the BH process of PUFA may be altered according to the origin of PUFA (triglycerides or phospholipids).

Hence, the aim of the present study was to evaluate the effect of the inclusion of a moderate amount (< 2%) of CHE or QUE tannin extracts in the diet of grazing ewes supplemented with soybean oil, on the blood plasma and milk fatty acid (FA) profile and on the milk yield and quality. Moreover, since some toxic effects of dietary tannins have been previously reported in ruminants (Reed, 1995; Hervas et al., 2003), blood parameters as indicators of hepatic function and oxidative status were also considered in the present experiment.

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; ALB, albumin; BH, biohydrogenation; C, control concentrate; CHO, cholesterol; CHE, chestnut tannin; CI, casein index; CLA, conjugated linoleic acid; DM, , dry matter; DMI, dry matter intake; FA, fatty acid; FCM, fat corrected milk; GBL, globuline; γGT, γ-glutamil-transferase; GLU, glucose; α-LNA, alpha-linolenic acid; LA, linoleic acid; MDA, malonaldehyde; MTP, milk total polyphenols; NDF, neutral detergent fibre assayed with heat stable amylase and expressed inclusive of residual ash; PUFA, polyunsaturated fatty acids; QUE, quebracho tannin; RA, rumenic acid; SGPT, serum glutamic-pyruvic, transaminase; SGOT, serum glutamic-oxaloacetictransaminase; TAE, tannic acid equivalents; VA, vaccenic acid.

Material and methods

Experimental design

Animals

Eighteen multiparous Comisana ewes at 172 ± 6 days in milking (DIM) kept at the Experimental Section of the Department of Agriculture, Food and Environmental Science − University of Perugia

Italy, were randomly allotted into 3 experimental groups, blocked for body weight (68.1 ± 7.83 kg; BW), age, and milk yield. Each group was formed by 6 ewes and kept in a pen. All animals grazed together eight hour per day on a spontaneous pasture, following the rotational grazing technique. Grazing periods lasted 3 days each by the displacement of the electrified fence, yielding approximately 40 m^2/ewe/day while water was always available. The trial lasted 4 weeks after 15 days of adaptation to the feeding regimen. The handling of the animals was according to Institutional Animal Care and Use Committee (IACUC, 2014) of University of Perugia. The ewes were milked twice daily at 07:30 and 17:30 h using a milking machine (43 kPa; 150 pulsation/min) and daily individual milk yield was recorded.

Diets

The experimental diets were formulated according to the nutrient requirements of an ewe weighing 68 kg and producing 1 kg of milk at 6.5% of fat (Cannas et al., 2004):

$$MEl = \{[251.73 + 89.64 \times PQ + 37.85 \times (PP/0.95)] \times 0.001 \times Yn\}/kl$$

measured milk yield at a particular day of lactation, kg/d; PQ is measured milk fat for a particular day of lactation, %; PP is measured true milk protein for a particular day of lactation, %; and kl is efficiency of ME utilization for milk production, which is equal to 0.644.

Diets were composed by spontaneous pasture managed as described above, 250 g per head and day of chopped grass hay (particle size>4 cm of length) and 800 g per head and day of a concentrate, which contained 84.5 g of soybean oil/kg DM and 52.8 g/kg DM of bentonite (control diet), or 52.8 g/kg DM of chestnut tannins (CHE diet) or 52.8 g/kg DM of quebracho tannins (QUE diet). All the concentrates ingredients were incorporated into pellets using a pelletting machine (CMS-IEM − Colognola ai Colli, Verona, Italy), pellet diameter was 5 mm, and the pelleting temperature ranged between 35 and 40 °C. The chemical composition of feeds is presented in Table 1.

Table 1. Ingredients, chemical composition and fatty acids profile of the experimental concentrates, hay and of the pasture administered to the ewes.

			Experimental concentrates [1]		
	Hay	Pasture	Control	CHE	QUE
Ingredients (g/kg of dry matter)					
Barley			213.8	213.8	213.8
Corn			211.3	211.3	211.3
Wheat bran			158.5	158.5	158.5

Soybean meal (44 CP)			126.8	126.8	126.8
Beet pulp			89.8	89.8	89.8
Soybean oil[2]			84.5	84.5	84.5
Bentonite			52.8	-	-
Chestnut tannin extract[3]			-	52.8	-
Quebracho tannin extract[4]			-	-	52.8
Molasses			41.3	41.3	41.3
$CaCO_3$			10.6	10.6	10.6
Sodium bicarbonate			5.3	5.3	5.3
Di-calcium phosphate			5.3	5.3	5.3
Chemical composition (g/kg of DM)					
Chemical composition (g/kg of DM)					
Crude Protein	111.2	156.8	135.0	135.0	135.0
Ether extract	12.0	24.5	96.0	96.0	96.0
NDF	636.4	425.6	181.0	181.0	181.0
ADF	501.3	347.4	74.2	74.2	74.2
ADL	105.7	67.3	12.9	12.9	12.9
Ash	69.6	43.2	85.0	85.0	85.0
ME (MJ/kg DM)	7.8	8.3	10.3	10.3	10.3
NEl (MJ/kg DM)	4.7	5.0	6.6	6.6	6.6
Fatty acids (g/100g of total fatty acids)					
16:0	35.5	16.3	14.0	14.0	14.0
18:0	5.8	4.2	3.6	3.6	3.6
cis-9 18:1	9.3	11.8	23.3	23.3	23.3
18:2 n-6	28.5	22.2	51.4	51.4	51.4
18:3 n-3	2.8	37.5	5.8	5.8	5.8

[1]CHE: concentrate containing chestnut tannin extract; QUE: concentrate containing quebracho tannin extract. [2]Fatty acid profile of soybean oil (g / 100g of total fatty acids): 16:0, 11.0; 18:0, 3.6; *cis*-9 18:1, 22.1; *cis*-9, *cis*-12 18:2, 53.7; *cis*-9, *cis*-12, *cis*-15 18:3, 7.2. [3]Hydrolysable tannins extracted from Chestnut wood (*Castanea sativa*) containing 750 g of equivalent tannic acid/kg DM (provided by Gruppo Mauro Saviola srl Radicofani, Siena, Italy). [4]Condensed tannins extracted from quebracho (*Schinopsis lorentzii*) containing 456 g of equivalent tannic acid/kg DM (provided by Guido Lapi spa, Castel Franco di Sotto, Pisa, Italy).

The dose of tannins was chosen in order to obtain a tannin concentration in the diet of nearly 16 g/kg of expected DM intake, estimated according to Cannas et al. (2004). Based on results from previous studies published in literature, this dose was considered as safe for the animal and practical for the farmers (Buccioni et al., 2015a, b). One hundred g/head of rolled barley with the 800 g/head of experimental concentrates were individually offered during milking. Chestnut hydrolysable tannins (750 g/kg DM of equivalent tannic acid; by Gruppo Mauro Saviola srl Radicofani, Siena, Italy), and extract of quebracho condensed tannins (456 g/kg DM of equivalent tannic acid; by Guido Lapi spa Castel Franco di Sotto, Pisa, Italy) were titrated according to Burns (1963). The chromatographic profile of CHE tannin extract is

reported in literature by Campo et al. (2012) while the profile of QUE tannin extract is reported by Pash et al. (2001).

Sampling and analysis

Feed sampling and analysis

Pasture was sampled hand-plucking one square meter area before each grazing period while hay, experimental concentrates and their orts were measured daily, pooled and sampled weekly. All collected samples were stored at −80 °C until analysis. Samples were freeze-dried and then ground for chemical analysis by mill Cyclotec 1093 (PBI International, Milan, Italy), using a mesh size of 1 mm. Crude protein (CP), ether extract (EE) and ash were determined according to the AOAC methods 976.06, 920.39 and 942.05, respectively (AOAC, 1995). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin were determined according to Van Soest et al. (1991), using heat stable amylase and sodium sulphite, and expressed inclusive of residual ash. Metabolizable energy (ME) and Net energy for lactation (NEl) were calculated according to Cannas et al. (2004). Feed FA were extracted according to Folch et al. (1957), esterified according to Christie (1982) with 19:0 (Sigma Chemical Co., St Louis, MO) as the internal standard, and identified using the same procedure described below for FA of milk samples.

Milk sampling and analysis

Individual milk samples from morning and evening milking were collected weekly and allotted into three aliquots for analysis: the first aliquot was processed in order to assess fat, lactose, protein and casein content, by using Milkoscan 6000 FT (Foss Electric, Hillerød Denmark), and total somatic cell count (SCC) according to ISO 13366-2|IDF 148-2 (2006), by using a Fossomatic 5000 (Foss Electric, Hillerød Denmark) and expressed as linear score (linear score =$\log_2$ [SCC/12,500]; Shook, 1993).

The second aliquot was processed to determine the milk rennet characteristics at 35 °C by a Maspress apparatus (Foss Italia, Padua, Italy), according to Zannoni and Annibaldi (1981). The following rennet parameters were determined: clotting time (r) that is the time from rennet addition to the beginning of coagulation, firming time (k_{20}) that is the time needed for the amplitude to reach 20 mm on the recording chart, and curd firmness (a_{30}) that is the amplitude of the trace 30 min after rennet addition. The third aliquot of milk samples was stored at −80 °C until analysis for FA extraction and composition by gas-chromatography

according to Buccioni et al. (2010). Individual fatty acid methyl esters (FAMEs) were quantified using valeric acid (5:0) and nonadecanoic acid (19:0) methyl esters (cod W275204 and cod N5377, respectively; Sigma Chemical Co., St. Louis, MO) as internal standards and identified by comparison of the relative retention times of FAMEs peaks from samples, with those of the standard mixture 37 Component FAMEs Mix (Supelco, Bellefonte, PA, USA 4:0 − 24:0 (cod 18919 − 1AMP, Supelco, Bellefonte, PA, USA), individual *trans*-9 18:1 and *trans*-11 18:1 (cod 46903 and v1381 respectively, Sigma-Aldrich, St. Louis, Missouri, USA), individual cis-9, t*rans*-11 18:2 (cod 1255, Matreya Inc Pleasant GAP, PA, USA.), CLA mix standard (cod 05632; Sigma-Aldrich, St. Louis, Missouri, USA) and published isomeric profile (Kramer et al., 1997; Kramer et al., 2004; Cruz-Hernandez et al., 2006). The 18:1 isomers elution sequence was performed according to Kramer et al. (2004). Moreover, standard mix of α-linolenic acid (α-LNA) isomers (cod 47792, Supelco, Chemical Co., St. Louis, MO) and of LA isomers (cod 47791, Supelco, Chemical Co., St. Louis, MO) and published isomeric profiles (Destaillats et al., 2005) were used to identify the isomers of interest. Two bacterial acid methyl ester mix (cod 47080-U Supelco, Chemical Co., St. Louis, MO; GLC110, Matreya, Pleasant Gap, PA) and individual standard for methyl ester of *iso* 14:0, *anteiso* 14:0, *iso* 15:0 and *anteiso* 17:0 (cods 21–1211-11, 21–1210-11, 21–1312-11 and 21–1415-11, Larodan Malmo, Sweden) were used to identify branched FA profile. Inter and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Boureau of Reference, Bruxelles, Belgium) and detection threshold of FA was 0.01 g/100 g of FA (Contarini et al., 2013). All FA composition results are expressed as g/100 g of total lipids for milk fat.

At 28th day of the trial, milk samples were collected also for total polyphenol content (milk total polyphenol, MTP) determination. MTP, expressed as tannic acid equivalents (TAE), was measured according to the Folin- Ciocalteu method (Makkar et al., 1993).

Blood sampling and analysis

Samples of blood were collected from each animal at the end of every experimental week by punching the jugular vein. Blood was stored into tubes without anticoagulant and serum was immediately separated by centrifugation (5000 x g for 30 min at 25 °C).

For FA profile, blood samples were collected using tubes with anticoagulant (heparin) and plasma was immediately separated by centrifugation (5000 ×g for 30 min at 25 °C). One millilitre of plasma for each sample was directly methylated using a combination of methods according to Kramer et al. (1997) modified by Park et al. (2001). The first step consisted of an

alkaline methylation with sodium methylate/methanol (0.5 mL of 0.5 M-Sodium Methoxide) to esterify glycerides. The second step involved an acid methylation with HCl/methanol (4 mL of 5% methanolic HCl, 1 h at 50 °C) as catalyst to esterify free fatty acids. Fatty acid methyl esters (FAME) were extracted using n-hexane (2 mL) with 9:0 and 23:0 methyl ester (cod 76368 and T9900 respectively, Sigma Chemical Co., St. Louis, MO) as internal standards for quantification, and maintained in vials with hermetic closure to avoid the loss of volatile components. FAME were separated and identified by gas-chromatography using the same programming described above for milk. All FA composition results were expressed as g/100 g of FA.

For metabolic profile, total protein (Colorimetric method BIURET), urea (kinetic enzymatic method), albumine (ALB; colorimetric BCG method), γ-glutamil-transferase (γ-GT; kinetic SZASZ-tris method), serum glutamic-pyruvic-transaminase (SGPT; kinetic UV IFCC method), serum glutamic-oxaloacetic-transaminase (SGOT; kinetic UV IFCC method), glucose (GLU; enzymatic colrimetric method), cholesterol (CHO; enzymatic colrimetric method CHO-POD), creatinine (kinetic enzymatic method − Jaffe), triglycerides (enzymatic colrimetric method) and phosphorus (Ammonium Molybdate method − UV) were detected using diagnostic kits (cods ASR01120; ASR01143; ASR0128012; ASR01194; ASR01219; ASR01229; ASR01202; ASR01101; ASR01150; ASR01134; ASR01181 Assel s.r.l., Rome Italy) with an auto blood-analyzer for hematology (Vegavet AMS, Analyser Medical System, Rome, Italy). Globuline (GBL) content was estimated by the difference between total protein and albumin contents. Moreover, blood samples, collected at the end of the trial (4^{th} week), were used also to determine the lipid peroxidation in plasma using as indicator the malonaldehyde (MDA). The assays were carried out using commercial kits (QuantiChrom™ TBARS Assay Kit, Bioassay System, cod. DTBA-100) by means colorimetric method, monitoring the change of absorbance at 532 nm with a spectrophotometer (Placer et al., 1996). The MDA concentrations were expressed as micromoles per millilitre for plasma.

Statistical analysis

All data (e.g., animal performance, milk composition, blood metabolic parameters and milk FA profile) recorded over the course of the experiment were processed as completely randomized design with repeated measures using the MIXED procedure of SAS (SAS, 1999):

$$Y_{ijkl} = \mu + D_i + T_j + I_k(D) + (D \times T)_{ij} + e_{ijkl}$$

where y_{ijkl} is the observation; μ is the overall mean; Di the fixed effect of diet (i= 1 to 3); T_j the fixed effect of sampling time (j =1 to 4); I_k is the random effect of the ewe nested within the diet (k =1 to 6); $(Di \times T)_{ij}$ the interaction between diet and sampling time and e_{ijkl} the residual error. The covariance structure was compound symmetry, which was selected on the basis of Akaike's information criterion of the mixed model of SAS. Statistical significance of the diet effect was tested against variance of sheep nested within diet according to repeated measures design theory (Littell et al., 1998). Multiple comparisons among means were performed using the Tukey's test.

Data related to MTP in milk and MDA in plasma were processed using one-way analysis of variance (SAS, 1999) with a model that included diet and experimental error.

$$y_{ij} = \mu + D_i + e_{ij}$$

where y_{ij} is the observation; μ is the overall mean; D_i the diet (i =1 to 3) and e_{ij} the residual error. Multiple comparisons among means were performed using the Tukey's test.

Results

Animal performance, milk composition and milk total polyphenol content

The dietary supplementation with tannic extracts did not affect the palatability of concentrates, completely consumed by animals.

During the experiment, no refusal was obtained from concentrate consumed, irrespective of the treatment (800 g/head and day). Since the concentrate was individually administrated, the daily intake of both tannins was 40 g per head and per day and of soybean oil was 60 g per head and per day.

The integration with CHE and QUE did not affect the milk yield and composition (Table 2). Urea concentration showed a significant variation due to only a time effect. An effect due to the interaction between diet and time (DxT) is observed for total protein and casein content. In contrast, Casein Index (CI), expression of the percentage of casein respect the total protein, was affected by diet and it was significant higher in milk from animals fed QUE ($P < 0.0259$; Table 2). The clotting parameters with the exception of a_{30} were affected by tannins. In particular, r was higher for QUE milk while k*20* increased regardless the kind of tannin. The data are reported in Table 2.

Table 2. Milk yield and composition from ewes fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHE diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

Item		Diet				P value[1]		
		Control	CHE	QUE	SEM[4]	D	T	D x T
Milk yield	g / d	767	834	895	60.2	0.3293	0.9864	0.9985
Milk composition	g / 100 g							
Fat		7.08	7.45	7.22	0.303	0.7191	0.1606	0.0886
Lactose		4.77	4.72	4.88	0.068	0.3067	<0.0001	0.7247
Protein		5.69	5.94	5.83	0.170	0.6109	0.9527	0.0084
Casein		4.44	4.61	4.62	0.143	0.6148	0.9588	0.0084
Urea	mg / dl	14.92	17.58	17.64	2.340	0.6590	0.0009	0.5701
Total solids	g / d	0.13	0.15	0.16	0.020	0.6836	0.8930	0.2772
Casein Index[2]		78.01 b	77.57 b	79.21 a	0.375	0.0259	0.0817	0.8965
Linear Score[3]		4.89	3.82	3.64	0.727	0.4526	0.0016	0.0721
Clotting parameters								
r	min	14.33 b	15.11 b	18.08 a	0.519	<0.0001	0.0031	0.0795
k_{20}	"	1.44 b	1.69 a	1.85 a	0.112	0.0435	0.0010	0.1986
a_{30}	mm	41.40	44.16	42.70	3.337	0.8565	0.8111	0.4755

[1]Probability of significant effect due to experimental factors: diet (D), time (T), and their interaction (D X T); [2]Casein Index: total casein / total protein *100; [3]Linear Score = log2(SCC / 12,500) where SCC is Somatic Cell Count; [4]Standard Error Mean

The presence of tannins, regardless the kind of the extract (CHE *vs* QUE), did not affect the total polyphenol content of milk from ewes fed the supplementations. In fact, no significant differences were found among groups (Control, 1.86 g/TAE; CHE, 1.94 g/TAE; QUE, 2.15 g/TAE; S.E.M, 0.18; P= 0.4675).

Metabolic profile, oxidative status and fatty acid profile of blood

Blood parameters were not affected by diets with the exception of phosphorous that was lower in the blood samples of animals fed CHE diet. Significant differences due to time effect (T) are shown by urea content and by transaminase, total protein, globuline, glucose and triglycerides. The data are reported in Table 3.

Table 3. Blood parameters from ewes fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHE diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

					P value [3]		
Item[1]	**Control**	**CHE**	**QUE**	**SEM[2]**	**D**	**T**	**D X T**
P tot g / dl	8.21	8.27	7.85	0.340	0.6644	0.0011	0.8699

Urea mg / dl	44.14	43.19	48.37	2.144	0.2895	<0.0001	0.1484
ALB g / dl	3.79	3.73	3.73	0.056	0.6525	0.4641	0.1110
GLB g / dl	4.42	4.54	4.12	0.162	0.4952	< 0.0001	0.0256
γ-GT U / l	58.87	60.66	62.75	4.120	0.5347	< 0.0001	0.7359
SGPT U / l	18.50	19.39	22.78	2.632	0.5393	0.0117	0.2041
SGOT U / l	207.52	248.31	195.45	22.413	0.6049	0.0065	0.4033
GLU mg / dl	67.20	67.85	66.83	1.397	0.8865	<0.0001	0.7079
CHO mg /dl	83.25	83.75	86.29	6.890	0.9467	0.3081	0.0628
Creatinine mg / dl	0.73	0.72	0.79	0.026	0.1629	0.1438	0.6079
Triglycerides mg / dl	18.08	22.05	17.75	1.338	0.0935	0.0048	0.0614
Phosphorus mg/dl	4.58 a	4.29 b	4.52 a	0.492	0.0038	0.9297	0.9811

[1]P tot: total protein; ALB: albumine; GBL: globuline; γ-GT: γ-glutamil-transferase; SGPT: serum glutamic-pyruvic-transaminase; SGOT: serum glutamic-oxaloacetic-transaminase; GLU, glucose; CHO, cholesterol. [2]Standard Error Mean. [3]Probability of significant effect due to experimental factors: diet (D), time (T), and their interaction (D X T).

The oxidative status of ewes, determined using MDA as indicator, did not present significant differences among groups, regardless the concentrates fed by animals (Control, 1.48 μmol/ml plasma vs CHE, 1.49 μmol/ml plasma vs QUE, 1.43 μmol/ml plasma; S.E.M. 0.07; P = 0.8484).

Fatty acid profile of blood showed that tannins, regardless the typology, favored the increase of VA and, at the same time, the reduction of stearic acid (18:0; SA) concentration. Data are reported in Table 4. Interestingly, neither LA nor α-LNA concentration was enhanced by CHE and QUE. No significant effects of tannin supplementation were observed also for BH intermediates such as RA, *trans*-11, *cis*-15 18:2, *trans*-10, *cis*-15 18:2 and *cis* and *trans* isomers of 18:1 different to VA (Table 4). Saturated fatty acids (SFA) are lower in blood from animals fed concentrates integrated with tannins and this effect is stronger with CHE. Monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) reached higher concentrations in blood from animals fed QUE and minor percentages in C group. Finally, the content of fatty acids with a carbon chain longer than 16 units was comparable in C and QUE groups and lower in CHE (Table 4).

Table 4. Fatty acid composition of blood from sheep fed 800g /head and day of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g /kg DM of chestnut tannin extract (CHE diet) or 52.8 g /kg DM of a quebracho extract (QUE diet).

	Diet				P value[2]		
Fatty acid g/100g FA	Control	CHE	QUE	SEM[1]	D	T	D x T
2:0	0.30	0.28	0.24	0.033	0.4672	<0.0001	0.7232
3:0	0.20	0.11	0.11	0.040	0.2482	0.9142	0.5382
4:0	0.24 a	0.17 b	0.17 b	0.014	0.0203	0.0577	0.4421
6:0	0.29	0.31	0.32	0.012	0.5267	0.4325	0.8541
8:0	0.37	0.58	0.22	0.140	0.0846	0.0324	0.0008
10:0	0.26	0.32	0.22	0.072	0.3399	0.1285	0.0112
12:0	0.08	0.08	0.12	0.032	0.6288	0.4123	0.1495
14:0	0.29	0.29	0.28	0.016	0.8388	0.0053	0.7158
cis-9 14:1	0.06	0.06	0.05	0.011	0.8682	0.5891	0.9334
anteiso 15:0	0.13	0.13	0.11	0.010	0.2768	0.1472	0.8038
15:0	0.36	0.32	0.36	0.020	0.2686	0.0885	0.9054
iso 16:0	0.11	0.11	0.12	0.017	0.9836	0.0326	0.9866
16:0	13.44	13.27	13.48	0.385	0.9191	0.0327	0.7097
cis-9 16:1	0.18	0.16	0.15	0.016	0.5863	0.0884	0.7434
iso 17:0	0.26	0.24	0.26	0.015	0.4674	0.3260	0.3963
anteiso 17:0	0.30	0.32	0.31	0.017	0.875	0.1539	0.1144
17:0	0.84	0.78	0.80	0.034	0.5045	0.0030	0.1730
cis-9 17:1	0.11	0.11	0.10	0.010	0.7918	0.3345	0.6518
18:0	24.02 a	21.14 b	22.25 b	0.610	0.0264	<0.0001	0.0419
trans-6,8 18:1	0.17	0.27	0.23	0.034	0.2182	0.0810	0.0906
trans-9 18:1	0.39	0.39	0.37	0.038	0.8109	0.3785	0.1943
trans-10 18:1	0.32	0.41	0.37	0.037	0.2334	0.4428	0.0820
trans-11 18:1	2.21 c	2.73 b	3.18 a	0.213	0.0309	0.1761	0.8676
trans-12 18:1	0.65	0.63	0.77	0.076	0.4023	0.8504	0.5560
cis-7 18:1	0.30	0.37	0.83	0.337	0.5214	0.6267	0.5443
cis-9 18:1	11.14	10.32	10.6	0.480	0.5584	0.0635	0.8051
cis-11 18:1	0.47	0.50	0.48	0.024	0.5968	0.1606	0.6554
cis-12 18:1	2.62	2.06	2.19	0.146	0.0626	0.0116	0.1968
cis-15 18:1	0.08	0.09	0.06	0.016	0.6386	0.5605	0.3394
cis-9, *cis*-12 18:2	24.36	23.34	25.55	0.622	0.0849	<0.0001	0.9687
cis-9, *trans*-11 18:2	0.48	0.54	0.59	0.044	0.2822	0.9649	0.3093
cis-9, *cis*-12, *cis*-15 18:3	1.99	1.74	1.93	0.146	0.5268	0.0028	0.1669
trans-11, *cis*-15 18:2	0.23	0.21	0.22	0.013	0.5636	0.3153	0.2733
Other fatty acids	12.75 b	17.62 a	12.96 b	0.423	0.0426	0.4251	0.3271
SFA[3]	41.49 a	38.45 c	39.37 b	0.361	0.0485	0.9524	0.7259
MUFA[4]	19.00 b	18.42 c	19.69 a	0.263	0.0425	0.5246	0.2546
PUFA[5]	27.06 b	25.83 c	28.29 a	0.594	0.0345	0.2543	0.3581
< 16:0[6]	16.31	16.19	15.95	0.352	0.3526	0.2485	0.5214

> 16:0[7]	71.12 a	66.35 b	71.24 a	0.523	0.0425	0.2541	0.5963

[1]Standard Error Mean. [2]Probability of significant effect due to experimental factors: diet (D). time (T). and their interaction (D X T); means within a row with different letters differ (P < 0.05). [3]SFA: saturated fatty acids
[4]MUFA: monounsaturated fatty acids. [5]PUFA: polyunsaturated fatty acids. [6]< 16:0 *de novo* fatty acids calculated according to Fievez et al., 2012. [7]> 16:0 preformed fatty acids calculated according to Chilliard et al., 2000 and Fievez et al., 2012.

Milk fatty acid profile

Only few significant differences among fatty acids as consequence of the dietary tannin supplementation were found (Table 5). In particular, regardless the tannin kind, RA increased when the concentrates contained polyphenols and the stronger effect is reached with QUE (P< 0.0002). In milk from CHE and QUE groups *cis*-9, *cis*-15 18:2 decreased with tannin supplementation (P< 0.0001; Table 5).

Table 5. Fatty acid composition of milk from sheep fed 800 g / head / d of a concentrate containing 84 g of soybean oil / kg DM plus 0 (control diet) or 52.8 g / kg DM of a chestnut tannin extract (CHE diet) or 52.8 g / kg of DM of quebracho tannin extract (QUE diet).

	Diet				P value[2]		
Fatty acid g/100g FA	Control	CHE	QUE	SEM[1]	D	T	D x T
4:0	4.363	4.397	3.882	0.234	0.2647	<0.0001	0.6455
6:0	1.311	1.343	1.161	0.067	0.1682	0.0031	0.9678
8:0	0.918	1.047	0.992	0.064	0.4605	0.1087	0.8375
10:0	2.595	3.087	2.849	0.237	0.3521	0.0074	0.9408
cis-9 10:1	0.15	0.15	0.15	0.003	0.2598	0.3586	0.4528
12:0	2.013	2.065	1.883	0.093	0.4074	0.2027	0.7435
cis-9 12:1	0.006	0.008	0.011	0.002	0.3530	0.159	0.2033
iso 13:0	0.042	0.027	0.031	0.008	0.5137	0.2531	0.4457
anteiso 13:0	0.010	0.010	0.020	0.005	0.5246	0.8254	0.1562
13:0	0.030	0.030	0.030	0.012	0.4852	0.2589	0.5914
iso 14:0	0.075	0.048	0.047	0.011	0.2030	0.8325	0.6128
14:0	6.963	7.123	6.821	0.141	0.3799	0.0020	0.7559
iso 15:0	0.155	0.156	0.150	0.006	0.7114	0.5916	0.7471
cis-9 14:1	0.126	0.115	0.124	0.007	0.6010	0.6495	0.8473
anteiso 15:0	0.288	0.276	0.291	0.013	0.6752	0.3354	0.5820
15:0	0.593	0.627	0.608	0.021	0.5327	0.0043	0.4372
iso 16:0	0.085	0.103	0.090	0.014	0.7377	0.0005	0.3660
16:0	16.611	19.290	16.590	0.947	0.1279	<0.0001	0.1649
cis-9 16:1	0.521	0.430	0.471	0.041	0.4017	0.0118	0.7001
iso 17:0	0.296	0.327	0.333	0.027	0.5385	0.3159	0.9608
anteiso 17:0	0.270	0.310	0.325	0.024	0.3164	0.3739	0.9443
17:0	0.472	0.468	0.481	0.018	0.9110	0.0173	0.9841
cis-9 17:1	0.13 b	0.13 b	0.15 a	0.006	0.0319	0.3419	0.4526

18:0	13.011	12.612	12.121	0.276	0.1149	0.0313	0.8718
trans-6,8 18:1	0.770	0.733	0.728	0.051	0.8534	0.1072	0.8936
trans-9 18:1	0.648	0.776	0.754	0.035	0.0662	0.0141	0.3605
trans-10 18:1	1.534	1.396	1.476	0.073	0.5105	0.3596	0.8554
trans-11 18:1	5.361	5.263	5.592	0.292	0.7440	0.0125	0.7759
trans-12 18:1	0.985	1.076	0.985	0.052	0.4616	0.7079	0.6560
cis-7 18:1	0.411	0.395	0.378	0.090	0.9702	<0.0001	0.7934
cis-9 18:1	19.506	19.612	18.790	0.578	0.5856	0.0664	0.2015
cis-11 18:1	0.526	0.515	0.516	0.031	0.9694	0.3217	0.9537
cis-12 18:1	0.487	0.487	0.479	0.032	0.9743	0.9078	0.9884
cis-15 18:1	1.118	1.213	1.064	0.043	0.0865	0.0390	0.5914
cis-9, *cis*-12 18:2	3.838	3.766	3.782	0.112	0.9162	0.0001	0.8258
cis-9, *trans*-11 18:2	2.196 c	2.293 b	2.716 a	0.081	0.0002	0.0003	0.1\972
cis-9, *cis*-12, *cis*-15 18:3	0.507	0.526	0.550	0.020	0.3776	<0.0001	0.1861
cis-9, *trans*-12, *trans*-15 18:3	0.023	0.024	0.043	0.013	0.5457	0.0574	0.7528
trans-9, *cis*-12, *trans*-15 18:3	0.034	0.043	0.033	0.005	0.3871	0.0017	0.1998
cis-9, *cis*-15 18:2	0.064 a	0.017 b	0.011 c	0.006	<0.0001	0.5544	0.5435
trans-11, *cis*-15 18:2	0.194	0.226	0.225	0.024	0.6470	0.0378	0.8339
trans-10, *cis*-12 18:2	0.01	0.01	0.01	0.005	0.9523	0.2596	0.1458
20:0	0.255	0.302	0.242	0.022	0.2245	0.8747	0.4103
20:4	0.022	0.022	0.025	0.005	0.9127	0.0655	0.7641
22:0	0.112	0.088	0.097	0.013	0.5314	0.3006	0.8853
SFA[3]	50.246	53.038	48.206	1.781	0.1173	<0.0001	0.7641
MUFA[4]	32.435	32.124	31.216	0.841	0.5863	0.0057	0.3198
PUFA[5]	6.922	6.922	7.365	0.208	0.2635	0.0008	0.6908
OIAR[6]	0.803 b	0.824 a	0.784 c	0.037	0.7879	0.0302	0.1638
DI[7]	0.017	0.016	0.017	0.002	0.1926	0.1159	0.2166
< 16:0[8]	36.440	39.636	35.166	1.508	0.1432	<0.0001	0.6570
> 16:0[9]	70.440	72.324	68.629	1.705	0.3565	<0.0001	0.2010

[1]Standard Error Mean. [2]Probability of significant effect due to experimental factors: diet (D), time (T), and their interaction (D X T); means within a row with different letters differ ($P < 0.05$). [3]SFA: saturated fatty acids. [4]MUFA: monounsaturated fatty acids. [5]PUFA: polyunsaturated fatty acids. [6]Ratio odd-iso to odd-anteiso FA: (iso 15:0 + iso 17:0) / (anteiso 15:0 + anteiso 17:0). [7]Desaturation index, DI = (*cis*-9 14:1 / 14:0 + *cis*-9 14:1). [8]< 16:0 *de novo* fatty acids calculated according to Fiviez et al 2012. [9]> 16:0 preformed fatty acids calculated according to Chilliard et al., 2000 and Fiviez et al., 2012.

Discussion

In the present trial, quebracho tannins increased the CI of milk (P =0.0259), probably as consequence of the slight decrease of whey proteins. Literature reported that tannins, especially if condensed, are able to interfere with amino acid absorption at the gut level because they selectively complex amino acids, affecting protein synthesis in tissues (Min et al., 2003; Patra and Saxena, 2011). The higher clotting time and firming time observed in milk from ewes fed QUE respect milk from no tannins and CHE fed ewes (P< 0.05), could be probably due to different interaction between casein and bioactive monomers derived by the rumen microbial BH of quebracho proantocyanidins (Gladine et al., 2007) or chestnut gallic acid compounds (Bhat et al., 1998), reaching in small quantities the mammary gland after duodenal absorption. Moreover, commercial tannin extracts from quebracho are not often pure proantocyanidins and might contain other compounds with a simpler structure (monomers) which could be metabolized in the rumen. Indeed, several authors demonstrated that tannins can be partially metabolized by rumen microorganisms and that their metabolites can be absorbed at the gut level with a transferring in meat and milk of small ruminants (Lopez-Andres et al., 2013; Luciano et al., 2011; Jordan et al., 2010; Singh et al., 2001). The absorption of polyphenolic compounds generally depends on i) their molecular structure that, in turn, affects their solubility; ii) the ability of rumen microbiome in degrading them to compounds with a lower molecular weight; iii) their percentage of inclusion in the animal diet. Chestnut and quebracho tannins are characterized by a very complex structure with a high molecular weight (Campo et al., 2012; Pash et al., 2001).

In the present trial, the lack of significant differences in MTP concentration across groups suggested that the polyphenol substances contained in the tannin extracts were little absorbed in the intestine. This result could be also due to the dietary concentration of the tannin extracts in treated groups (about 1.6% on DMI). In literature, significant differences have been observed when the concentration of dietary polyphenols from condensed tannins contained in pasture *Hedysarum coronarium* L. has been up to 6.0% of the DMI (Di Trana et al., 2015), hence with a percentage of inclusion more than three folds higher respect to that used in the present experiment. Nevertheless, a key role seems to be played by the kind of metabolite deriving from BH of tannins at the rumen level. In fact, in literature, previous findings report a higher clotting time of milk when grape extracts (Felix da Silva et al., 2015) or tea catechins (Haratifar and Corredig, 2014) are added, as consequence of the interaction between polyphenols and proline residues close to the cleavage point of k-casein, reducing the enzyme

accessibility. Han et al. (2011) observed that milk clotting parameters is affected by the structure and source of polyphenols while O'Connel et al. (1998) observed an increase of the interference on milk clotting behavior with the increase of the polymeric size of phenolic compounds.

No significant differences were found in oxidative status of blood among groups, probably because animals were at late lactation. A previous study on dairy cows, in fact, suggested that chestnut tannin might exert antioxidant properties when animals were in the transition period (Liu et al., 2013). In contrast, other studies suggested that condensed tannin, as QUE, may exhibit toxic effect in liver (Min et al., 2003; Patra and Saxena, 2011; Reed, 1995; Hervas et al., 2003). In the present study, data related to blood parameters showed that CHE and QUE did not exert a toxic effect in liver at short term according to optimal values of transaminase.

Fatty acid profile of blood suggested that tannins were able to interfere with BH of dietary PUFA. In fact, plasma samples from CHE and QUE groups contained higher concentration of VA and lower concentration of SA if compared to plasma samples from C group. This pattern suggested that tannins interfere with the last step of BH of α-LNA and of LA affecting the activity of microorganisms responsible of VA hydrogenation, as reported also by previous studies (Buccioni et al., 2015a; Vasta et al., 2010, Hervas et al., 2013). Moreover, our data confirmed that the effect of quebracho is stronger than that of chestnut tannin in modulating rumen fermentation of PUFA (Table 4).

In the present study, LA mainly is originated from soybean oil triglycerides whereas α-LNA from structural lipids of pasture grasses. Since rumen BH of LA and α-LNA produces different pattern of intermediates (Shingfield et al., 2013), the comparison of plasma lipid fatty acid profile between CHE and QUE sheep might provide information about difference in the BH pattern due to the effect of the type of tannin included in the diet. According to Table 4, among the BH intermediates only VA content significantly varied, suggesting that the effect of CHE and QUE did not differ in the BH of PUFA contained in triglycerides or structural lipids because VA is originated either from LA either from α-LNA, main FA in oil and roughage respectively.

The addition of tannin extract to the diet CHE and QUE did not result in an enhancement of the rumen outflow of α-LNA and LA, as confirmed by the lack of differences in the fatty acid profile of both blood plasma and milk samples. This result did not agree with findings obtained in a previous trial by Buccioni et al. (2015b). These authors found that the intake of α-LNA from pasture and linseed increased concentration of α-LNA in milk from ewes fed a diet supplemented with chestnut tannins, suggesting an effect of CHE extract on the BH

process of α-LNA starting from the first step of the pathway. In a previous trial, Buccioni et al. (2015a) found that the addition of similar amounts of quebracho and chestnut tannin extracts to the diet of dairy ewes resulted in an increase of LA in milk fat, as a consequence of the reduction of LA BH process. Also in this case, therefore, the results of the present experiment did not confirm a reduction of the LA BH from the first step of the process. Since the previous study adopted diets containing hay as forage basis instead of pasture, the role of the forage typology should be better understood in order to interpret the effect of tannin extracts on rumen BH of LA.

In the present experiment, the amount of VA escaped from rumen BH was probably enhanced when dairy sheep are fed diet supplemented with tannin extract, especially in the case of QUE diet (Table 4). This aspect was confirmed by the higher concentration of VA in plasma samples from QUE animals. Since *cis*-9, *trans*-11 CLA is mainly produced in mammary gland by Δ^9desaturation of VA (Bauman and Griinari, 2003), higher concentration of circulating VA probably led to higher amounts of *cis*-9, *trans*-11 CLA in milk fat. Hence, comparing plasma and milk fatty acid composition, it seemed that the activity of Δ^9desaturase (SCD) increased RA concentration in milk reducing the differences in VA concentration found in blood samples (Tables 4 and 5). Interestingly, also the concentration of SA in milk fat did not differ across treatments, despite the percentage of SA in plasma samples did. Similarly, to VA, the content of SA in milk fat is strictly regulated by the uptake of mammary tissue and by Δ^9desaturase enzyme activity, which converts SA to *cis*-9 18:1. In particular, almost 50% of *cis*-9 18:1 secreted in sheep milk originates from SCD activity (Frutos et al., 2014). However, several authors found that rumen fluid fatty acid profile may not fully reflect those of digesta in duodenum when dairy lactating cows fed a diet supplemented with a blend of soybean and fish oils together with tannins from *Vaccinium vitis idaea* (Szczechowiak et al., 2016). Moreover, Szczechowiak et al. (2016) found also that the treatment did not change the expression of five genes regulating the FA metabolism in mammary gland (codifying for acetyl-CoA carbossilase 1, ACACA; fatty acid synthase, FASN; stearoyl-CoA desaturase, SCD; fatty acid desaturase 1, FADS1; fatty acid elongase 5, ELOVL5) and of the gene codifying for lipoprotein lipase (LPL) regulating the lipoprotein absorption catalyzing the hydrolysis of triglycerides transported in blood stream by very low density lipoproteins and by chilomicrons at the gut level (Malgorzata Brzozowska and Oprzadek, 2016).

Conclusion

The supplementation of diet of dairy ewes with chestnut or quebracho tannin extracts was effective in perturbing the last step of rumen BH of LA from soybean oil and α-LNA from pasture, as suggested by the higher amounts of VA in the plasma of animals fed QUE and CHE diets. The lack of effect on BH intermediates other than VA (the last step of the process) may suggest that rumen BH of PUFA was not influenced by the origin of PUFA. The higher amount of circulating VA probably enhanced the RA synthesis in mammary gland by SCD activity, resulting in higher percentages of RA in milk fat from animals fed QUE and CHE diets. Although more studies are needed in order to better understand why in some cases tannin extracts act at the first step of the BH whereas in other cases, as in the present study, the interference happened at the last step of BH, the use of tannin extracts might be an efficient way to improve PUFA content of sheep milk.

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2.2 Effect of chestnut tannin extract, vescalagin and gallic acid on fatty acid biohydrogenation and microbial community profile in rumen liquor: an *in vitro* study.

(Manuscript in progress)

Personal contribution: Concieved the experiment, FAME and DMA analysis, collaboration in statistical analisis and wrote the manuscript

A B S T R A C T

The aim of this trial has been to study, in an *in vitro* experiment, the potential effects of chestnut tannin, vescalagin and gallic acid on microbial community profile of rumen liquor, on dimethylacetals characterization, on polyunsaturated fatty acid biohydrogenation and on fiber degradability. Four feeds (basal diet - diet C - formulated with tannin free ingredients; basal diet supplemented with chestnut tannin extract - diet CHT - at the inclusion level of 1.6 g/100g of dry matter; basal diet supplemented with vescalagin - diet VES - at the inclusion level of 0.24 g/100g of dry matter; basal diet supplemented with gallic acid - diet GAL - at the inclusion level of 0.032 g/100g of dry matter) were inoculated with ewe rumen fluid and fermented for 24h. At the end of fermentation, samples of rumen liquor were analysed by means of high-throughput sequencing (HTS) of the 16S rRNA gene for microbiota characterization, by GC-MS for fatty acid and dimethylacetal (DMA) profile. Results showed that CHT, VES and GAL did not exert strong detrimental effects on microbiota that presented only a little perturbation. The genera *Anaerovibrio*, *Bibersteinia*, *Escherichia/Shigella* and *Streptococcus* were enriched in the fermenters supplied with CHT compared to the other conditions. The relative abundance of the genus *Pseudobutyrivibrio* increased with CHT, compared to the control and to the fermenters supplied with VES. Conversely, the relative abundance of the genus *Arcobacter* decreased with CHT. Furthermore, a slight decrease in the relative abundance of the genus *Treponema* was observed in the fermenters supplied with GAL compared to the fermenters supplied with VES. This trend was consistent with chemical data in which no effect on fatty acid profile was found among groups. In contrast, the presence of CHT extract, VES and GAL in the basal diet modified the concentration of several DMA. At 24h DMA 12:0, DMA 13:0, DMA 14:0, DMA 15 iso, DMA 18:0 and DMA 18:1 trans11 were lower in C fermenters than in the others ($p \leq 0.05$).

Introduction

Chestnut tannin (CHT) is a hydrolysable polyphenol that can be extract from chipped chestnut wood obtained by distillation in water. of chipped chestnut wood Iand it is composed by several fractions of which vescalagin (VES) and gallic acid (GAL) represent about 20% and 6% of total tannins, respectively (Bargiacchi et al., 2017). CHT exhibits antimicrobial activities against *Clostridium perfringens* (Tosi et al., 2013) or *Cryptosporidium parvum* (Bonelli et al., 2018) and VES and GAL seem to play an important role as antimicrobials (Ekambaram et al., 2016). Quideau et al. (2004) studied several nonahydroxyterphenoyl-containing C-glycosidic ellagitannins (castalagin, vescalagin, from Castanea spp.) and these

authors found that the most effective was vescalagin, which showed an 50% inhibitory concentration (IC50) of 0.04 nM against both Acyclovir-susceptible and Acyclovir–resistant *Herpes Simplex Virus* strains. Panizzi et al. (2002) demonstrated the efficacy of GAL as antimicrobial against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Saccharomyces cerevisiae* and *Candida albicans*.

In literature, studies regarding the activities of bioactive compounds contained in functional foods on human health increased (Sofi et al., 2010; Pintus et al., 2013; Benjamin et al., 2015). Consequently, food industry is focused in developing foods with healthful properties and health claim appeal. In animal feeding, strategies have been set up to increase, in milk and meat, the level of polyunsaturated fatty acids (PUFAs), level as α-linolenic acid (α-LNA), considered by the European Food Safety Authority a healthful fatty acid (European Food Safety Authority, 2009, 2010; Mele et al., 2011; Buccioni et al., 2015a; Cappucci et al., 2018; Mannelli et al., 2018). Data reported in literature, showed that the use of CHT in ruminant diet may be an efficient tool to modulate microbial biohydrogenation (BH), favouring the increase of bioactive moleculesa in ruminant derived food (Buccioni et al., 2015b; a, 2017). VES represented the main component present in CHT (Bargiacchi et al., 2017) while GAL is quickly free in rumen liquor (RL) by hydrolysis (Murdiati et al., 1992).

The aim of this *in vitro* trial was to verify if the modulator activity of CHT in RL is due to the potential effects of VES or GAL or of a synergic effect of all components contained in CHT.

The main obstacle in understanding microbiota structure or function is that only few bacteria are isolable and cultivable respect to the whole rumen ecosystem. Recently, next generation sequencing technologies offered powerful tools to study rumen microbiome and high-throughput sequencing (HTS) of the 16S rRNA gene has emerged as an effective strategy to characterize the microbial communities (Brulc et al., 2009; Mannelli et al., 2018). No metataxonomic data are available in literature on the microbiota structure affected by CHT or by components of it. Hence, another aim of this trial was to study microbial community profile of rumen liquor (RL) by means of HTS of the 16S rRNA gene.

Keyword: chestnut tannin, vescalagin, gallic acid, rumen microbiota, biohydrogenation, Dimethyl acetals, HTS-technology

Abbreviations

ADF, acid detergent fiber; ADL, acid detergent lignin; ASV, amplicon sequence variance; BH, biohydrogenation; C, control; CHT, chestnut tannins; CP, crude protein; DM, dry matter; DMA, dimethylacetals; EE, ether extract; FA, fatty acids; FAME, fatty acid methyl esters; GAL, gallic acid; HTS, high-throughput sequencing; α-LNA, α-linolenic acid; NDF, neutral detergent fiber; NDFdeg, NDF degradability; NDFundeg, NDF residuals; OM, organic matter; PUFA, polyunsaturated fatty acids; qPCR, quantitative PCR; RL, rumen liquor; VES, vescalagin.

Material and method

Feeds

The control feed was formulated using tannin-free ingredients. The formulation of diets was the same used in an in vivo trial (Buccioni et al., 2017b), to have a real diet tested previously with animals. Feeds involved a control (diet C) composed of chopped grass hay (60.00 g/100g on DM), barley (8.56 g/100g on DM), maize meal (8.46 g/100g on DM), wheat bran (6.35 g/100g on DM), soybean meal (5.08 g/100g on DM), beet pulp (3.60 g/100g on DM), soybean oil (3.39 g/100g on DM), molasses (1.70 g/100g on DM), mineral-vitamin supplement (1.26 g/100g on DM), and of three other diets obtained by adding to C, either 1.60 g/100g on DM of CHT extract (diet CHT) or 0.24 g/100g on DM of VES (diet VES) or 0.032 g/100g on DM of GAL (diet GAL).

Chestnut tannin powder was provided by Gruppo Mauro Saviola srl (plant in Radicofani, Siena, Italy) and contained 750 g equivalents of tannic acid/kg on DM, determined according to the method of Burns (1963). VES and GAL were purchased by Sigma-Aldrich (cods 76418 and G7384, respectively; Sigma-Aldrich, St. Louis, MO). The concentrations of VES and GAL inclusion in the diets were calculated after characterization of CHT extract according to the method reported by Bargiacchi et al. (2017).

Proximate analyses of feed samples

Samples of diet C were oven-dried at 60°C for 24h. The dry samples were analyzed for crude protein (CP), ether extract (EE) and ash according to AOAC methods (1995; 976.06, 920.39 and 942.05 procedures, respectively). Fiber fractions were determined according to van Soest et al. (1991) as follow: neutral detergent fiber (NDF) was determined using sodium

sulphite and heat stable amylase; acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined in a sequential analysis. All data were expressed inclusive of residual ash.

The chemical composition of the control diet was DM 92.00 g/100g of feeds, CP, 12.40 g/100g on DM; EE, 4.87 g/100g on DM; NDF, 46.63 g/100g on DM, ADF, 33.28 g/100g on DM; ADL, 6.43 g/100g on DM.

The concentrations of VES and GAL inclusion in the diets were confirmed as previously reported (Bargiacchi et al., 2017).

Rumen inoculum

The whole rumen content from one ewe sacrificed at slaughterhouse was used to provide the liquor as inoculum for the fermentation, according to Denek et al. (2006) and Lutakome et al. (2017). Animal was fed for 1 month before the slaughtering with the diet C used in the in vitro trial. The ewe was not sacrificed specifically for the experiment but slaughtered as replacing quote in farm.

The RL was immediately mixed and transferred to the laboratory in a thermostatic box (39°C). Then, it was filtered through four layers of cheese cloth into a flask under a flux of CO2, as described by Buccioni et al. (2011). Six hundred millilitres of rumen liquor were buffered (1 : 3, v/v) by adding an artificial saliva solution (McDougall, 1947). Feeds (1.0 g on DM) were incubated (6 fermenters per diet) with 100 ml of buffered inoculum and pH was monitored continuously during the whole period of the trial (Buccioni et al., 2011). Incubation times were 0 and 24 h. At 24 h, all fermenters were used for rumen content analysis as follow: 3 fermenters per diet were used for fatty acids (FA), dimethylacetals (DMA) and microbial characterization; the other 3 fermenters per diet were used for the determination of NDF degradability, as described below. Moreover, after filtration and buffering at time 0, three samples of fresh rumen fluid (100 ml) were freeze-dried and analyzed as blank to control the present of possible artefacts.

Fatty acids analysis and DMA

Immediately after collection, each sample was measured for pH and stored at −80°C until analysis for FA and DMA composition. Before the analysis, samples were freeze-dried and then trans-esterified by using a combined basic and acid procedure, according to Jenkins (2010) as modified by Alves et al. (2013). Briefly, 250 mg of freeze-dried rumen sample was put in a centrifuge tube and added with 1 mL of toluene and 1 mL of hexane containing internal standard (C19:0, 1 mg/mL). The basic trans-esterification was performed by the

addition of 2 mL of sodium methoxide in methanol (0.5 M). The solution was vortexed and left to react for about 10 min at 50°C. Subsequently, the mixture was cooled to room temperature and then added with 3 mL of HCl solution in methanol (10:90 vol:vol). The solution was allowed to react for 10 min at 80°C. Once cooled, 2 mL of a K2CO3 solution (6:94 wt:vol) was added, followed by the addition of 2 mL of hexane to extract fatty acid methyl esters (FAME) and DMA. The solution was vortexed, centrifuged, and finally, the supernatant phase with FAME and DMA was transferred to another tube. The extraction step was repeated twice. The identification of single FAME and single DMA was obtained by GC/MS to detect the retention times, according to Alves et al. (2013). After, FAME and DMA in each sample were separated by thin-layer chromatography, as described in Alves et al. (2013) and the profile was determined using a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD) equipped with a flame-ionization detector and a high polar fused-silica capillary column (Chrompack CP-Sil 88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 μm). Hydrogen was used as the carrier gas at a flow of 1 mL/min. Split/ split less injector was used with a split ratio of 1:80. An aliquot of the sample was injected under the following GC conditions: the oven temperature started at 60°C and held at that level for 1 min; it was then increased to 173°C at a rate of 2°C/min, and held at that level for 30 min, before being once again increased to 185°C at 1°C/min and held for 5 min, and then to 220°C at a rate of 3°C/min, and held for 19 min. The injector temperature was set at 270°C and the detector temperature was set at 300°C. All FAME and DMA composition results were expressed as g per 100 g of DM.

Rumen degradability

NDF degradability (NDF_{deg}) was determined according to Tilley and Terry method (1980) limiting the procedure only to the first step and not considering the digestibility with pepsin. NDF residuals (NDF_{undeg}) were analyzed according to Van Soest et al. (1983). NDF_{deg} was calculated by difference between NDF of feeds before the fermentation and NDF residuals after 24h of incubation:

$$NDF_{deg} = (NDF_{feed} - NDF_{undeg})/ NDF_{feed} * 100$$

Amplification of 16S rRNA gene, sequencing and sequence analyses

DNA was extracted with the Fast DNA Spin kit for soil (MP Biomedicals, Solon, OH) according to Mannelli et al. (2018). DNA purity and quantity were measured using a ND-

1000 Spectrophotometer (NanoDrop Technologies, Labtech, Ringmer, UK) and standardized to a concentration of 10 ng/µl. The V3-V4 hypervariable regions of the 16S rRNA gene were PCR-amplified using the Pro341f and Pro805R primers (Takahashi et al., 2014). Sequencing was performed at BMR Genomics (Padova, Italy) by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) using a 300 bp x2 paired end protocol. Bioinformatic elaborations were performed in R 3.5.1 (R Core Team 2018) with DADA2 package (Callahan et al., 2015), version 1.8.0. The first 20 bases were removed from both forward and reverse reads, additionally forward reads were truncated at 280 bases and reverse reads were truncated at 250 bases. The reads with expected errors higher than 0.5 were discarded. Specific error rates were estimated for the forward reads and for the reverse reads. Filtered reads were dereplicated, the estimated error rates were used to infer the Amplicon Sequence Variants (ASV) (Callahan et al., 2017) and the reads pairs were merged with default parameters. Chimeric sequences were removed. Taxonomic assignment for each ASV was performed against the ribosomal database project (RDP) database (confidence 80%).

Quantification of the 16S rRNA gene by quantitative PCR

Abundance of total bacteria was estimated by quantification of the copy number of the 16S rRNA gene by quantitative PCR (qPCR) as previously reported (Buccioni et al., 2015a) using universal primers to target partial 16S rRNA gene of total bacteria (Maeda et al., 2003). The analysis was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hertfordshire, UK). Amplification conditions were 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 30 s.

Statistical Analysis

Data for FAME, DMA, NDF_{deg} and microbial relative abundance at genus level (previously tested for normal distribution) were processed by General Linear Model of Statistical Analysis System (SAS, 9.2, 2008) using the following linear model with diet as fixed factor:

$$Y_i = \mu + Diet_i + e_{ij}$$

where Y is the observation, μ is the overall mean, Diet is the fixed effect of i^{th} diet (i = 1 to 4), and e_{ij} is the residual error. For the sake of simplicity, only one level of probability ($P \leq 0.05$) was adopted for the significance of differences between means.

Pairwise correlation among bacterial taxa and FA or DMA composition was performed by multivariate analysis (SAS, 9.2, 2008).

A Non Metric Multidimensional Scaling (NMDS) and a permutational multivariate analysis of variance (PERMANOVA) based on Hellinger transformed genus relative abundance data, calculated removing the unclassified reads, were performed using the vegan package (Oksanen et al. 2018) in R 3.5.1 (R Core Team 2018) using the metaMDS and the adonis2 functions, respectively.

Results

Microbial community composition

The number of copies of the 16S rRNA gene per mL of RL was around 10^8 in all the tested conditions (Fig. S1).

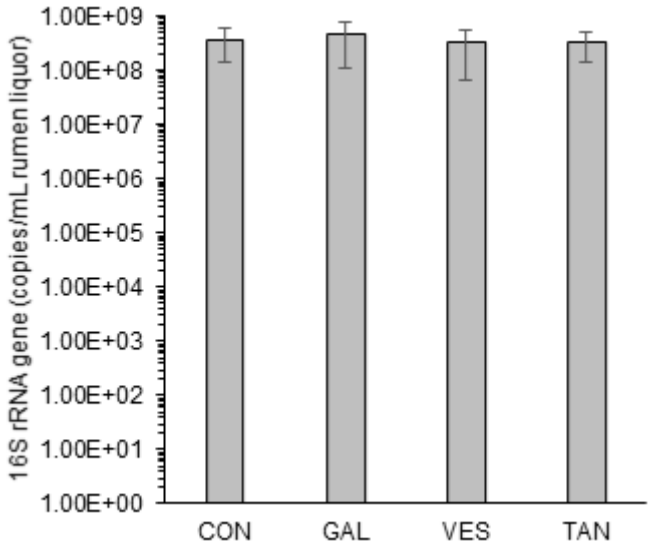

Figure S1. Results of the quantitative PCR on the 16S rRNA gene.

The taxonomic composition of the microbial communities selected at the end of the *in vitro* trial was investigated by HTS of the 16S rRNA gene. ASV rarefaction analysis (Fig. S2) indicated that the sequencing depth was enough to describe the biodiversity within the dataset.

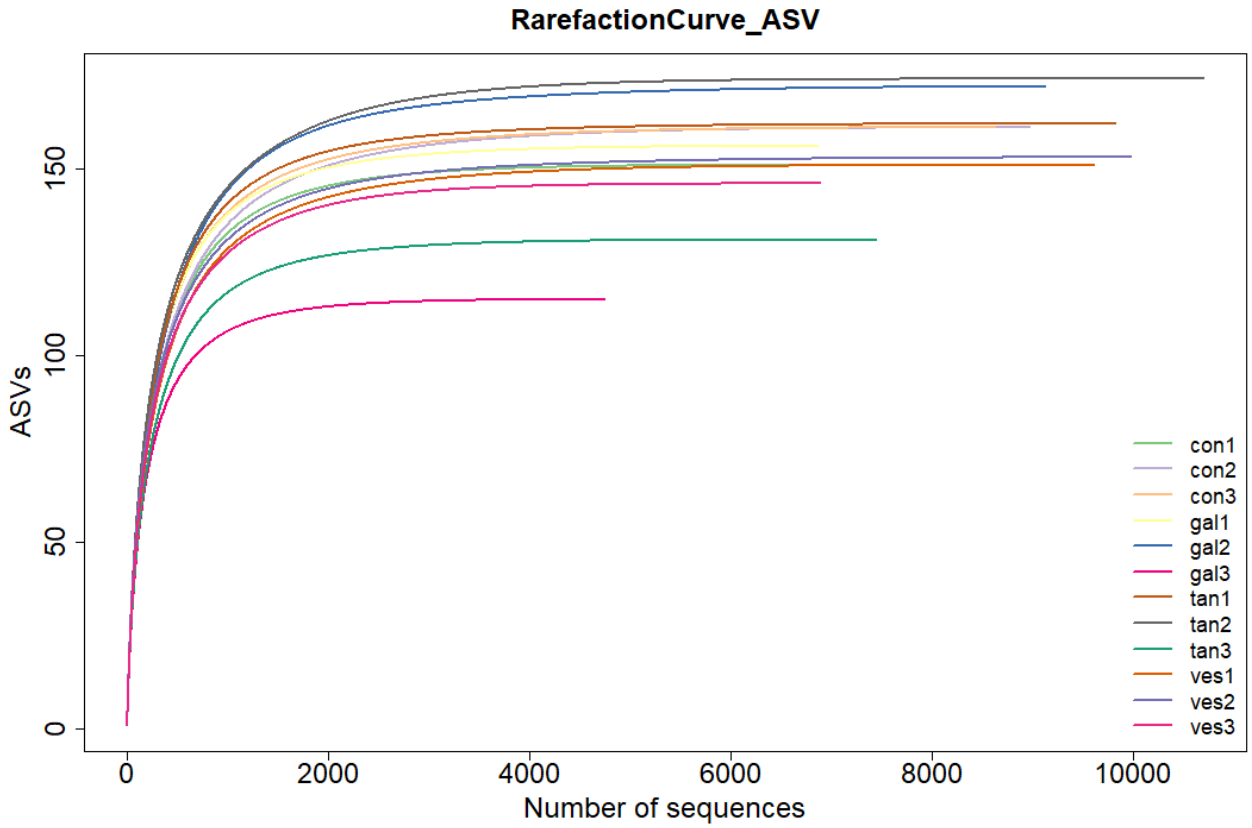

Figure S2. Rarefaction curve calculated at genus level.

The Shannon Index (H') and the ASV richness were used as an estimation of the alpha-diversity within each samples group (Table 1).

	C	GAL	VES	CHT
ASVs	158 ± 3	148 ± 17	150 ± 2	156 ± 13
Shannon Index (H')	4.32 ± 0.03	4.2 ± 0.1	4.29 ± 0.06	4.3 ± 0.1

Table 1. ASVs number and Shannon Index (H') calculated for each tested condition. Values are reported average ± standard error

H' ranged between 4.2 ± 0.1 (GAL) and 4.32 ± 0.03 (C), while the ASV richness ranged between 148 ± 17 (GAL) and 158 ± 3 (C). In total 14 phyla, 22 classes, 24 orders, 31 families and 44 genera were identified within the whole dataset. A core microbiota (i.e. taxonomic groups shared by all conditions) was identified (Table 2) and was comprised of 10 phyla, 15 classes, 17 orders, 21 families and 25 genera.

Table 2. Taxonomic groups shared by all conditions

Phylum	Class	Order	Family	Genus
Bacteroidetes	*Bacilli*	*Aeromonadales*	*Acidaminococcaceae*	*Acinetobacter*
Candidatus_Saccharibacteria	*Bacteroidia*	*Anaeroplasmatales*	*Anaeroplasmataceae*	*Alloprevotella*
Euryarchaeota	*Betaproteobacteria*	*Bacteroidales*	*Bacteroidaceae*	*Anaeroplasma*

Firmicutes	*Clostridia*	*Burkholderiales*	*Campylobacteraceae*	*Anaerovibrio*
Fusobacteria	*Deltaproteobacteria*	*Campylobacterales*	*Clostridiales_Incertae_Sedis_XIII*	*Arcobacter*
Proteobacteria	*Epsilonproteobacteria*	*Clostridiales*	*Desulfovibrionaceae*	*Bacteroides*
Spirochaetes	*Erysipelotrichia*	*Desulfovibrionales*	*Erysipelotrichaceae*	*Bibersteinia*
SR1	*Fusobacteriia*	*Erysipelotrichales*	*Fusobacteriaceae*	*Campylobacter*
Tenericutes	*Gammaproteobacteria*	*Fusobacteriales*	*Lachnospiraceae*	*Desulfovibrio*
Verrucomicrobia	*Methanobacteria*	*Lactobacillales*	*Methanobacteriaceae*	*Fusobacterium*
	Mollicutes	*Methanobacteriales*	*Methanomassiliicoccaceae*	*Mannheimia*
	Negativicutes	*Methanomassiliicoccales*	*Moraxellaceae*	*Methanobrevibacter*
	Spirochaetia	*Neisseriales*	*Neisseriaceae*	*Methanomassiliicoccus*
	Subdivision5	*Pasteurellales*	*Pasteurellaceae*	*Moraxella*
	Thermoplasmata	*Pseudomonadales*	*Porphyromonadaceae*	*Paraprevotella*
		Selenomonadales	*Prevotellaceae*	*Prevotella*
		Spirochaetales	*Ruminococcaceae*	*Pseudobutyrivibrio*
			Spirochaetaceae	*Roseburia*
			Streptococcaceae	*Ruminobacter*
			Succinivibrionaceae	*Saccharofermentans*
			Veillonellaceae	*Selenomonas*
				Streptococcus
				Succiniclasticum
				Succinivibrio
				Treponema

More than 70% of the microorganisms classified at phylum level in each group was represented by the phyla *Proteobacteria* (mainly *Gammaproteobacteria* of the families *Succinivibrionaceae*, *Moraxellaceae* and *Pasteurellaceae* – order *Aeromonadales*, *Pseudomonadales* and *Pasteurellales*, respectively – and, in minor extent, *Betaproteobacteria* of the family *Neisseriaceae* – order *Neisseriales*) and *Bacteroidetes* (mainly of the family *Prevotellaceae*, order *Bacteroidales*, class *Bacteroidia*) (Fig. S3).

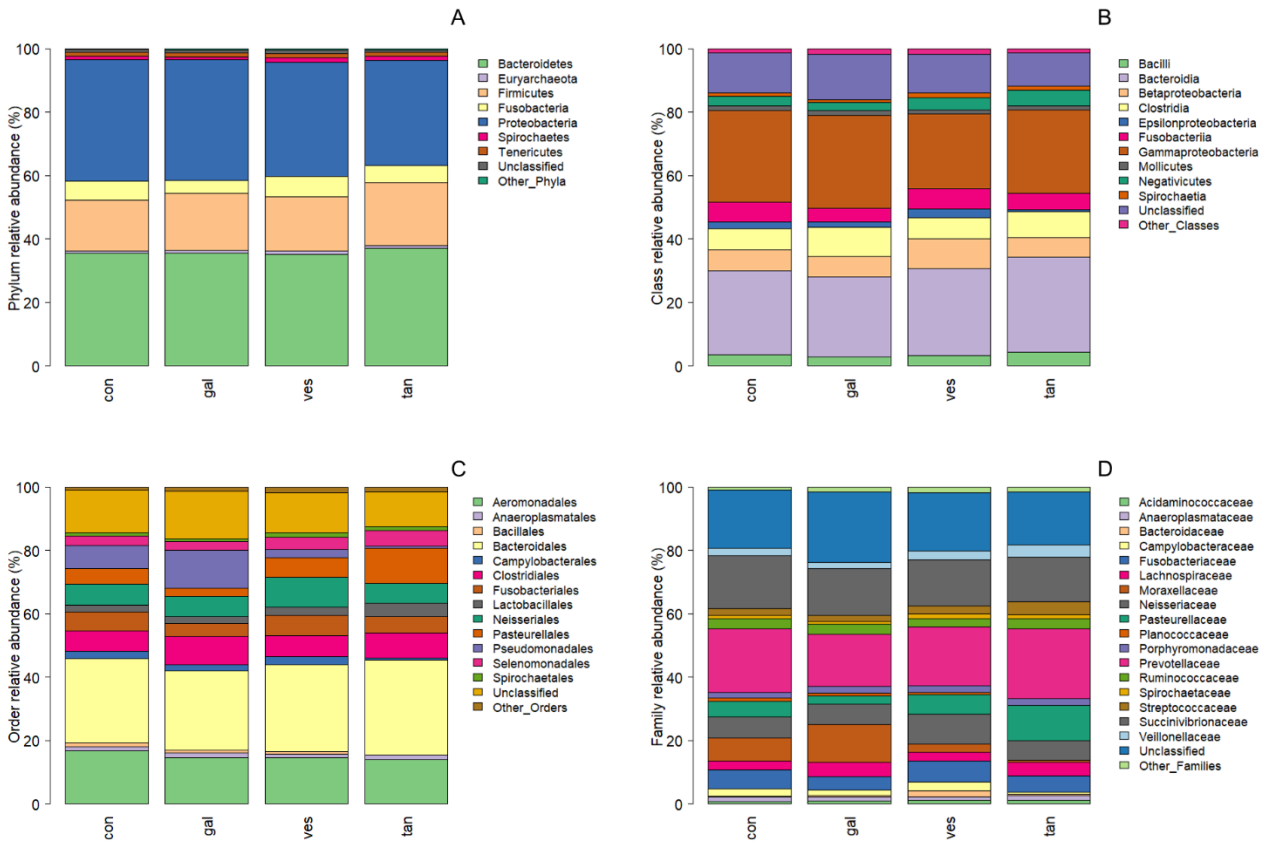

Figure S3. Taxonomic composition at phylum (A), class (B), order (C) and family (D) level of the microbial communities enriched during the in vitro trial. Average abundances are reported for each tested condition. Only the groups with an average relative abundance of 1% (or higher) in at least one condition are reported.

The most abundant genera (Fig. 1 and Table 3) within the core microbiota were *Prevotella* (between ~11% – GAL – and ~16% – CHT), *Ruminobacter* (between ~8% – CHT – and ~11% – C) and *Fusobacterium* (between ~4% – GAL – and ~7% – VES).

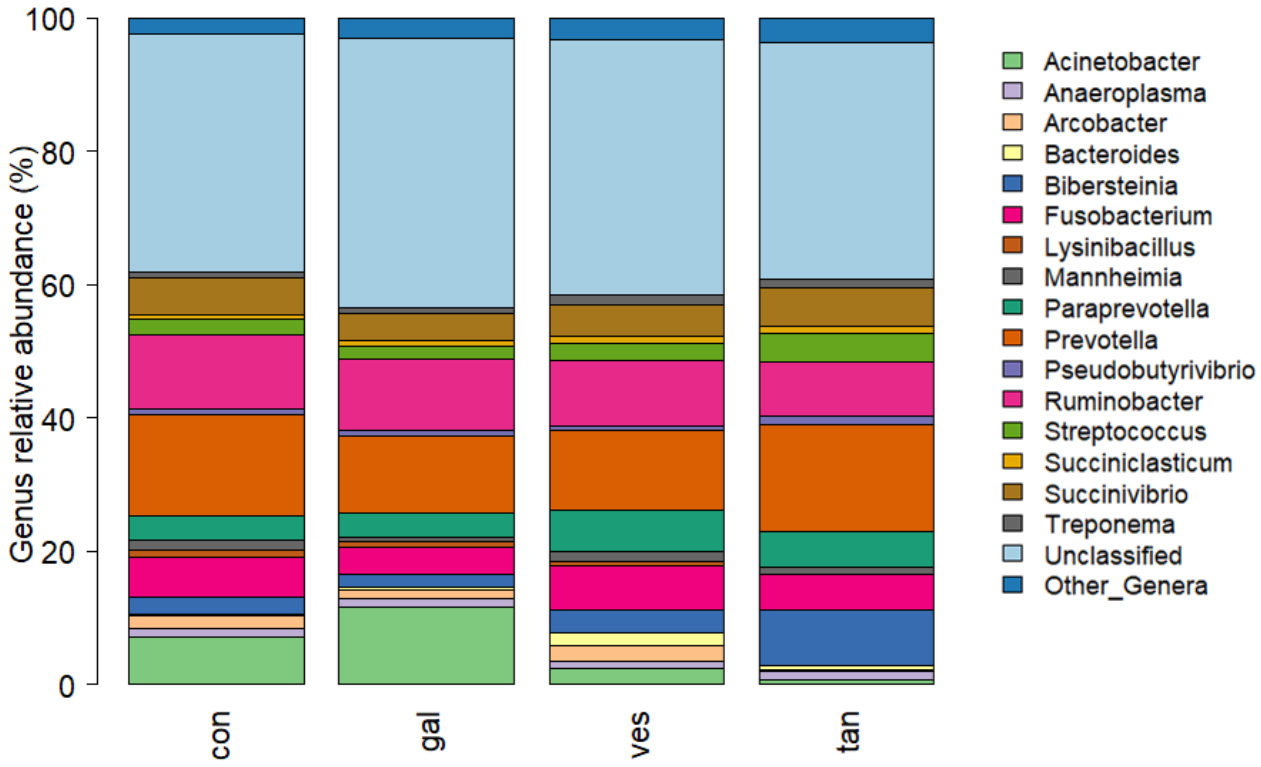

Figure 1. Taxonomic composition at genus level of the microbial communities enriched during the in vitro trial. Average abundances are reported for each tested condition. Only the genera with an average relative abundance of 1% (or higher) in at least one condition are reported.

Table 3. Relative abundance of the microorganisms classified at genus level (confidence 80%). For each genus the taxonomic classification at *phylum* level and at order level is reported.

Phylum	Order	Genus	C (%)	GAL (%)	VES (%)	CHT (%)	SEM	p value
Euryarchaeota	*Methanobacteriales*	*Methanobrevibacter*	0.34	0.20	0.48	0.37	0.15	0.6309
		Methanosphaera	N.D.	0.03	N.D.	N.D.	0.01	0.4411
	Methanomassiliicoccales	*Methanomassiliicoccus*	0.19	0.54	0.48	0.29	0.13	0.2790
Bacteroidetes	*Bacteroidales*	*Bacteroides*	0.28	0.38	1.96	0.57	0.48	0.1218
		Petrimonas	0.03	N.D.	0.04	N.D.	0.03	0.5880
		Porphyromonas	N.D.	N.D.	0.02	N.D.	0.01	0.4411
		Alloprevotella	0.04	0.07	0.09	0.05	0.03	0.6864
		Paraprevotella	3.81	3.67	6.16	5.25	0.91	0.2399
		Prevotella	15.15	11.46	11.97	16.16	1.53	0.1528
Elusimicrobia	*Elusimicrobiales*	*Elusimicrobium*	0.01	0.01	N.D.	N.D.	0.01	0.5948
Fibrobacteres	*Fibrobacterales*	*Fibrobacter*	0.02	0.03	N.D.	0.01	0.02	0.7279
Firmicutes	*Bacillales*	*Kurthia*	N.D.	0.18	N.D.	N.D.	0.09	0.4411
		Lysinibacillus	1.16	0.69	0.67	N.D.	0.39	0.2896
	Lactobacillales	***Streptococcus***	**2.19^{a}**	**1.99^{a}**	**2.51^{a}**	**4.22^{b}**	**0.24**	**0.0006**
	Clostridiales	*Butyrivibrio*	N.D.	N.D.	N.D.	0.17	0.09	0.4411
		Clostridium_XlVa	N.D.	0.09	0.12	0.13	0.09	0.7078
		Clostridium_XlVb	N.D.	N.D.	0.03	N.D.	0.01	0.4411
		Pseudobutyrivibrio	**0.71^{a}**	**0.99^{ab}**	**0.68^{a}**	**1.31^{b}**	**0.10**	**0.0086**
		Roseburia	0.21	0.22	0.22	0.14	0.10	0.9232

		Peptostreptococcus	N.D.	N.D.	0.02	< 0.01	0.01	0.4411
		Anaerofilum	N.D.	N.D.	0.03	N.D.	0.01	0.4411
		Clostridium_IV	0.06	N.D.	0.06	0.02	0.03	0.5830
		Ruminococcus	0.14	0.10	N.D.	0.03	0.06	0.4272
		Saccharofermentans	0.04	0.11	0.06	0.11	0.06	0.7622
	Selenomonadales	*Succiniclasticum*	0.78	0.8	1.06	1.15	0.18	0.4620
		Anaerovibrio	**0.12[a]**	**0.11[a]**	**0.15[a]**	**0.53[b]**	**0.09**	**0.0317**
		Selenomonas	0.58	0.47	0.56	0.94	0.12	0.1015
Fusobacteria	*Fusobacteriales*	*Fusobacterium*	6.07	4.20	6.56	5.28	0.69	0.1647
Proteobacteria	*Burkholderiales*	*Brachymonas*	N.D.	N.D.	N.D.	0.03	0.01	0.4411
		Comamonas	N.D.	N.D.	N.D.	0.08	0.04	0.4411
	Desulfovibrionales	*Desulfovibrio*	0.05	0.05	0.03	0.02	0.04	0.8945
	Campylobacterales	***Arcobacter***	**1.84[a]**	**1.39[a]**	**2.23[a]**	**0.29[b]**	**0.25**	**0.0028**
		Campylobacter	0.42	0.50	0.51	0.24	0.12	0.3658
	Aeromonadales	*Ruminobacter*	11.28	10.59	9.91	8.12	1.36	0.4438
		Succinivibrio	5.41	4.00	4.59	5.76	0.51	0.1443
	Enterobacteriales	***Escherichia/Shigella***	**0.02[a]**	**N.D.[a]**	**0.07[a]**	**0.24[b]**	**0.03**	**0.0009**
	Pasteurellales	*Actinobacillus*	0.06	N.D.	N.D.	0.14	0.04	0.1154
		Bibersteinia	**2.39[a]**	**1.86[a]**	**3.49[a]**	**8.30[b]**	**0.87**	**0.0030**
		Mannheimia	1.33	0.72	1.55	1.15	0.41	0.5536
	Pseudomonadales	*Acinetobacter*	7.08	11.49	2.33	0.62	3.36	0.1752
		Moraxella	0.17	0.21	0.18	0.13	0.09	0.9269
Spirochaetes	*Spirochaetales*	***Treponema***	**1.04[ab]**	**0.82[b]**	**1.56[a]**	**1.19[ab]**	**0.17**	**0.0778**
Synergistetes	*Synergistales*	*Pyramidobacter*	N.D.	0.22	0.19	0.13	0.07	0.2345
Tenericutes	*Anaeroplasmatales*	*Anaeroplasma*	1.40	1.33	1.16	1.37	0.19	0.8156

SEM = standard error of the mean; N.D. = not detected (i.e. relative abundance < 0.01). The sum of the relative abundances for each sample is lower than 100% because unclassified are not included in the table. Genera with a significative different relative abundance between the tested conditions ($p < 0.1$) are bolded. Significative different relative abundances ($p < 0.1$) are reported with letters a and b.

A PERMANOVA performed on the Hellinger transformed genus relative abundance data indicated a difference between the communities enriched in the tested conditions ($P < 0.01$). The difference was highlighted also by the NMDS plot (Fig. 2) in which the samples collected from the fermenters supplied with CHT formed a cluster separated from the samples collected from the other fermenters.

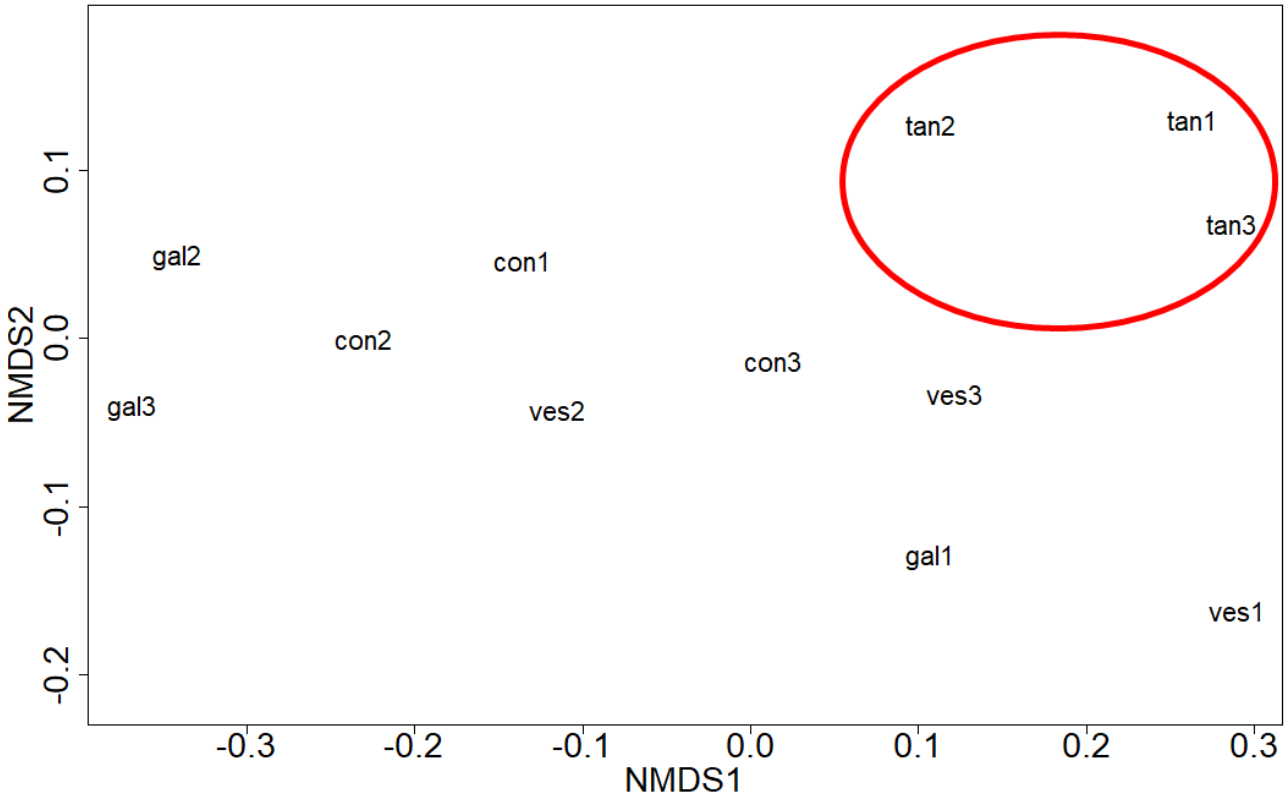

Figure 2. Non Metric Multidimensional Scaling (NMDS) based on Hellinger transformed genus relative abundance data. The circle highlights the communities enriched in the vessels where chestnut tannin extract was administered.

Seven genera (*Anaerovibrio*, *Arcobacter*, *Bibersteinia*, *Escherichia/Shigella*, *Pseudobutyrivibrio*, *Streptococcus*, *Treponema*) out of the 44 genera detected within the dataset showed significant different relative abundance between the tested conditions (Table 3). The post-hoc comparisons of the average genus relative abundance clearly showed a significant effect of the CHT in shaping the microbial communities (Table 3). The genera *Anaerovibrio*, *Bibersteinia*, *Escherichia/Shigella* and *Streptococcus* were enriched in the fermenters supplied with CHT compared to the other conditions. The relative abundance of the genus *Pseudobutyrivibrio* increased with CHT compared to C and to the fermenters supplied with VES. Conversely, the relative abundance of the genus *Arcobacter* decreased with CHT. Furthermore, a slight decrease in the relative abundance of the genus *Treponema* was observed in the fermenters supplied with GAL compared to the fermenters supplied with VES.

Pairwise correlations were performed on the relative abundance of bacterial genera (Table 4) indicating several co-occurrence patterns in particular for the genus *Anaerovibrio*. Furthermore, pairwise correlations evidenced a positive relation between *Anaerovibrio* and *Actinobacillus* or *Escherichia/Shighella* or *Pseudobutyrivibrio* or Streptococcus or

Bibersteinia but negative between *Bibersteinia* or *Escherichia*/*Shighella* or *Pseudobutyrivibrio* and *Arcobacter* (Table 4).

Table 4. Parwise correlation among bacteria genus of interest

bacteria genus	bacteria genus	correlation	P
Anaerovibrio	*Actinobacillus*	0.6195	0.0317*
Arcobacter	*Anaerovibrio*	-0.6917	0.0127*
Bibersteinia	*Actinobacillus*	0.7378	0.0062*
Bibersteinia	*Anaerovibrio*	0.5828	0.0467*
Bibersteinia	*Arcobacter*	-0.5981	0.0400*
Brachymonas	*Bibersteinia*	0.6767	0.0157*
Campylobacter	*Anaerovibrio*	-0.6219	0.0308*
Escherichia/Shigella	*Actinobacillus*	0.6227	0.0306*
Escherichia/Shigella	*Anaerovibrio*	0.8671	0.0003*
Escherichia/Shigella	*Arcobacter*	-0.7018	0.0110*
Escherichia/Shigella	*Bibersteinia*	0.7733	0.0032*
Escherichia/Shigella	*Campylobacter*	-0.5806	0.0478*
Prevotella	*Anaerovibrio*	0.5844	0.0460*
Prevotella	*Escherichia/Shigella*	0.6047	0.0373*
Pseudobutyrivibrio	*Anaerovibrio*	0.7778	0.0029*
Pseudobutyrivibrio	*Arcobacter*	-0.6666	0.0179*
Pseudobutyrivibrio	*Escherichia/Shigella*	0.6133	0.0339*
Ruminobacter	*Bibersteinia*	-0.5938	0.0418*
Selenomonas	*Anaerovibrio*	0.913	<.0001*
Selenomonas	*Arcobacter*	-0.6057	0.0369*
Selenomonas	*Escherichia/Shigella*	0.7631	0.0039*
Selenomonas	*Pseudobutyrivibrio*	0.6818	0.0146*
Streptococcus	*Anaerovibrio*	0.7075	0.0101*
Streptococcus	*Arcobacter*	-0.7288	0.0072*
Streptococcus	*Bibersteinia*	0.8576	0.0004*
Streptococcus	*Escherichia/Shigella*	0.8146	0.0013*

Streptococcus	*Lysinibacillus*	-0.6172	0.0325*
Succiniclasticum	*Streptococcus*	0.6067	0.0365*
Succinivibrio	*Anaerovibrio*	0.581	0.0476*
Succinivibrio	*Escherichia/Shigella*	0.618	0.0322*

Rumen degradability, FAME and DMA profile

Dietary supplementation with CHT did not affect NDF degradability, showing not significant difference compared to C while VES and GAL decreased it respectively about 4 and 6 g/100g of DM (Table 5).

Table 5. Differences in degradability of basal diet among treatments.

	C	CHT	VES	GAL	SEM[1]	P[2]
NDF g/100g $_{DM}$	41.73 b	41.88 b	43.71 a	44.79 a	0.75	0.0380
Degradability g/100g $_{DM}$	10.49 a	10.17 a	6.25 b	3.94 c	1.61	0.0380

[1] Standard Error Mean. [2] Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

At the end of the trial, no significant differences were found in FAME profile of RL fermenting the 4 feeds (Table 6).

Table 6. Fatty acid profile of rumen liquor at 24h

Fatty Acid g/100g $_{DM}$	C	CHT	GAL	VES	SEM[1]	P[2]
12:0	0.065	0.063	0.063	0.078	0.009	0.5999
13:0 *iso*	0.008	0.011	0.000	0.007	0.005	0.4784
13:0	0.010	0.005	0.000	0.009	0.006	0.6385
14:0 *iso*	0.047	0.052	0.054	0.063	0.006	0.3839
14:0	0.226	0.180	0.224	0.259	0.033	0.4627
15:0 *iso*	0.115	0.101	0.108	0.126	0.010	0.4681
15:0 *ante*	0.132	0.200	0.194	0.225	0.036	0.3765
15:0	0.228	0.225	0.213	0.261	0.023	0.5346
16:0 *iso*	0.134	0.125	0.126	0.133	0.016	0.9663
16:0	8.066	8.371	7.876	9.060	0.576	0.9165
16:1 *cis*9	0.061	0.063	0.062	0.080	0.010	0.5954
17:0 *iso*	0.079	0.078	0.086	0.089	0.008	0.7200
17:0 *ante*	0.148	0.143	0.154	0.171	0.019	0.7689
17:0	0.153	0.152	0.155	0.158	0.018	0.9951
*cyclo*17:0	0.029	0.036	0.017	0.034	0.006	0.1920
18:0 *iso*	0.012	0.016	0.008	0.017	0.006	0.7611
18:0	12.209	11.840	10.865	12.440	0.968	0.9220

18:1 *trans*4	0.026	0.037	0.045	0.052	0.010	0.4109
18:1 *trans*5	0.025	0.030	0.041	0.032	0.005	0.2584
18:1 *trans*6/trans7/trans8	0.279	0.299	0.263	0.268	0.037	0.9047
18:1 *trans*9	0.183	0.171	0.172	0.161	0.022	0.9187
18:1 *trans*10	0.294	0.301	0.293	0.317	0.043	0.9773
18:1 *trans*11	4.094	4.263	3.153	3.190	0.729	0.608
18:1 *trans*12	0.296	0.311	0.287	0.308	0.036	0.9637
18:1 *trans*15	0.221	0.242	0.197	0.264	0.034	0.5896
18:1 *cis*9	6.888	6.930	6.453	7.492	0.533	0.9566
18:1 *cis*11	0.518	0.512	0.458	0.554	0.086	0.8869
18:1 *cis*12	0.167	0.171	0.151	0.174	0.023	0.9054
18:1 *cis*13	0.020	0.024	0.023	0.025	0.004	0.8297
18:1 *cis*14+trans16	0.202	0.186	0.181	0.186	0.022	0.9083
18:1 *cis*15	0.023	0.035	0.030	0.028	0.004	0.3214
18:1 *cis*16	0.042	0.037	0.040	0.033	0.005	0.6939
18:2 *cis*9 *trans*12	0.032	0.031	0.035	0.035	0.006	0.9706
18:2 *trans*9 *trans*12	0.032	0.032	0.036	0.033	0.005	0.9472
18:3 *cis*9 *trans*11 *cis*15	0.078	0.077	0.065	0.058	0.014	0.7264
18:2 *cis* 9 *cis* 12	13.980	14.399	13.580	16.429	0.974	0.9098
20:0	0.267	0.252	0.218	0.263	0.390	0.8147
18:3 *cis*9 *cis*12 *cis*15	1.856	1.880	1.760	2.114	0.391	0.9283
18:2 *cis*9 *trans*11	0.150	0.121	0.096	0.096	0.031	0.5990
18:2 *trans*10 *cis*12	0.010	0.006	0.015	0.019	0.004	0.3451
18:2 *trans*11 *cis*13	0.013	0.006	0.013	0.014	0.003	0.3995
21:0	0.022	0.027	0.034	0.026	0.005	0.5106
18:2 *trans*10 *trans*12	0.048	0.042	0.052	0.054	0.011	0.8673
22:0	0.254	0.241	0.211	0.149	0.049	0.4883
23:0	0.044	0.037	0.038	0.123	0.045	0.5000
24:0	0.149	0.139	0.125	0.144	0.019	0.8450

[1] Standard Error Mean. [2] Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

In contrast, the presence of CHT extract, VES and GAL in the basal diet modified the concentration of several DMA (Table 7).

Table 7. DMA profile of rumen liquor at 24h

DMA	C	CHT	GAL	VES	SEM[1]	P[2]
DMA 12:0	0.052 b	0.257 a	0.266 a	0.234 a	0.044	0.0294
DMA 13:0 *iso*	0.025	0.124	0.103	0.095	0.024	0.0868
DMA 13:0	0.044 b	0.128 a	0.124 a	0.132 a	0.020	0.0458
DMA 14:0 *iso*	0.365 b	0.244 b	1.318 a	1.132 a	0.152	0.0075
DMA 14:0	0.281 b	0.824 a	0.921 a	0.800 a	0.122	0.0238
DMA 15:0 *iso*	0.257 b	0.652 a	0.816 a	0.700 a	0.110	0.0329

DMA 15:0 *ante*	0.639	4.688	2.107	2.026	1.096	0.1464
DMA 15:0	0.280	0.593	0.477	0.530	0.223	0.7784
DMA 16:0 *iso*	0.575	12.137	2.3820	2.689	3.179	0.1173
DMA 16:0	1.957	5.225	5.628	5.454	0.956	0.0767
DMA 16:1	0.074 c	0.316 a	0.212 b	0.190 b	0.035	0.0088
DMA 17:0 *iso*	0.041	0.122	0.145	0.144	0.029	0.1061
DMA 17:0 *ante*	0.054 c	0.212 a	0.214 a	0.168 b	0.024	0.0054
DMA 17:0	0.030 d	0.181 a	0.114 b	0.083 c	0.025	0.0200
DMA 18:0	0.186 b	0.518 a	0.572 a	0.567 a	0.093	0.0544
DMA 18:1 *trans11*	0.030 b	0.122 a	0.087 a	0.094 a	0.015	0.0208
DMA 18:1 *cis9*	0.173 b	0.571 a	0.408 b	0.417 ab	0.077	0.0389
DMA 18:1 *cis11*	0.087	0.294	0.166	0.227	0.048	0.0751
DMA 18:1 *cis12*	0.024	0.081	0.026	0.040	0.020	0.2493
DMA 17:1	0.097	0.360	0.276	0.247	0.098	0.3558
DMA 18:2	0.099	0.904	0.250	0.185	0.284	0.2489
DMA 26:0	0.552	1.3700	0.961	1.3570	0.290	0.2283
Total DMA	5.9340	30.2550	17.5830	17. 523	5.4760	0.0791

[1] Standard Error Mean. [2] Probability of significant effect due to experimental diets; means within a row with different letters differ ($P < 0.05$).

At 24h DMA 12:0, DMA 13:0, DMA 14:0, DMA 15 *iso*, DMA 18:0 and DMA 18:1 *trans*11 were lower in C fermenters than in the others ($P \leq 0.05$). VES and GAL enhanced DMA 14 *iso*, while DMA 16:1 increased with all 3 treatments compared to C. All treatments increased DMA 17:0 and DMA 18:1 *cis*9 and the highest values were reached with CHT.

The genus *Pseudobutyrivibrio* showed negative correlation with 18:1 *trans*9 and 18:1 *cis*6, and the genus *Anaerovibrio* was correlated negatively with 18:1 *trans* 11 *cis*15 (Table 8).

Table 8. DMA and FA parwise correlation with bacteria genus of interest.

item	**bacteria genus**	**correlation**	**P**
DMA-12:0	*Anaeroplasma*	-0.6079	0.0360*
DMA-12:0	*Elusimicrobium*	-0.6401	0.0250*
DMA-12:0	*Ruminococcus*	-0.5966	0.0406*
DMA-i13:0	*Brachymonas*	0.6482	0.0226*
DMA-i13:0	*Mannheimia*	0.601	0.0387*
DMA-i13:0	*Ruminobacter*	-0.6446	0.0236*
DMA-13:0	*Methanobrevibacter*	0.626	0.0294*
DMA-13:0	*Methanosphaera*	-0.5933	0.0420*
DMA-13:0	Unclassified	-0.5922	0.0425*

DMA-i14:0	*Methanobrevibacter*	0.7128	0.0093*
DMA-i14:0	*Methanosphaera*	-0.6591	0.0197*
DMA-14:0	*Methanosphaera*	-0.6137	0.0338*
DMA-i15:0	*Methanosphaera*	-0.6197	0.0316*
DMA-a15:0	*Methanomassiliicoccus*	0.6579	0.0200*
DMA-a15:0	*Methanosphaera*	0.9002	<.0001*
DMA-16:1	*Methanobrevibacter*	0.7204	0.0082*
DMA-16:1	*Pseudobutyrivibrio*	-0.6256	0.0296*
DMA-i17:0	*Brachymonas*	0.6235	0.0303*
DMA-a17:0	*Lysinibacillus*	0.6551	0.0208*
DMA-a17:0	*Methanobrevibacter*	0.7152	0.0089*
DMA-17:0	*Moraxella*	0.6093	0.0354*
DMA-18:1t	*Comamonas*	0.6437	0.0239*
DMA-17:1	*Roseburia*	-0.5985	0.0398*
DMA-18:2	*Acinetobacter*	0.6212	0.0311*
DMA-18:2	*Bibersteinia*	-0.6315	0.0276*
DMA-18:2	*Methanosphaera*	0.7189	0.0084*
DMA-18:2	*Streptococcus*	-0.642	0.0244*
DMA26:0	*Moraxella*	-0.5824	0.0469*
Total DMA	*Methanosphaera*	0.8181	0.0011*
iC17:0	*Streptococcus*	0.0722	0.8237
C18:1t9	*Pseudobutyrivibrio*	-0.6459	0.0233*
C18:1t11	*Escherichia/Shigella*	-0.6759	0.0158*
C18:1c14+t16	*Pseudobutyrivibrio*	-0.6099	0.0352*
cyclo17:0	*Treponema*	-0.7685	0.0035*
C18:1c16	*Pseudobutyrivibrio*	-0.6428	0.0242*
C18:2c9t12 2	*Pseudobutyrivibrio*	-0.6308	0.0279*
C18:2t11c15	*Anaerovibrio*	-0.7749	0.0031*
C18:2t11c15	*Escherichia/Shigella*	-0.7883	0.0023*
C21:0	*Treponema*	0.6087	0.0357*

C22:0	*Bibersteinia*	-0.7161	0.0088*
C23:0	*Bibersteinia*	0.6805	0.0149*

Considering DMA, *Pseudobutyrivibrio* and *Streptococcus* were negatively correlated with DMA 16:1 and DMA 18:2 *cis*9 *cis*12, respectively (Table 9).

Discussion

Recently, a global survey on 742 rumen and camelid foregut samples collected from 32 animal species in 35 countries allowed to determine a "core bacterial microbiome" (Henderson et al., 2015). Key microorganisms detected in all samples were *Prevotella*, *Butyrivibrio*, *Ruminococcus*, unclassified members of the families *Lachnospiraceae* and *Ruminococcaceae*, as well as unclassified *Bacteroidales* and *Clostridiales* (Henderson et al., 2015). In another study the genera *Paraprevotella*, *Succinivibrio*, *Treponema*, *Fibrobacter* and *Oscillibacter*, in addition to the above mentioned *Prevotella*, *Butyrivibrio* and *Ruminococcus* were identified as core genera in rumen bacterial communities of pre-ruminant dairy calves and cows and beef steers (Wu et al., 2012). Considering the sequences classified at genus level in this study, only the genera *Prevotella*, *Paraprevotella*, *Succinivibrio* and *Treponema* were detected in all conditions. Furthermore, members of the genus *Oscillibacter* were not detected in the whole dataset. Despite similarities can be identified between the core microbial community identified during this *in vitro* trial and the core rumen communities described during *in vivo* studies some difference can be observed. A possible reason of this shift can be the absence of microorganisms-host interactions (e.g. absence of immune system) that characterize *in vitro* trials. However, even if some difference can be observed during *in vitro* experiments compared to *in vivo* experiments, the overall behavior can be considered reliable, and *in vitro* experiments are widely used (Buccioni et al., 2011; Jayanegara et al., 2015; Cantalapiedra-Hijar et al., 2018). In our trial *Streptoccus* spp. increased with CHT in accordance with Costa et al. (2018) findings that showed a largely higher content of this bacterium in RL from ewes fed CHT respect to its concentration in RL from ewes fed mimosa tannins or a mix of CHT and mimosa. However, the explanation for differential responses of bacteria to tannins is unclear and factors as dose, *in vivo vs in vitro* trials, kind of tannin, extract grade of purity, concur to explain the inconsistence of results reported in literature.

Parwise correlations among microbial species, confirmed that rumen bacteria are synergic in their activities and that metataxonomic approach could be an efficient method to study bacteria in their environment because the study of single species in pure cultures may not lead to a global interpretation of data.

The inclusion of CHT did not affect the degradability of dietary NDF. Commonly, condensed tannins decrease the rumen degradation of fiber as consequence of important changes in microbial community profile while, hydrolysable ones are less strong (Costa et al., 2018). CHT employ a less detrimental action on cellulolytic bacteria than condensed tannins as quebracho or mimosa (Deaville et al., 2010; Buccioni et al., 2015a, 2017a; Costa et al., 2018). A plausible explanation of this behavior could be related to the higher grade of depolymerization of hydrolysable tannins as CHT in rumen respect to condensed ones (McSweeney et al., 1999). Recently, Costa et al. (2018) comparing mimosa tannins *vs* CHT found a reduced growth of cellulosolytic bacteria with condensed than hydrolysable tannins which resulted gentler. This result is consistent with our microbiological findings that do not show a great perturbation of microbial community and with *iso* and *anteiso* FAME profile (marker of rumen microbial activities according to Fievez et al., 2012 that was similar among treatments. However, Zimmer and Cordesse (1996) found that CHT decreased *in vivo* apparent organic matter (OM) digestibility in ewes and goats and Tabacco et al. (2006) confirmed that lucerne ensiled with CHT reduced *in vitro* OM digestibility by 5%. It is well known that tannins are able to modify the ratio among microbial species in RL or to inhibit microbial enzymes being proteins or to complex plant cell wall components (Smith et al., 2005). Certainly, the percentage of tannins inclusion in the diet or their source plays an important role and this could explain the inconsistence of many data reported in literature. In our experiment the CHT inclusion is about 1.6g/100g of DM, lower than in the cited trials.

VES and GAL reduced the NDF degradability even if no considerable changes in microbial community were found. This finding could be explained considering the possibility of VES and GAL to inhibit microbial enzymes involved in NDF degradation or complexing the fiber making it unavailable without being strongly detrimental with bacteria (Reed, 1986, 1995).

Considering the fatty acids involved in BH pathway, PUFA are not preserved by any treatment. The BH occurred normally showing the same content of 18:0 among groups at 24h. Many *in vivo* trials found an increase of PUFA in milk or meat when tannins are included in the diet of ruminants, especially with condensed ones. Hydrolysable tannins are less strong in inhibiting BH than condensed ones and they can be partially metabolized by several bacteria

species as *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986), reducing their antimicrobial activities. Mc Sweeney et al. (1999) demonstrated that hydrolysable tannins are susceptible to depolymerization in the rumen, forming monomeric derivatives with a less complex structure and with a lower affinity with proteins, enzymes or bacteria membrane, respect to the polymeric precursor. Several *in vivo* studies found that tannin addition in dairy ewes diet are not able to modify milk fatty acid profile indicating that the perturbation of BH at the rumen level could be low (Toral et al., 2011, 2013). In contrast, Carreño et al. (2015) in an *in vitro* trial found that CHT was able to modulate BH of PUFA even if the high dose used in this experiment limits the supplementation in animal feeding for possible detrimental effects on animal health.

In another study, Mannelli et al. (2018) showed that polyphenols from olive oil pomace decreased BH, limiting the lipolysis process that is the prerequisite for the next BH. These authors found a reduction of *A. lipoliticus* in RL from animals fed supplemented diets with olive oil by-product. In contrast, in our trial CHT increased the relative abundance of *A. lipoliticus*. These findings confirmed that the activities of polyphenols are strictly related to their chemical structure and consequently to their vegetal source. Moreover, this result showed that the efficacy of CHT is not due to the activity of VES or GAL singularly.

DMA are derived by plasmalogen lipids during the methylation process of RL fatty acid analysis and their composition is similar to fatty acid profile showing odd, even, saturated and unsaturated chains from C12 to C18 length. Their variation is strongly linked to the ability of bacteria to be resilient to the environmental changes, modifying their membrane fluidity as defense (Kaneda, 1991; Goldfine, 2010). Recently Alves et al. (2013) put in evidence how DMA can be an efficient tool as microbial marker. DMA 15:0 *iso* increase could be symptomatic of adaptation of cellulosolitycs to the stimuli induced by CHT, VES and GAL present in RL. Similar considerations may be applied to DMA 17 *ante* for amilolytics. Our findings are consistent with results of Alves et al. (2013), confirming that the presence of DMA 18:1 isomers is indicative of BH intermediate incorporation in structural lipids. Considering data reported by Costa et al. (2018), our findings are consistent in noting that the inclusion of CHT or its components in the diet is able to affect DMA 13:0, DMA 14:0 *iso*, DMA 16:1 and DMA 18:0 concentration.

Since the 70s, the presence of plasmalogens (alk-1'-enyl glyceryl ethers) is proved in several species of anaerobic bacteria. Our study showed a correlation among several plasmalogen derivatives and microbial species regardless the fermented diets, confirming the importance of DMA composition as tool of microbial characterization. Moreover, our results

were in accordance with a study of Langworthy (1975) on anaerobic mycoplasma, showed that *Anaeroplasma* is related to DMA12:0.

5. Conclusion

At inclusion level used in this trial, CHT, VES and GAL did not show detrimental effect on rumen microbial communities. These treatments did not interfere with PUFA BH processes in vitro. A correlation among several plasmalogens and microbial species regardless the fermented diets were found, confirming the importance of DMA composition as tool of microbial characterization. Moreover, the activity of CHT is likely due to its complex structure, rather than its single components (e.g. VES or GAL). More studies are need to investigate the interaction of tannins with rumen microbial community to enhance the synthesis of bioactive compounds at rumen level.

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CONCLUSIONS

Data obtained in the trials carried out in this thesis, showed that the kind of poliphenol plays an important role on the effect on rumen microbiota. In fact, the absorption of PP generally depends on: molecular structure; RL microbiota degrading capacity; percentage of inclusion in animal diet.

Polipenols from olive oil bio-waste favoured an increase of PUFA in RL and in milk especially of α-LNA and RA. Our data seems demonstrate that they affect the lipolysis because A. lipolyticus decreases its concentration. Instead, A. lipolyticus, in presence of CHT, was enhanced, suggesting another mechanism of action of this PP. Considering tannins, from chestnut and quebracho, they affect the last step of BH of α-LNA and of LA, interfering with the microorganisms responsible of VA hydrogenation. Moreover, QUE seems to be stronger than CHT.

Considering our results and what is reported in literature, the use of PP in ruminants feeding could be a valuable tool for manipulating rumen metabolism. However, the knowledge about the nature and the suitable quantities of PP is the starting base. It is necessary to know the chemical and structural PP characteristics to study their interactions with the ruminal metabolism, since each kind of polyphenol has specific mechanism of action. In vitro trials may be a valid tool to test matrices rich in PP or polyphenolic extracts that can be used in animal feeding. So, testing how these substances interact with the ruminal microbiota and estimate healthy inclusion for in vivo trials, may be possible.

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