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USE OF BY-PRODUCTS IN DAIRY SHEEP NUTRITION

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CHAPTER 1

1. GENERAL INTRODUCTION

1.1Agro-industrial byproducts

By-products are obtained from an asset processing during food processing activity. By-products must be used without other treatment after its industrial processing, satysfing all rules about health and environment safety (European Union, 2008). In Europe the production of by-products and wastes, amounts to about2.5 10^8 $year^{-1}$ (AWARENET, 2004). Wastes management is one of the most main goal of European Union's policy as it could have advantage on reduction of air and water pollution, greenhouse gas emission and health human problems. An important objective indicated by the "7th Environment Action Programme of EU to 2020" is to maximize Recycle and Reuse of the Resources (European Union, 2014).

In the world, developed and underdeveloped countries want to adapt to the political and social importance of reducing pollution, resulting from industrial activities; in fact, the consequence is that many industries consider their waste as raw material for other industries(Mirzaei-Aghsaghali and Maheri-Sis,2008).

By-products can be classifiedon the basis of the industry origin: agro-food industry, no agro-food industry, crop residues and animal waste (Mirzaei-Aghsaghali and Maheri-Sis,2008).The agro-food industries can be classified in fruit and vegetable industry, grain processing industry, brewery and winery industry, marine industry, meat industry, dairy industry (Helkar et al., 2016).The disposal of agro-food industries waste is important to the ecosystem, because this waste is highly perishable, it has high water activity, poor oxidative stability and

optimum enzymatic activity. Processing costs of waste disposal have an economic impact to the agro-food industry. However, food-industry waste could be an important source of functional compounds thanks to their favorable nutritional and rheological properties (Helkar et al., 2016).

These waste are only partially valorized, because few amounts are used as soil physico-chemical properties improvers, animal feed and composting, while the main volumes are managed as waste of environmental concern. Byproducts of agro-food industry might provide natural antioxidant, antimicrobial agent vitamins and they might contain macromelecules such as cellulose, starch, lipids, protein, plant enzymes or pigments. For these reasons pharmaceutical, cosmetical, food industry and biofuels industries are interested to by-products (Federici et al., 2009). There are some economically limitingg factor tothe use of by-product related to storage, transport, expensive treatment and these treatment, often, produce other waste (Federici et al., 2009). Thus agro-industrial byproduct are used as feed or fertilizer (Schieber et al., 2001).

The industry of food production generate large quantities of food waste that includes different categories: crop waste and residues; fruit and vegetables by-products; sugar, starch and confectionary industry by-products; oil industry by-products; grain and legume by-products; and distilleries' and breweries' by-products (Kasapidou et al., 2015). There are many agricultural wastes which are used traditionally in animal nutrition, such as oil cake meals, bran, middlings, brewers' grains, beet pulp and molasses (Correddu, 2015).

1.2 Grape Marc, Tomato Pomace and Exhausted Myrtle Berries

1.2.1 Chemical composition and fatty acid profilein grape marc, tomato pomace and exhausted myrtle berries

In the last years the attention has been focused on no-conventional by-productsderived fromfruit and vegetable industry, which could be interestingfor animal nutrition as source of bioactive compounds (Schieber et al., 2001; Mirzaei-Aghsaghali e Maheri-Sis, 2008). Among the no conventional by-products, the attention of this thesis has been directed on: grape marc (**GM**), by-products resulting by wine industry that is composed of skin, pulp and seeds (Spanghero et al., 2009); tomato pomace (**TP**), result from the processing of juice, paste and/or ketchup(Ventura et al., 2009); exhausted myrtle berries (**EMB**) derived from the maceration process of myrtle berries used to produce a commercial liqueur called "Mirto rosso".

In the world, there is a large production of grape and tomato, and in Italy, their production represents 14% and 9% of the world production (Table 1). The process generates large amount of by-product increasing the interest in their recycling. Sardinia is the only producer of Myrtle liqueur, and the by-product EMB contains alcohol that makes it a special waste with costly disposal. However, it is a good source of polyphenols and therefore could be recycled as source of bioactive compounds (Nudda et al., 2017).

The percentages of by-products from the winery, tomato industry and by the myrtle processing summarized in Table 2. Processing of grape, tomato and myrtle generates various types and quantities of by-products. The quantity of by-products

obtained from winemaking and tomato industry depends on raw material; GM is on average18.72 % (±9.69) and TP is about 3.40 % (± 1.19).

Several by-products have been included in the diets of different ruminant species and therefore most of them have been characterized in chemical composition as shown in Table 3. The processing by-products of grape (Table 3) used in ruminant diets, present an average content of 12% in CP, 43% in NDF and 6.7% of EE. The tomato processing by-products present an average content of 17% CP, 47.7% NDF and 10.5% EE. The chemical composition of these by-products is largely variable and the different processing methods or the mixing of different parts of the by-products cause several differences within the same type of by-product (Mirzaei-Aghsaghali and Maheri-Sis, 2008). The type of by-products included in the diet is influenced by the cost of traditional feedstuff and the safety of the by-product for animals (Mirzaei-Aghsaghali and Maheri-Sis, 2008).

The fatty acid profile of grape and tomato by-products is reported in Table 4. The most abundant FA in both by-products is linoleic acid belonging to the omega-6 family (C18:2 *n*-6), followed by oleic acid (C18:1 *cis*-9) and palmitic (C16:0).

1.2.2 Bioactive compound in grape marc, tomato pomace and exhausted myrtle berries

The polyphenol composition and main functional components presents in by-products are reported in Table 5. Grape marc are rich of phenolic compound that are natural antioxidant (Yi et al., 2009; Spanghero et al., 2009), primarily tannins compounds that have an important role in quality of wine influencing color, taste and body (Bombardelli and Morazzoni, 1995). Tomato pomace is composed mainly to peels and seed (Fondevila et al., 1994) which are a good source of protein, vitamins and minerals (Shdaifat et al., 2013). Moreover TP are rich in antioxidant compounds, such as lycopene, β-carotene (carotenes), and phenolic compounds (Del valle et al., 2006). Flavonoids are the main group of phenols in tomatoes and include flavonols, flavanols, flavanones such as naringenin, anthocyanidins and stilbenes. These compounds are located in the tomato peel (Raiola et al., 2015).

The residue of Mirto liqueur processing is composed by seeds and peels; the Myrtle berries contain a large number of biologically active components (Alipour et al., 2014) as flavonols, flavanols and phenolic acid (Barboni et al., 2010).

1.3 Biological properties of bioactive compound present in by-products.

1.3.1 Antioxidant activity of Polyphenols

Phenolic compounds (Polyphenols) are produced of the secondary metabolism of plants. There are 8000 different structures of polyphenols that are characterized by an aromatic ring bearing one or more hydroxyl groups, and chemical structure ranges from simple molecules as phenolic acids to more complex structure such

as tannins. During the normal development of plant, or under different stress conditions the synthesis of this compounds occur (Naczk and Shahidi, 2004). The polyphenols have a role relative to plant defence mechanism incontrasting pathogens, herbivorous, insects and solar radiation (Quideau et al., 2011). Several studies highlighted the beneficial role of polyphenols on human health. In particular, in a recent study of Grzesik et al. (2018), authors observed excellent antioxidant properties of catechins and other flavonoids.Naringenin, flavonoid contained in tomato peel (Navarro-González et al., 2011), has antioxidant and anti-inflammatory activities, low toxicity and consequently it has potential to be used as a therapeutic tool (Cavia-Saiz et al., 2010; Martinez et al., 2015).

The important role of phenolic compounds and lycopene is represented by the antioxidant activity that is associated with their ability to act as free radical scavengers (Sanchez-Moreno et al., 1999; Zhang et al., 2014) and to donate atoms or electrons, or chelate metal cations (Bravo, 1998). Also low concentrations of polyphenols could modulate some cell functions; in fact, they can influence the expression or enzyme activities. Furthermore they are involved in antioxidant enzyme activity function: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and the concentration of glutathione (GSH) (Molina et al., 2003; Koyama et al., 2013).

1.3.2Bioactive compound and human health

Based on animal models, the polyphenols canbe used to treat acute and chronic conditions such as ischemia, neurodegeneration, diabetes, and cancer (Shay et al., 2015).

The polyphenols of grape have potential benefits for the treatment of cardiovascular diseases and diabetes (Rasines-Perea and Teissedre, 2017).

Flavonoids, class of phytochemicals, thanks to their anti-inflammatory properties promote bone health; both in the bone disorder in later life and as a therapy during high oxidative stress or chronic inflammation of bone (Weaver et al., 2012).

Lovegrove et al. (2017) suggest that dietary flavonoids, in particular flavonols and anthocyanidins, improve vascular function and lower blood pressure at doses achievable in diets contain high level of fruit, vegetables, cocoa and teas; the intake of flavanolshasa beneficial effects in the visual function (Milbury, 2012).

Proanthocyanidins, known as condensed tannins, are a group of phenolic compounds that have beneficial health effect on cardiovascular and metabolic disorders and on some carcinogenic diseases (Bladé et al., 2016). In addition, in recent studies was observed that the proanthocyanidins could be a safe intervention for the clinical treatment of Parkinson's disease (Chen et al., 2018) andseveral sudies observed a reversing action on the osteoporosis by stimulating bone formation or by regulating bone reabsorption in animal model (Oršolić et al., 2018)

Resveratrol is known to have several health-promoting effects on animals and humans. This compound hassome beneficial effects on animals with insulin-deficient diabetes and it has anti-oxidant and anti-inflammatory properties.

Preliminary studies about human showed the effects of resveratrol on type 2 diabetes (Szkudelski and Szkudelska,2015).Furthermore, resveratrol studies

showed that this phenol exerts neuroprotective effects on central features of Alzheimer's disease (Lange and Li, 2018).

Jin et al. (2017) suggest that the naringenin may represent a potential therapeutic agent for the inflammation-related diseases. In a recent study of inflammatory pain in mice, the authors suggest that naringenin inhibits both inflammatory pain and neurogenic inflammation (Pinho-Ribeiro et al., 2016). Rashmi et al. (2018) in a study on mice, the naringenin showed potent free radical scavenging activity. In particulars, naringenin helps to mitigate streptozotocin induced liver complications. Park et al. (2017) suggest that naringenin may be a potent inducer of apoptosis of pancreatic cancer cells.

Lycopene, a carotenoid, is synthesized by plant and microorganism only (Agarwal and Rao, 2000).This carotenoid has potential anticancer properties and it is antioxidant agent (Brown et al., 2004; Chen et al., 2015). The intake of food containing lycopenesis inversely related to risk of cancer of the lung, and stomach (Giovannucci, 1999).

The intake of tomato products and lycopene has positive effects on blood lipids, blood pressure and endothelial function. These effects suggest that the tomato products can tackle cardiovascular diseases (Cheng et al., 2017). The intake of green tea, fruit and vegetable, which contained lycopene, is able to reduce prostate cancer risk (Jian et al., 2007). Carotenoides in the diet could reduce risk of breast cancer (Eliassen et al., 2012; Gloria et al., 2014) and esophageal cancer (Ge et al., 2013; Ba Ngoc et al., 2018).

Furthermore lycopene has a greater effect than α- and β-carotene to decrease the cancer cell growth (Levy et al., 1995).

1.4 By-products in animal nutrition

Byproducts from agro-food industry may be a resource for animal feeding if their chemical profile permits their reuse (Laufenberg et al., 2003; Wyman, 2003). The production of waste derived from the agro-food industry has been estimated approximately about 30% of processed raw material (Kasapidou et al. 2015).The utilization of fruit and vegetable by-products in animal nutrition might reduce livestock nutrition cost and the environmental impact. The large use of several agro-industrial byproducts in ruminant nutrition is due and the presence of functional ingredients. As shown in Table 5, GM and EMB are rich in polyphenols, while TP is rich in carotenoids, in particular lycopene. The composition of the basal diet could affect concentration of fat-soluble micronutrients, mainly β-carotene and α-tocopherol (Martin et al., 2004), and phenolic compounds (O'Connell and Fox, 2001). These bioactive compounds are involved in the prevention of the photoxidation the milk fat globule membrane (site of auto-oxidation) (Lindmark-Månsson and Åkesson, 2000; O'Connell and Fox, 2001), affecting nutritional and organoletic properties of milk (O'Connell and Fox, 2001; Nozière et al., 2006).

1.4.1 Effects of bioactive compound on animal performance and health

The presence of polyphenols in ruminant diets affected milk production (Woodward et al., 2001;Hymes-Fecht et al., 2013), and milk quality (Hilario et

al., 2010; Liu et al., 2013;Correddu et al., 2016), evidenced anthelmmintic (Athanasiadou et al., 2000) and immunomodulatory effects (Min et al., 2005;Nudda et al., 2015). Furthermore several studies evidenced the effect of polyphenols in reducing the rumen ammonia production (Bhatta et al., 2009; Theodoridou et al., 2010) and methane emissions (Dschaak et al., 2011; Liu et al., 2011).

Several studies showed beneficial effect of polyphenols in ruminant diet on health condition, ruminal metabolism, milk production and quality. Polyphenols in ruminant diet may have negative or positive effects, depending on dose, chemical structure, molecular weight and physiological stage of the animal (Hagerman and Butler, 1991). In fact, there are different results have been reported in literature, about the correlation between concentration of phenolic compounds and effects on animal performance.

The inclusion of moderate concentration of phenolic compounds in ruminant diets (intake under 50 g/kg DM) could improve animal production, likely due to a better utilization of nutrients, in particular protein, with a consequent greater availability of amino acids at intestinal level. But the results reported in literature are inconsistent about the inclusion of polyphenol in diet on animal performance (Correddu, 2015). Several studies show that the low concentration of tannins in the diet can perturb the rumen fermentation (Barry and Duncan, 1984; Bhatta et al., 2000), as well as the microbial protein synthesis (Makkar et al., 1995; Bhatta et al., 2001) with positive effects on animal performance. In contrast, similar low doses of these phenolic compounds showed negative effects on rumen

fermentation (McSweeney et al., 2001; Min et al., 2002). Abarguei et al. (2010) suggest that these results about ruminal depend both by the level of tannins and the type of tannins, such as condensed or hydrolysable tannin and nature of plant.

Lamb diets added with 4.2 g condensed tannins per kg of DM increased animal live weight (Montossi et al., 1997). The increase of live weight in sheep has been observed with a diet contain higher dose of condensed tannins (34 g tannins/kg DM; Wang, 1996). On the other hand, reduction of lamb weight has been reported with diets supplemented with 25 and 27 g of condensed tannins/kg DM of diet (Priolo et al., 2000; Vasta et al., 2007).

The milk production can be influenced by the inclusion of polyphenols in the diet. Negative effect of polyphenol on milk yield has been related with a reduction of total feed intake (Grainger et al., 2009; Griffiths et al., 2013). The correct dose of polyphenols that could improve the milk production seem related to different factor, such as the source of these compounds, the chemical composition, and the interaction with the others ingredients included in the diet. Similar doses of phenolics, if deriving from different sources, could have different effects on milk production (Correddu, 2015).

The inclusion of polyphenols in ruminant diet could be positive for animal health and in particular there are many studies that showed the interaction between the intake of these components and the reduction of parasitism. Azaizeh et al. (2013) showed the anthelmintic potential of polyphenols extract. Sheep diet added with tannin-rich foliage of Havardia albicans (400g /kg DM) showed antiparasitic

effect (Galicia-Aguilar et al., 2012). Athanasiadou et al. (2000) showed the effect of condensed tannins on the development of intestinal parasitic infection.

The toxicity of polyphenols in ruminant regards tannic acid, gallic acid and ellagic acid (Zhu et al., 1992), which derived compounds from degradation of hydrolysable tannins are known to be toxic for ruminant at high dietary inclusion level (Pryor et al., 1972; Murdiati et al., 1992). Studies on sheep (Hervás et al., 2003) and goats (Silanikove et al., 1996) did not showed any toxic effect of several types of tannins.

Tannins are able to bind proteins contained in the diet, causing a decrease of rumen fermentation and NH_3 production. This is supported by *in vitro* fermentation of pomenagrade extract (Abarghuei et al., 2014a). An improving of the N utilization has been reported replacing the conventional silage in dairy cow diet with tannins sources (Hymes-Fecht et al., 2013). The increase of levels of quebracho tannins in ewe diet with alfalfa hay caused different effects on fiber digestibility: in fact high level of tannins (22.5 g / kg of DM) decreased fiber digestibility (Al-Doibaib, 2009; Hymes-Fecht at al., 2013).

The presence of polyphenols ruminant diets can influence biohydrogenation process by reducing or inhibiting the activity and growth of rumen microbes (Cabiddu et al., 2009; Vasta et al., 2009). The extent of the effects of polyphenols on ruminal biohydrogenation depends on several factors, such as chemical characteristics, the amount and phenolic profile and the other dietary components. Several works (Jones et al., 1994; Min et al., 2005) showed the selective activity of tannins on ruminal bacteria population. Vasta et al. (2010) showed that the

inclusion of tannins has influenced the biohydrogenation with an increase of vaccenic acid (C18:1 trans-11; **VA**) and a decrease of stearic acid (C18:0; **SA**) to vaccenic acid ratio. The authors have explained that there was a change in rumen microbial population with an increase in *Butyrivibrio fibrisolvens* bacteria [responsible for conversion of linolei acid (C18:3 cis-9, cis-12, cis-15; **LA**) to rumenic acid (C18:2 cis-9, trans-11; **RA**)] and a decrease in *Butyrivibrio proteoclasticus* bacteria (responsible for conversion of VAto SA). Furthermore, in a in vivo experiment Vasta et al. (2010) observedthe ability of condensed tannins to inhibit the last step of biohydrogenation of unsaturated fatty acids (**UFA**), consisting in the enzymatic reduction of VA to SA, as suggested by previous in vitro studies (Khiaosa-Ard et al., 2009; Vasta et al., 2009).The importance of the inclusion of polyphenols in ruminant diet could be due to the reduction of polyunsaturated fatty acids(**PUFA**) biohydrogenation and the increase of VA in rumen, and increase the quantity of these FA that reach the mammary gland and thus increase the amount of PUFA, VA and (conjugated linoleic acid (**CLA**) in milk. The linolenic acid (**LNA**) transfer rate from diet to cows milk was affected by the different essence in the diet, and the polyphenols content (Kälber et al., 2011). Cows fed Lotus cornicolatus, as source of tannins, had an increase of *n*-3 FA and in particular LNA in milk (Turner et al., 2005).The inclusion of pomegranate extract, as source of polyphenols, in the diet of lactating dairy cows decreased SFA and the *n*-6:*n*-3 ratio, while increasedeicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) (Abarghuei et al., 2014b).Toral et al. (2011) observed that the supplementation with two extracts of quebracho rich in

condensed tannins (CT) and chestnut rich in hydrolysable tannins (HT) did not increase the amount of VA and RA in milk. Subsequently, Toral et al. (2013) confirm that the addition of 20g /kg on DM of tannins did not increase the amount of beneficial fatty acids concentration in milk. The lack of increase of RA in milk when in ruminant diet are present the polyphenols, could be given to a reduction in the rate of conversion of VA into RA in mammary gland (Vasta et al., 2008); in fact, in severalstudiesthe inhibitory effect of PUFA on the Δ^9-desaturase enzyme expression was observed (Sessler and Ntambi, 1998; Vinknes et al., 2013).

1.4.1.1Tomato pomace

The by-product of tomato processing (seeds and skin) is usually included in animal feed after it has been pulverized (Ashes et al., 1992).In fact there are many studies on the utilization waste of tomato processing (Tables 3, 6 and 7) in poultry (Mansoori et al., 2008), dairy cows (Weiss et al., 1997), ewes (Abbeddou et al., 2011a, 2011b, 2015) andgoats (Razzaghi et al., 2015). However, the energy of TP is limited due to the high fiber content (Shdaifat et al., 2013). In cows fed different percentage of whole tomato seeds (4, 2.4 and 1.1 % of DM of total mix ratio), did not change milk yield, milk fat concentration, MUN and PUN decreased and C18:3 and C18:2fatty acids increased (Cassinerio et al., 2015). Tomato wasteinclusion in goats diet decreased N excretion in urine and CH_4 emissions, improvingthe quality of the FA profile in milk (Romero-Huelva et al., 2012), increasingtotal CLA and VA content ofmilk (Razzaghi et al., 2015). Tomato pomace in ewes diets (about 30% of DM) did not affect ruminal fermentation (Abbeddou et al., 2011b) andincreasedmilk C18:1*cis*-9 (Abbeddou

et al., 2011b). The inclusion of tomato pomace (30% of the diet DM) in broiler diet, as a source of α-tocopherol, reduced lipid oxidation in meat (King and Zeidler, 2004). Tomato pomace added in lamb diet (20% of the total diet), did not change growth performance of the animals compared to a control group was fed soybean (Fondevila et al., 1994), evidencing the role of some by-products in replacing more expensive and competitive foods with human nutrition.

1.4.1.2Grape marc

The effects of by-products of winemaking process used in ruminant nutrition are summarized in Table6 and 7. The inclusion of GM in the diet of cows (Chedea et al., 2017; Moate et al., 2014) and ewes (Mokini et al., 2017; Manso et al., 2016) evidenced not univocal results. Supplementation of grape pomace in ewes diet, decrease milk yield and increased fat, protein and urea content in milk (Congiu and Congiu 2003).Different results have been reported by Manso et al. (2016) supplementing grape pomace (5 and 10 % of total mix ratio) in ewes diet: it did not affect on DMI and milk yield, but decreased lactose concentration and did not modify the milk fatty acids profile. The inclusion of grape seeds (in a dose of 274g per day of DM) in Sarda dairy sheep decreased saturated fatty acids (**SFA**) and increased UFA, PUFA, RA and VAin milk (Correddu et al., 2016). In addition, grape seed in ewes diet could have an immunomodulatory effect (Nudda et al., 2015).

Feeding dairy cows with ensiled grape marc (4.9 kg of DM/day) and dried grape marc (4.1 Kg of DM/day) did not change milk yield, decrease milk fat content and increased RA, monounsaturated fatty acids (**MUFA**) and PUFA concentration in

milk, and decrease environmental methane emissions (Moate et al., 2014) and improved the antioxidant capacity in milk (Santos et al., 2014). The supplementation of grape seed and grape marc meal (1200 g of DM/day) in cow diet did not influence metabolic and antioxidant parameters in blood plasma (Gessner et al., 2015).

Ishida et al. (2015) fed wethers with winery sediment and grape pomace (75g/ kg of DM vs 166 g /kg of DM), observed no effects on DMI but decrease rumen NH_3.

Studies on lamb showed that whole dried red wine grape pomace (5% of concentrate) can be used in the diet without negative effects on the shelf life of meat (Guerra-Rivas et al., 2016).This is supported by the increasing in antioxidant activity (DPPH and ABTS) of meat from lamb fed diet containing grape pomace (9% of grape pomace in corn silage).In the same study an improvement of the growth of facultative probiotic bacteria and the inhibition of the growth of pathogen populations by using grape pomace has been reported (Kafantaris et al., 2017).

1.4.1.3Exhausted myrtle berries

There are several biologically active components in myrtle berries (Alipour et al., 2014) and in particular flavonols, flavanols and phenolic acid (Barboni et al., 2010).

The secondary metabolites of *Myrtus communis* are the essential oil and polyphenols.The species of Myrtus are rich in volatile oils (Satrani et al., 2006, Shikhiev et al., 1978; Tuberoso et al.,2006), phenolic acid (Romani et al.,1999),

flavonoid (Romani et al., 1999; Joseph et al., 1987) and tannins (Diaz and Abeger 1986). Regarding the polyphenols compound in myrtle berries some studies showed the presence of tannins, anthocyanins (0.2-54%), fatty and organic acids (9-52%) (Barboni et al., 2010). The quantity of polyphenols dependfrom the degree of berries maturation, while the extraction amounts of these substance can be influenced by the analitical solvent used (Martin et al., 1990; Tuberoso et al., 2010; Messaoud et al., 2012).

The myrtle berries are used in Sardinia to produce the liquer Mirto and the by-products of this processing are the exausted myrtle berries (EMB). To date there is only one study about composition of exhausted myrtle berries and the use of this by-product on ewes diet; in this study the authors showed that the inclusion of this by-product reduced urea concentration in blood and milk (Nudda et al., 2017).This could be related to the presence of polyphenols that has been found in a content of 5.30 g of Gallic Acid equivalent (GAE)/100 g of DM (Nudda et al., 2017). In this study a depressive effect of EMB on milk yield has been reported. Several studies indicate negative impact of polyphenols on intake and production, as likely consequence of their low palatability (Jöbstl et al., 2004). Some negative effects of tannins were associated to their interaction with digestive enzyme and epithelium lining digestive tract (Silanikove et al., 2001).

The attention of food/feed scientists and food/feed industry to by-products with high content in nutrients and bioactive compounds, and the need to recycle waste characterized by high disposal costs is constantly growing. Several both

widespread and local by-products are commonly undervalued and thrown away due to lack of profitable alternative uses or because are highly perishable.

Their inclusion in ruminant diets could be a practical and economic option for their recycle and valorization. The use of some by-products in dry form could also be an interesting source to the feed industries for their inclusion in pellet/concentrate formulations. However, studies on dairy sheep on the use of dry by-products on the productive performance, rument function and health status of the animal are limited or absent.

1.5 TABLES

Table 1. Production of Co-byproducts from tomato, wine and myrtle industries

			World	Italy	Sardinia	Sardinia (tonnellate)
Tomato industry						
Production Tomato[1] (Mt)			38	5.32	0.038	38279.4
Co/by-production [2]g / 100 g of raw material	4 - 4.5 %					
Co/By-product quantity (Mt)		min	1.52	0.21	0.0015	1531.2
Co/By-product quantity (Mt)		max	1.710	0.24	0.0017	1722.6
Co/By-product quantity (Mt)		min- max	1.52 - 1.71	0.21- 0.24	0.0015 - 0.0017	
Grape wine						
Production Grape[3] (Mt)			67.32	5.8	0.165	165318.4
Co/by-production[2]g / 100 g of raw material	15 - 30 %					
Co/By-product quantity (Mt)		min	10.10	0.87	0.025	24797.8
Co/By-product quantity (Mt)		max	20.20	1.74	0.050	49595.5
Co/By-product quantity (Mt)		min- max	10.10 -20.20	0.87 - 1.74	0.025 - 0.050	
Exhausted myrtle berries [4]						
Production Myrtle Berries (Mt)					0.0004	400.0
Co/by-production g / 100 g of raw material	45%					
Co/By-product quantity (Mt)						
Co/By-product quantity (Mt)						
Co/By-product quantity (Mt)					0.00018	

[1] Ismea, 2017 ;[2] Table 2; [3] Wadhwa et al., 2013; [4] Nudda et al., 2017

5 **Table 2**. Different part of by-products obtained from grape and tomato
6

Products	By product (g / 100 g of raw material)	References
Grape pomace	20	*Chedea et al., 2017*
Grape pomace dry	15	*Wadhwa et al., 2013*
Grape pomace wet	25 - 45	*Wadhwa et al., 2013*
Grape pomace	18 - 20	*Spanghero et al., 2009*
Grape pomace	20	*Nielsen and Hansen, 2004*
Grape seed	3 - 6	*Wadhwa et al., 2013*
Grape stalks	2.5 - 7.5	*Wadhwa et al., 2013*
Red and white wine production	20 - 30	*Kasapidou et al., 2015*
Tomato pomace	4	*Aghajanzadeh-Golshani et al. 2010*
Tomato pomace	3 - 5	*Celma et al., 2009*
Tomato pomace	4.5	*Mirzaei-Aghsaghali and Maheri-Sis, 2008*
- peels	3	
- seeds	1.5	
Exhausted myrtle berries	45	*Nudda et al., 2017*

Table 3. Chemical composition of by-products used in ruminant nutrition

By-product	Animal	Chemical composition[1]									References
		DM	OM	CP	NDF	ADF	ADL	EE	NFC	Ash	
Grape pomace	Lamb	95.5	-	11.9	37.6	31.7	-	7.3	-	8.93	*Guerra-Rivas et al,. 2016*
Grape pomace	Sheep	-	86.6	12.2	37.6	31.7	20.7	6.4	-	-	*Manso et al., 2016*
Grape pomace	Wethers	43.9	91.8	9.5	47.4	44.0	-	8.5	26.3	8.2	*Ishida et al., 2015*
Dried grape marc	Cow	-	-	13.1	50.7	47.7	36.9	-	18.3	8.5	*Moate et al,. 2014*
Ensiled grape marc	Cow	-	-	13.3	53.5	53.1	42.2	-	17.7	7.7	*Moate et al,. 2014*
Grape pomace	Lamb	89.0	-	12.8	47.1	31.2	-	-	-	-	*Bahrami et al,. 2010*
Grape pomace	Sheep	52.5	94.0	9.4	56.8	47.6	20.0	5.2	-	-	*Abarguei et al,. 2010*
Grape pulp	Sheep	-	81.1	13.8	24.3	19.3	74.7	3.17	-	-	*Guerra-Rivas et al., 2016*
Grape seed	Sheep	97.4	-	9.3	53.9	-	41.1	10.9	23.1	2.7	*Correddu et al,. 2015*
Grape seeds	Sheep	-	92.7	10.4	52.3	45.4	35.3	9.9	-	-	*Guerra-Rivas et al., 2016*
Winery sediment	Wethers	31.2	78.6	19.8	6.4	4.3	-	2.8	49.6	21.4	*Ishida et al., 2015*
Tomato pomace	Goats	94.1	95.5	21.7	55.4	42.2	-	9.3	-	-	*Razzaghi et al., 2015*
Tomato pomace	Sheep	85.1	96.6	19.4	50.0	34.0	-	-	-	-	*Shdaifat et al., 2013*

Table 3. (Continued)

By-product	Animal	Chemical composition[1]									References
		DM	OM	CP	NDF	ADF	ADL	EE	NFC	Ash	
Tomato pomace	Sheep	-	95.2	19.1	55.2	46.2	25.9	10.0	10.9	-	*Abbeddou et al,. 2011a*
Wet tomato pomace	Sheep	14.2	96.2	19.5	63.6	43.5	-	-	-	-	*Denek and Can, 2006*
Tomato pomace	Cow	24.7	-	20.0	61.1	43.9	25.8	-	-	3.9	*Weiss et al., 1997*
Whole tomato seeds	Cow	-	-	23.5	50.3	30.8	-	20.3	-	3.8	*Cassinerio et al,. 2015*
Corn + pomace silage	Cow	32.3	-	9.8	44.5	25.7	6.1	-	-	-	*Weiss et al., 1997*
Tomato pomace	Fattening rabbits	23.5	-	4.4	1.3	1.2	-	2.2	-	1.3	*Peiretti et al., 2013*
Exhausted myrtle berries	Sheep	97.0	-	8.0	67.0	53.34	34.8	11.0	29.2	2.8	*Nudda et al,. 2017*

[1]Data expressed as g/kg DM.

Table 4. Fatty acid profile of by-products included in ruminant diets

By-product	g/ 100 gof total FAME									References
	C14:0	C16:0	C16:1	C18:0	C18:1cis 9	C18:2n6	C18:3n3	C20:0	C22:0	
Grape pomace	0.10	6.20	0.10	4.90	21.90	14.80	51.30	0.20	0.10	*Guerra-Rivas et al., 2016*
Grape pomace	0.30	11.10	0.60	4.40	16.00	61.30	3.70	0.50	-	*Manso et al., 2016*
Dried Grape marc	0.10	9.40	0.30	4.60	15.80	66.70	1.50	0.50	-	*Moate et al., 2014*
Ensiled Grape marc	0.30	9.20	0.20	4.60	15.20	66.30	1.10	0.40	-	*Moate et al., 2014*
Grape seed	-	8.50	-	4.90	9.60	74.00	0.30	-	-	*Correddu et al., 2015*
Grape seeds	0.74	19.30	1.70	5.62	1.27	40.2	1.25	1.38	1.57	*Guerra-Rivas et al., 2016*
Grape pulp	0.10	8.07	0.20	3.97	17.20	69.00	0.52	0.20	0.10	*Guerra-Rivas et al., 2016*
Tomato pomace	-	11.50	0.30	3.10	20.70	57.10	2.90	0.30	0.50	*Razzaghi et al., 2016*
Tomato pomace	0.12	14.11	0.24	5.88	-	53.33	2.65	0.40	-	*Peiretti et al., 2013*
Tomato pomace	-	14.50	0.38	3.22	13.70	36.50	-	-	-	*Abbedoou et al., 2011*
Whole tomato seed	0.12	13.38	-	4.48	-	55.45	2.21	0.34	-	*Cassinerio et al., 2015*
Exhausted myrtle berries	-	8.17	-	3.80	9.11	76.24	0.94	-	-	*Nudda et al., 2017*

17 **Table 5.** Functional ingredients identified in grape and tomato by-products and polyphenol contents in grape, tomato and myrtle by-products
18

	Poliphenols (u.m./kg DM)	Main functional Ingredient	References
By-product of winemaking			
Grape pomace	5.96 g GAE	Polyphenols	*Chedea et al., 2017*
Grape pomace	-	Flavonoids, polyphenols, anthocyanins	*Nassiri-Asl and Hosseinzadeh, 2016*
Grape pomace	44.10 g	Polyphenols, flavonoids: anthocyanins, flavonols and flavanols (condensed tannins)	*Guerra-Rivas et al., 2016*
Grape pomace	42.8 g	Polyphenols (condensed tannins, anthocyanins)	*Manso et al., 2016*
Grape pomace	10.0 g CAE	Polyphenols	*Ishida et al., 2015*
Grape pomace	-	Polyphenols	*Kasapidou et al., 2015*
Grape pomace	4.8 g	Polyphenols, tannins	*Bahrami et al., 2010*
Grape pomace	70.50 g	Polyphenols, tannins	*Abarghuei et al., 2010*
Grape pulp (reed wine Italy)	31.00 g	Procyanidins, anthocyanidins	*Spanghero et al. 2009*
Grape pomace	-	Polyphenols, tannins	*Alipour and Rouzbehan, 2007*
Grape pomace	-	Polyphenols, flavonoids, tannins	*Nielsen and Hansen, 2004*

19

Table 5. (Continued)

	Poliphenols (u.m./kg DM)	Main functional Ingredient	References
Grape pomace	-	Polyphenols, resveratrol, catechin, epicatechin, procyanidins	*Torres et al., 2002*
Grape pulp and seeds	-	Polyphenols	*Guerra-Rivas et al., 2016*
Grape seed	3.30 g GAE	Polyphenols	*Correddu et al., 2015*
Grape seed (red wine)	58.20 g	Polyphenols	*Lachman et al., 2013*
Grape seed (white wine)	32.20 g	Polyphenols	*Lachman et al., 2013*
Grape seed (reed wine Italy)	51.00 g	Procyanidins, anthocyanidins	*Spanghero et al. 2009*
Grape seed extract	67.00 g	Polyphenols, flavonoids, anthocyanidins, condensed tannins	*Mokini et al., 2017*
Grape skin extract	51.00 g	Polyphenols, flavonoids, anthocyanidins, condensed tannins	*Mokini et al., 2017*
Grape seed meal extract	-	Flavonoids, gallic acid, catechin, epigallocatechin-3-galate, epigallocatechin, epichatechin-3-gallete, epicatechin and proanthocyanidins	*Gessner et al., 2015*
Grape marc meal extract	-	Procyanidins, anthocyanidins	*Gessner et al., 2015*
Grape seed extract	85.80 g GAE	Polyphenols, flavonoids, Proanthocyanidins	*Arvanitoyannis et al., 2006*
Ensiled Grape pomace	-	Condensed tannins	*Moate et al., 2014*
Grape residue silage	19.50 GAE	Polyphenols, flavonoids	*Santos et al., 2014*

22 **Table 5. (Continued)**
23

	Poliphenols (u.m./kg DM)	Main functional Ingredient	References
By-product of Tomato industry			
Whole tomato	0.68 g	Polyphenols	*Kaur and Kapoor, 2002*
Tomato pulp	0.13 g GAE	Polyphenols, flavonoids, ascorbic acid, lycopene (28.00 g / Kg DM)	*Toor and Savage, 2005*
Tomato products	-	Lycopene	*Cassinerio et al., 2015*
Tomato pomace	6.1 g	Polyphenols, tannins	*Razzaghi et al., 2015*
Tomato pomace	-	Lycopene (0.10 g / kg of DM)	*Shao et al., 2013*
Tomato pomace	-	Carotenoid (0.16 g / kg of DM)	*Peiretti et al., 2013*
Tomato pomace	0.95 g GAE	Polyphenols, lycopene (0.41 g / kg of DM), β-Carotene (0.15 / kg of DM)	*Kalogeropoulos et al., 2012*
Tomato pomace	6.43 g TAE	Polyphenols, tannin	*Abbeddou et al., 2011a*
Tomato pomace	-	Lycopene, ß-carotene	*Del Valle et al., 2006*
Tomato skin	0.29 g GAE	Polyphenols, flavonoids, ascorbic acid, lycopene (87.00 g / kg DM)	*Toor and Savage, 2005*
Tomato peel	7.16 g GAE	Polyphenols	*Valdez-Morales et al., 2014*
Tomato skin	-	Lycopene	*Kasapidou et al., 2015*

24

25 **Table 5. (Continued)**
26

	Poliphenols (u.m./kg DM)	Main functional Ingredient	References
Tomato seed	6.73 g GAE	Polyphenols	*Valdez-Morales et al., 2014*
Tomato seed oil	-	Lycopene (0.06 g /kg DM)	*Shao et al., 2013*
Tomato seed	0.22 g GAE	Polyphenols, flavonoids, ascorbic acid, lycopene (84.00 g /kg DM)	*Toor and Savage, 2005*
By-product of Myrtle liqueur process			
Exhausted myrtle berries	53.00 g GAE	Polyphenols	*Nudda et al., 2017*

27 TAE: Tannic acid equivalents; CAE: Caffeic acid equivalent; GAE: Gallic acid equivalents

28 **Table 6.** Effects of tomato, grape and myrtle by-products on yield and composition of milk in ruminants
29

By-product	Animal species	Milk							References
		Yield	fat (%)	Protein (%)	Casein (%)	Lactose (%)	Urea (mg/dL)	Somatic Cell Count (cell/mL)	
Grape pomace	Cow	ns	↓	ns	-	ns	-	-	*Moate et al., 2014*
Grape pomace	Ewes	ns	ns	ns	-	↓	-	-	*Manso et al., 2016*
Grape pomace	Cow	↑	ns	ns	-	ns	-	-	*Gessner et al., 2015*
Grape pomace	Cow	ns	ns	↓	-	-	-	ns	*Nielsen and Hansen, 2004*
Dried grape pomace	Cow	ns	-	-	-	-	-	-	*Chedea et al., 2017*
Grape seed	Ewes	↑	-	-	-	-	-	-	*Mokni et al., 2017*
Whole tomato seed	Cow	ns	↓	-	-	ns	-	-	*Cassinerio etal., 2015*
Tomato pomace sun dried	Ewes	-	↑	↓	-	↓	-	-	*Abbeddou et al., 2015*
Tomato pomace	Goats	-	ns	ns	-	ns	-	-	*Razzaghi et al., 2015*
Tomato pomace	Ewes	ns	ns	ns	-	-	-	-	*Shdaifat et al., 2013*
Tomato pomace	Goats	ns	ns	ns	ns	↓	-	-	*Romero-Huelva et al., 2012*
Ensiled Tomato pomace with corn	Cows	ns	ns	ns	-	-	-	-	*Weiss et al., 1997*
Exhausted myrtle berries	Ewes	ns	ns	ns	ns	ns	↓	ns	*Nudda et al., 2017*

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

Table 7. Biological effects observed in different speciesusing by-products from grape, tomato and myrtle

By-product	Animal	Effect[1]	Refernces
Grape pomace ensiled with silage corn	Lambs	Increased antioxidant mechanisms. Enhanced the growth of facultative probiotic bacteria. Inhibited the growth of pathogen populations such as Enterobacteriacae and *E. coli*	*Kafantaris et al., 2017*
Grape seed extract	Lamb	Did not negative effects on the shelf lifeof meat.TBARS meat values were numerically lower.	*Guerra-Rivas et al., 2016*
Grape pomace	Ewes	Did not substantially modify the milk fatty acids	*Manso et al., 2016*
Grape marc	Cows	Reduced CH4 emission. Enhanced concentration of MUFA, PUFA and CLA.	*Moate et al., 2014*
Grape pomace and winery sediment	Wethers	Can alter nitrogen metabolism and act as antioxidants for ruminants.	*Ishida et al., 2015*
Grape marc and seed meal extract	Cows	Did not influence the metabolic and antioxidant paramethers in plasma blood.	*Gessner et al., 2015*
Grape seeds	Ewes	Decreased SFA of milk increased UFA, PUFA, Rumenic acid and vacenic acid of milk.	*Correddu et al., 2016*
Grape seeds	Ewes	Immunomodulatory effect	*Nudda et al., 2015*
Grape residue silage	Cows	Improve the antioxidant capacity in milk	*Santos et al., 2014*
Tomato waste	Goats	Improved milk fatty acid composition, decrease N in urine and CH_4emission.	*Romero-Huelva et al., 2012*
Tomato pomace	Cows	Decreased MUN and PUN. Increased milk concentration of C18:3 and C18:2	*Cassinerio et al., 2015*
Tomato pomace	Goats	Improved total CLA and C18:1*trans*11 contents in milk fat.	*Razzaghi et al., 2015*

Table 5. (Continued)

By-product	Animal	Effect	Refernces
Dried Tomato pomace	Ewes	Improved milk fatty acid composition, increase C18:1c9	*Abbeddou et al., 2011a*
Dried Tomato pomace	Ewes	Did not effect on ruminal fermentation	*Abbeddou et al., 2011b*
Tomato pomace	Ewes	Improved the milk fat composition. Increased PUFA, n3/n6 and CLA. Decreased cholesterol content of milk	*Romano e t al., 2010*
Exhausted myrtle berries	Ewes	Reduction urea concentration in blood and milk	*Nudda et al., 2017*
Tomato pomace	Broiler	Minimize oxidation lipid in meat	*King and Zeidler. 2004.*
Tomato pomace	Rabbits	Improved fatty acids composition on meat	*Peiretti et al., 2013*
Fermented Grape pomace	Pig	Improved fatty acid composition on meat.	*Yan and Kim, 2011*
Tannins from winery by-product	Rats	Gastric protective activity	*Saito et al., 1998*

[1] TBARS: thiobarbituric acid-reactive substances; MUFA:monounsaturated fatty acids; PUFA: polyunsaturated fatty acids;

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OBJECTIVE OF THE THESIS

The objectives of the present thesis were:

- to study the effects of use of small amounts of dried tomato pomace, grape pomace and exhausted myrtle berries on milk yield and composition, and blood biochemical parameters (Chapter 2);

- to enhance information on nutrient and polyphenol composition of the by-products from tomato, wine and myrtle industries processing supplementing existing feedstuff tables. (Chapter 2);

- to evaluate if tomato pomace, grape pomace and exhausted myrtle berries could be appropriate supplements to counteract oxidative stress and improve the oxidative stability of milk and plasma blood from Sarda sheep (Chapter 3);

- to study the effectsof these by-products on milk fatty acid profile (Chapter 3);

- to evaluate rumen function of dairy ewes fed by-products (Chapter 4);

CHAPTER 2

Small amounts of agro-industrial byproducts in dairy ewes diets affects milk production traits, hematological profile

ABSTRACT

The aim of this study was to evaluate the effect of diets containing different dried by-products on milk yield and composition, and blood biochemical parameters of lactating ewes. Thirty-six Sarda dairy sheep at about 120 ±10 days in milk and with an average pre-trial milk yield of 1720 ±430 g/d were assigned to 4 experimental groups and fed diet containing: no by-product (CON), exhausted myrtle berries (EMB), dry tomato pomace (TP) and dry grape marc (GM). Feed intake, milk yield, milk composition and biochemical parameters were affected by the inclusion of by-product in the diet. Ewes fed by-products consumed less dry matter than CON (1.88 *vs* 1.79 in GM and 1.71 kg in EMB and TP groups). The GM group yielded more milk(+200 g/d), 8.4 g/d more protein, and 5.5 g/d more fat than CON group. The EMB group produced less milk than CON (1050 *vs* 1220 g/d). The addition of TPdid not affect production performances compared to CON. No interaction effects between diet and sampling timeon daily intake and dairy performance were observed. Values of plasma biochemical parameters were within the physiological range for the species in all groups, demonstrating the good health conditions of ewes throughout the experiment. In conclusion, the GM

596 at tested amounts turned out to be the most appropriate by-product to improve
597 milk yield, prevent considerable decrease in dry matter intake, and maintain the
598 health status of animals.
599

1. INTRODUCTION

Around 2.5 million tons of food waste and residues from food processing industryare generated every year in the EU, and only 40 % is recycled (European Commission, 2016). Because of the increased attention to circular economy in the agrifood system (Jurgilevich et al., 2016), there are many examples of re-use of agro-industrial by-products. Among the most popular and easy to apply for recycling system is the use in animal nutrition (Laufenberg et al., 2003; Federici et al., 2009) thanks to their sources of high digestible fiber, protein and lipids. Recently, the agro-industrial byproducts are receiving interest for their chemical composition because source ofbioactive compounds, such as polyphenols,that can exert positive effects on production performance (Santos et al., 2014; Nudda et al., 2015; Kotsampasi et al., 2018), milk nutritional composition (Tsiplakou and Zervas, 2008; Buccioni et al., 2015; Cappucci et al., 2018), in the decrease of nitrogen excretion (Bhatta et al., 2009; Theodoridou et al., 2010; Cappai et al., 2013, 2014), and methane emission (Dschaak et al., 2011; Liu et al., 2011; Moate et al., 2014). Some by-products are largely widespread in several countries, as tomato pomace and grape marc, whereas others are produced at local level as results of processing of typical products (Nudda et al., 2017).

Tomato pomace (**TP**) is composed mainly by peels and seeds. Its energy content is limited because of the high fiber content and it is highly perishable due to the high moisture content. For this reason, TP is usually ensiled and used as supplement of highly fermentable fiber (Del Valle et al., 2006; Shdaifat et al., 2013). Moreover, TP is a source of protein, mineral and antioxidant compounds.

623 The available information regarding the inclusion of TP in dried form in
624 ruminant diets is limited to the study of Abebddou et al. (2011a,b), whodid not
625 show relevant effects on milk yield and composition. However, TP exerted an
626 antioxidative activity in milk suggesting that a transfer of some active
627 metabolites from feed to milk occurred (Abebddou et al. 2011a).
628 Grape marc (**GM**) is a by-product of the wine industry, made by the remaining
629 skins, seeds, and stems of grapes. Only 3% of produced GM is re-used in animal
630 feeding (Beres et al., 2017). Different by-products from winery industry have
631 been tested in dairy cows (Santos et al., 2014) and sheep (Correddu et al., 2015;
632 Manso et al., 2016), without any detrimental effects on milk yield, except when
633 used as silage in dairy cow feeding(Moate et al., 2014). The use of high doses of
634 fresh GM resulted in negative effects on rumen digestibility and retained nitrogen
635 in sheep (Abarghuei et al., 2010). However, the dose and the physical form of by-
636 products could be a reason of the different response observed in the trials. For
637 example, Moate et al. (2014) found a higher milk yield when GM was included in
638 the diet of cows in a dried rather than ensiled form. Moreover, GM is an
639 interesting source of phenolic compounds (Peixoto et al., 2018) which can exert
640 antioxidant properties in ruminant products (Santos et al., 2014; Guerra-Rivas et
641 al., 2016).
642 Several local by-products are commonly undervalued due to lack of profitable
643 alternative uses. Their inclusion in ruminant diets could be a practical and
644 economic option for their recycle and valorization. An example is represented by
645 exhausted myrtle berries (**EMB**), a by-product of the maceration process of

myrtle (*Myrtuscommunis L.*) berries used to produce a commercial liqueur called Mirto, EMB is a typical product of Mediterranean area and it is characterized by interesting antioxidant properties (Tuberoso et al., 2010). A preliminary work carried out on lactating sheep supplemented with 50 and 100 g/day of EMB showed a linear reduction of blood and milk urea (Nudda et al., 2017), suggesting effects on nitrogen metabolism and excretion.

The attention of food/feed scientists and food/feed industry to by-products with high content in nutrients and bioactive compounds, and the need to recycle waste characterized by high disposal costs is constantly growing. The aim of the present study was to evaluate the effects of addition of small amounts of tomato pomace, exhausted myrtle berries and grape marc in dried form, on milk yield and composition and blood biochemical parameters of dairy ewes. Moreover, additional information on nutrient and polyphenol composition of the tested by-products will be provided, supplementing existing feedstuff tables.

2. MATERIALS AND METHODS

2.1 Animals and Diets

The animals were handled following the European Union Guidelines on animal care (European Union, 2010). Thirty-six Sarda dairy ewes were assigned to four experimental groups, consisting of nine animals each. They had averaged for milk yield (1720 ±430 g/d), body weight (45.5 ±4.83 kg), body condition score (BCS, 2.77±0.11), parity (4.2±0.25) and DIM (120±10 days). Sheep were housed to have

669 3 animals per pen on straw litter. After an adaptation period of 2 weeks, animals
670 were fed the following experimental dietary treatments administered according to
671 groups. The experimental feeding consisted of a base diet offered as TMR
672 formulated to meet energy and protein requirements of the dairy ewes calculated
673 by the Small Ruminant Nutrition Model (Tedeschi et al., 2010). Ingredients and
674 chemical composition of the experimental diets are reported in Table1. One group
675 was fed TMR only and served as control group (**CON**), the second group was fed
676 TMR with a supplement of ground exhausted myrtle berries (EMB group),
677 whereas the third group was fed a supplement of ground dry tomato pomace to the
678 TMR (TP group); and the fourth group was fed a supplement of ground grape
679 marc to the TMR (GM group). Throughout the feeding trial ewes were milked
680 twice daily at 07:30 and 16:30. Body weight (BW) and body condition score
681 (BCS) were recorded at the beginning, in the middle and at the end of the
682 experimental. Individual milk yield was recorded twice per week.
683 The TMR was offered 3 times a dayat pen level allowing for 20% daily refusals in
684 order to not limiting total daily intake. The by-product was provided grinded (75
685 g/d per animal of EMB, 100 g/d per animal of TP and 100 g/d per animal of GM)
686 and mixed with 50 g of soybean meal and 200 g of beet pulp (by-product mix) to
687 each animal by using individual feeders and their daily intake was monitored
688 individually to ensure total consumption.The experimental trial lasted 8 weeks.

689 ***2.2 Sampling and Analysis***
690
691 ***Feed sampling and analysis***. Daily amount of feed offered and orts was weighed
692 and recorded to calculate individual average daily intake. All by-products
693 combined in the diet were supplied by agro-industrial factories producing in
694 Sardinia (Italy). The TP were collected from regional tomato industry (Casar,
695 Cagliari, Italy), and was composed by seeds and peels allowed firstly to air dry
696 and then dried in air oven at 45°C for 12h. The GM were collected from different
697 wineries of red wine (Sardinia, Italy) and were dried in air oven at 65°C for 12h.
698 The exhausted myrtle berries (*Myrtus communis)* were obtained from a local
699 winery (Sella&Mosca, Alghero, Italy) as by-products of the process for "Myrtus
700 liqueur" production as detailed previously (Nudda et al., 2017).
701 The dry matter (DM) content of samples was determined by oven-drying at 105
702 °C for 24 h. The fiber fractions content [neutral detergent fiber (NDF), acid
703 detergent fiber(ADF), acid detergent lignin(ADL)] were determined following the
704 sequential procedure described by Van Soest. (2015), using the filter bag
705 equipment of Ankom (Ankom Technology Corp., Fairport, NY). Ash, protein and
706 ether extract contents were determined following the analytical AOAC (2000;
707 2005) procedures (methods 942.05, 988.05 and 920.39, respectively). Organic
708 matter (% DM) was obtained by calculation: 100 – ash; NFC was calculated as
709 follows: NFC (% DM) = 100 − (NDF + CP + ash + EE). Metabolizable energy
710 concentration of offered diets (Mcal/kg of DM) was calculated using the Small
711 Ruminant Nutrition Model (Tedeschi et al., 2010). These parameters (except for
712 energy) were expressed as percentage of DM. Protein fractions were determined

713 with the method described by Licitra et al., (1996) and these were calculated as
714 follows: A (NPN), B_1 (buffer-soluble true protein), B_2 (buffer-insoluble protein –
715 neutral detergent soluble protein), B_3 (neutral detergent insoluble protein – acid
716 detergent insoluble protein) and C (acid detergent insoluble protein) .
717 The FA of feeds were extracted and analyzed as outlined by Correddu et al.
718 (2016), with the gas chromatograph Agilent and a CP-Sil88-fused silica capillary
719 column SP™-2560 (100 m × 0.25 mm ID, 0.20-µm film, Supelco, Bellefonte, PA,
720 USA). The concentration of phenolic compounds in the by-products was
721 determined by the Folin-Ciocalteu method as described by Kim et al. (2003), here
722 modified on purpose as detailed by Nudda et al. (2017). The phenol composition
723 of by-products was determined by HPLC-DAD. By-products samples were
724 extracted using a mixture of aqueous ethanol (70% v/v). An aliquot of 0.5 g was
725 added 5 mL of aqueous ethanol and sonicated for 60 min at 20°C and centrifuges
726 for 30 min at 4000 rpm at 10°C. The pellet was re-extracted with aqueous ethanol
727 and the surnatant was combined and filtered. An aliquot of the extract was diluted
728 with two parts of phosphoric acid at 0.2 M.The HPLC analysis was performed on
729 an Agilent 1100 system (Agilent Technologies, Milan, Italy) equipped with a
730 quaternary pump, a degasser, an autosampler, a thermostated column
731 compartment, and coupled with a DAD detector UV 6000 (Thermo Finnigan,
732 Milan, Italy). The chromatographic separation of phenols compounds was
733 achieved on a reversed-phase Kinetex column (5u, C18, 100 A, Phenomenex) at
734 room temperature. Acetonitrile and 0.22M aqueous solution of phosphoric acid
735 were used as mobile phases A and B, respectively, with a flow rate of 0.6

736 mL/min. The linear gradient started with 95% of solvent B reaching 85% solvent
737 B at 35 min, 70% solvent B at 70 min and 90% solvent B at 100 min. The initial
738 conditions were re-established within 1 min and isocratic conditions were
739 maintained up to 15 min. The injection volume was 10 µL. The concentrations of
740 each phenol was obtained against external calibration curves and expressed as
741 expressed as milligrams of active ingredient per kg of dry samples.
742 The chemical compositions of the diets and of the by-products used in this
743 experiment are reported in tables 1 and 2. Concentration of CP in tomato by-
744 product was greater than that in GM whereas EMB had the lowest value.
745 However, the TP product was the richer in the proportion of lignified protein as
746 evidenced by the high value of B3 protein fraction characterized by CNCPS
747 method. The lipid content was similar among all by-products and were
748 characterized by high proportion of linoleic acid (18:2 *cis*-9,*cis*-12; *n*-6), being the
749 highest in EMB where it exceeded 70% of the total FA. In TP and GM, oleic acid
750 (18:1 *cis*-9; *n*-9) and palmitic acid (16:0) were in a similar proportion. The GM
751 had the lower amounts of NDF and ADF, whereas the value of NFC was similar
752 to that of EMB. The total phenol content was the lowest in TP samples. The
753 phenol composition of by-products is reported in Tables 3, 4 and 5.
754
755 ***Milk sampling and analysis.*** Individual milk samples were collected weekly from
756 morning plus afternoon milking and were analyzed separately for chemical
757 composition (fat percentage, protein percentage, lactosepercentage, pH, urea,
758 NaCl) by Fourier transform mid-infrared (FTMIR) spectroscopy equipment

(Milkoscan 6000, Foss Electric, Hillerød, Denmark) and for somatic cell count (SCC) (Fossomatic 360, Foss Electric). The value of each parameter was calculated as weighted average of the morning and afternoon data.

Samples were also processed to determine milk coagulation properties (MCP) according to the procedure detailed by Manca et al. (2016), by using the Formagraph instrument (Foss Electric A/S, Hillerød, Denmark) which recorded the 3 coagulation traits: rennet coagulation time (RCT, min), curd firming time (k20, min), and curd firmness (a30, mm).

Blood Sampling and Analysis. Blood samples were taken from the jugular vein of each fasting ewe, after the morning milking on d 0 (before starting the experimental period), and on d 15, 30, and 45 of the experiment. The white blood cell count (WBC), red blood cell count (RBC), hemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, lymphocytes (LYM), monocytes (MONO), neutrophil granulocytes (NEU), eosinophils granulocytes (EOS) and basophiles granulocytes (BASO) were determined in blood samples added with K3EDTA using an automatic cell counter instrument (Hematology analyzer Alcyon Mindray BC-5000, Shenzhen,China).

Whole blood samples were stored in EDTA-K_2 containing tubes and transported refrigerated to the laboratory where hematology profile was analyzed within 6 hours from sampling. In brief, 15 µL of each sample were needed for the determination of 23 hematological parameters through capillary analysis based on

782 tri-angle scattering and chemical dying read through flow cytometry technology.
783 It were analyzed following parameters: white blood cell count (WBC), red blood
784 cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume
785 (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin
786 concentration (MCHC), platelet (PLT), neutrophil granulocytes (NEU),
787 lymphocytes (LYM), monocytes (MON), eosinophils granulocytes (EOS),
788 basophiles granulocytes (BAS), mean platelet volume (MPV), platelet distribution
789 width (PDV) and plateletcrit (PCT).
790 Both absolute and relative values for leucocyte formula interpretation were
791 analyzed for each sample.
792

793 ***2.3 Statistical analysis***
794
795 All data (i.e., animal performance, milk composition, and biochemical blood
796 parameters) were analyzed as a completely randomized design with repeated
797 measures using the PROC MIXED procedure of SAS version 9.2 (SAS Institute,
798 2008). The model included the fixed effects of treatment, sampling date and the
799 treatment × sampling date interaction plus the random effects of pen. Somatic cell
800 counts were log-transformed before statistical analysis (Ali and Shook, 1980).
801

802 **3. RESULTS**

803 ***3.1 Animal Performance***

804 The by-products doses supplied individually were completelyconsumed by the
805 animals by the animals of all groups. Experimental diets affected the daily feed
806 intake, which was lower in all groups receiving by-products compared to CON.
807 The lowest intake has been observed in EMB and TP (P<0.001; Table 6) groups.
808 A depression of daily intake in GM compared to CON has been also evidenced
809 (P<0.05) even if to a lower extent compared to EMB and TP.
810 The BW was not affected byby-products supplementation (P = 0.77). It increased
811 in all ewes during the experiment (P<0.05), even if with a very low rate
812 considering the long duration of the experiment (Table 6). No differences were
813 observed amongexperimental groups for BCS value (P = 0.749).
814

815 ***3.2 Milk Yield and Composition***
816
817 Milk yield and composition of the experimental groups are reported in Table 6.
818 By-products supplementation significantly affected milk yield and composition
819 (*P*< 0.05). The milk yield was higher in GM (P<0.05), andlower in EMBand
820 TPcompared to CON group, respectively.
821 GM inclusion in the diet reduced significantly milk fat, protein and casein
822 contents compared to CON diet. The same effects on milk composition have been
823 observed with the inclusion of TP when compared with the CON diet. The EMB
824 did not change milk fat, protein and casein contents compared to control, except
825 for lactose content that was reduced (P<0.0001).

826 The GM diet increased yield of protein, casein and lactose compared to CON
827 (P<0.0001), while fat yield did not change. The EMB inclusion reduced yield of
828 fat and casein compared to CON.
829 The inclusion of all 3 by-products did not influence SCC, whereas the bacterial
830 count was reduced in milk of EMB and GM groups (P<0.0001).
831 The urea yield was not changed by GM, whereas was numerically lowest in EMB
832 group.
833 The MCP showed not differences of supplemented groups compared to CON
834 (Table 6). The RCT was lower, whereas the average values for A30 were similar
835 to previous report on Sarda dairy ewes (Mele et al., 2006; Manca et al., 2016).
836 Among the treated groups, milk from EMB group showed the highest values of
837 A30 compared to other by-products, as consequence ofhigher milk fat and casein
838 content.
839 Almost all milk parameters were influenced by period (*P*< 0.001), except the SCC
840 log.
841 Diet × period interaction was not significant for any of the parameters reported.
842

843 ***3.3 Blood parameters***

844 The haematological parameters are reported as mean ± standard error in Table 7.
845 Values turned out to be within the physiological range for the sheep (Keneko et al,
846 2008; Latimer, 2011).

847 A significant effect of the interaction of diet x sampling time was found as to Eos,
848 Neu, RBC and HGB concentration in whole blood, both for absolute and relative
849 values ($P < 0.05$).
850 The level of several hematological parameters changed with sampling date ($P <$
851 0.05), except for RBC and HBG, RDW_SD, MCH, MPV and PDW. Neu, MCHC
852 increased with time, whereas Lym, HCT and RDW_CV showed an opposite
853 pattern. Mon and MCV did not show a defined trend over time: they change from
854 the first to the second sampling and then tended to return to the level of first
855 sampling ($P < 0.05$).
856 Almost all hematological parameters were not affected by treatments, except from
857 Eos concentration and percentage ($P > 0.05$) which were lower in GM group than
858 CON, and Neu percentage which were higher ($P > 0.05$) in TP than CON group,
859 respectively.
860 The interaction of fixed factors (diet x sampling) highlighted a decrease of Eos,
861 RBC and HGB on the 3rd sampling period for all treated groups whereas CON
862 showed an increase (data not shown).
863

864 **4. DISCUSSION**

865 In all experimental groups the body weight of each animal at the end of the
866 experiment was not different from the initial BW. By-product intake was
867 individually monitored to ensure its total consumption.Therefore differences in
868 DMI observed in all groups fed by-products were due to reduction of TMR intake.

869 Reduction of daily intake when EMBwasincluded in the diet of dairy sheep has
870 been observed in our previous study (Nudda et al., 2017), and it could be
871 partially related to its polyphenol composition, that is characterized by lignified
872 compounds and hydrolysable tannins which represented 77.4% of total
873 polyphenol (Table 3). Hydrolysable tannins can be toxic to ruminants when given
874 in large amount and with insufficient time for microbial adaptation. Usually, a
875 significant proportion of hydrolysable tannins is degraded in the rumen to lower
876 molecular weight compounds, such as gallic and ellagic acid (Waghorn, 2008),
877 which exert lower toxicity at dose rates of <0.4 g/kg live weight per day (Murdiati
878 et al., 1992). This could be only a partial explanation of the depressive effects on
879 intake, because the dose of hydrolysable tannins in EMB used in this trial is low,
880 not detrimental to liver and kidney activities (Nudda et al. 2015), and the duration
881 of the trial was long enough to allow an adaptation of the animal to the presence
882 of hydrolysable tannins in the diet.
883 On the other hand, undigestible protein(C fractions) in EMB represented more
884 than 40%and the effects of tannins on degradability of protein fractions could
885 have reduced the CP degradable at ruminal level depressing the activity of rumen
886 microorganisms. And moreover the higher ADL content of the byproducts might
887 have reduced fiber degradability and contributed to reduce the DM intake (Van
888 Soest, 1994). These aspects could also have contributed together with the
889 reduction of the daily intake in ewes fed diet supplemented with EMB.
890 The reduction of daily intake in GM supplemented group compared to CON was
891 not in agreement with previous reports forewes (Correddu et al., 2015; Manso et

892 al., 2016) and cows (Moate et al., 2014; Santos et al., 2014; Gessner et al., 2015)
893 where no difference in DM intake was observed compared to control when dried
894 GM and/or grape seeds were added to the diet, also in higher amount than used in
895 our experiment.

896 The composition of TP, evidenced that flavanone naringenin is the main
897 polyphenol detected (Table 5), followed by flavonol (mainly quercetin and
898 kaempferol) which accumulates almost specifically in the peels (Stewart et
899 al.,2000). All these compounds have been reported in literature to have potential
900 antibacterial (Diniz-Silva et al., 2017) and antiprotozoal (Calzada et al., 1999)
901 activity, and therefore a potential interference in the activity of rumen microflora
902 should be considered. In a previous experiment by using dried TP(Abbeddou et
903 al., 2011b), the authors observed a slight trend for refusals showing that
904 palatability was not as high as with the control feeds. Similar problems have been
905 observed in our trial, especially during the adaptation period for TP and EMB.
906 The tendency to refuse was not observed for the GM, which were consumed
907 quickly by all ewes.

908

909 ***4.1 Milk yield and composition***

910 The EMB group exhibited the lowest milk yield compared to CON group. In a
911 previous study were 50 and 100 g of EMB in sheep diet were used, no variation of
912 milk yield was observed, even if it was evidenced a numerically lower milk yield
913 in group receiving the highest EMB dose (Nudda et al., 2017). The dose used in

914 this trial was 75 g/d, lower that the dose of 100 g/d previously tested, but probably
915 always too high. The depressive effect of EMB on MUN that has been previously
916 observed (Nudda et al., 2017), it has not been confirmed in this trial.

917 The inclusion of TP in the diet did not affect milk yield and FCM compared to
918 CON group in according to other studies on ewes (Shadaifat et al., 2013), goats
919 (Razzaghi et al., 2015) and cows (Cassinerio et al., 2015) fed by-product from
920 tomato processing conserved in different forms. In this case an increase on milk
921 yield was expected as TP in dry form is characterized by a high content of
922 beneficial nutritive and bioactive compounds (Nour et al., 2018). A dilution of fat
923 and protein contents has been also observed in TP compared to CON despite no
924 change in milk yield. The depressive effect of tomato by-product on fat and
925 protein content has been previously observed in sheep (Abbeddou et al., 2015)
926 and cows (Cassinerio et al., 2015). Even if the TP is characterized by high protein
927 content, its rumen degradability could be low due to high lignification (Ventura et
928 al., 2009), as evidenced by the high value of B3 protein fraction in our TP (Table
929 2). Therefore, considering that TP addition to diet provided an additional little
930 amount of CP, mainly undegradable, this could probably must have reduced the
931 action of proteolitic bacteria reducing the synthesis of microbial protein and
932 consequently of the aminoacid at mammary gland level.
933 Grape marc increased milk yield compared to CON group. The additional yield of
934 milk and milk components in GM group did not occur at the expense of body

935 reserves because change in BW and BCS was not affected by GM
936 supplementation.

937 Differently to our results, no effect of similar dose of GM on milk yield has been
938 observed in Churra breed ewes (Manso et al., 2016). Otherwise in dairy cows an
939 increase of milk with grape marc supplementation has been observed (Gessner et
940 al., 2015). The authors hypothesized that increase in milk yield could depend from
941 the effect of an improvement of the rumen fermentation due to the physical form
942 of the by-product. This is supported by results of Drosou et al. (2015) who
943 reported that dried GM favoured higher milk production than the wet GM, since
944 drying causes breakage and destruction of cell walls and consequently large
945 cavities and intercellular spaces are formed allowing to the cellular substances to
946 be easily extracted. In addition, the polyphenol of GM could have generated
947 complexes with proteins that cause a lower protein solubility and ruminal protein
948 degradation, and may have increase the quantity of protein digested in the
949 smallintestine (Patra and Saxena, 2011).

950

951 ***4.2 Blood analysis***

952 The interpretation of the hematological profile of experimental animals is crucial
953 for the correct interpretation of health and homeostasis as well of homeorhesis of
954 ewes during lactation, in view of the potential effects from experimental dietary
955 treatments.

956 In this trial, the effect of the diet modified the circulating concentration of EOS
957 and BASO fractions. Whilst EOS appeared to be the highest in CON group
958 compared with other groups supplemented with by-products, BASO showed the
959 opposite trend being the highest fraction in the GM group. In particular, among
960 the three diets where the TMR was supplemented with the by-products mix, ewes
961 of the GM group turned out to significantly reduce circulating EOS concentration
962 in blood, after 3 weeks from the beginning of the trial and contemporarily
963 increase the BASO concentration. Both EOS and BASO belong to the WBC
964 group and are both involved in the inflammatory response towards in the
965 modulations of both acute and chronic hyperimmune reactions. Both those two
966 sub-populations of granulocytes possess cytoplasmic granules rich in enzyme
967 released upon antigenic stimulation. BASO are less represented in normal
968 conditions than other granulocyte sub-populations of WBC. EOS are also
969 involved in the regulation of the immune response of the host against parasites.
970 Often, subclinical parasitosis in sheep flock raised on pasture are responsible for
971 an increase of EOS in the blood above the upper limit of the physiological range,
972 with a relatively high prevalence in relation to the positive testing to internal
973 parasites, like in the case of pulmonary or intestinal Strongylosis, or with hepatic
974 infestation like in cases of Dicrocoeliosis and Fascioliasis. On the other hand, a
975 positive linear effect following the daily consumption of biologically active
976 compounds contained in the experimental diet administered to the GMgroup
977 cannot be established with certainty, in the light of the fact that all animals
978 appeared healthy and no direct effects on parasite search in feces was assessed in

this trial. However, in case of subclinical infestation the positive effect on the significant reduction of EOS concentration in blood of sheep from GMgroup, the indirect effect on of the etiological agent (parasite?) thanks to the biologically active compound contained in the diet cannot be excluded. In any case, values of EOS did not exceed the upper limit value of the physiological range. From a nutritional point of view, consumption ofdiets rich in polyphenols can lead to different episodes in humans and animals, depending on the biology and the consumer on one side and on the polyphenolic spectrum on the other. The recent literature pointed out a potential direct effect of polyphenols on degranulation of BASO and EOS *in vitro*and *in vivo* in rats (Magrone et al., 2017; Pérot et al., 2017), emphasizing the effect on polyphenols from cranberries and wine. However, experimental evidence is far to explain in an exhaustive way the possible effect of dietary polyphenols on the modulation of degranulation of BASO and EOS as well as on the systemic proportion of granulocyte circulating in blood of large animals at different stages of production.

5. CONCLUSIONS

The addition of small amounts of dried tomato pomace, exhausted myrtle berries and grape marc, to a TMR fed to dairy ewes highlighted a generalized depression in DMI, with no influence on BW and on BCS variations but with different responses of milk yield and quality. Moreover, no interesting differences were found in serum biochemical parameters indicating that detrimental effects should

1001 not be expected from the use of small doses of these by-products. In particular,
1002 compared to CON diet, GM showed a higher yield and lower milk quality, TP a
1003 similar milk yield, but lower quality, whereas EMB lower yield and similar milk
1004 quality. Regarding the hematology, the possible effect of dietary polyphenols on
1005 the modulation of degranulation of BASO and EOS as well as on the systemic
1006 proportion of granulocyte circulating in blood of large animals at different stage
1007 of production, were found in this trial but further investigations may help to
1008 elucidate the diet effects. On the basis of this experiment, GM and, in a lower
1009 extent, TP can be conveniently used as a supplement in the rations of dairy ewes,
1010 whereas EMB, at tested amounts in this experiment, could seriously decrease milk
1011 yields. The employment of by-products tested in our investigation did not point
1012 not adverse effects on health of dairy ewes.

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1192 **7. TABLES**

1193 **Table 1.** Ingredients and chemical composition of offered diets

1194

	Diet[1]			
Item	CON	EMB	TP	GM
Ingredient (kg/d per animal, as fed)				
TMR[2]	2.210	2.210	2.210	2.210
By-product mix				
Soybean[3]	0.050	0.050	0.050	0.050
Beet pulp[4]	0.200	0.200	0.200	0.200
Exhausted myrtle berries		0.075		
Tomato Pomace			0.100	
Grape Pomace				0.100
Total DM supplied	2.460	2.535	2.560	2.560
Chemical composition (g/100gof DM unless otherwise noted)				
DM (g/100g of fresh feed)	88.33	88.51	88.50	88.53
NDF	33.47	34.46	34.62	34.27
NFC	39.73	39.06	38.60	38.94
ADL	3.97	4.82	5.09	4.88
CP	17.42	17.12	17.35	17.16
Ash	7.55	7.43	7.43	7.60
Ether extract	1.82	1.93	2.00	2.02
ME, Mcal/kg of DM [5]	2.34	2.32	2.32	2.33
ME supplied, Mcal/d	5.76	5.88	5.94	5.96

[1]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape pomace

[2]TMR composition: pellet hay = 19.67%; soybean meal = 14.21%; flaked corn = 12.53%; medium chop straw = 11.80%; meal corn = 5.83%; Beet pulp = 4.44%; molasse = 3.64%; premix = 0.73%; medium chop hay = 9.05%; commercial pellet = 18.10%. Chemical composition: DM = 88.05% ; NDF = 32.39% of DM; NFC = 40.74% of DM; ADL =3.86% of DM; CP = 17.38% of DM; Ash = 7.54% of DM; Ether extract = 1.94% of DM.

[3]Soybean, chemical composition: DM = 89.56%; NDF = 18.59 % of DM; NFC = 21.11% of DM; ADL = 0.44% of DM; CP = 52.14% of DM; Ash = 7.06% of DM; Ether extract = 1.10% of DM. [4]Beet pulp, chemical composition: DM = 90.59%; NDF = 48.79% of DM; NFC = 33.48% of DM; ADL =6.09% of DM; CP = 9.32% of DM; Ash = 7.82% of DM; Ether extract = 0.60% of DM.

[5]Calculated using the Small Ruminant Nutrition Model (Tedeschi et al., 2010).

Table 2. Chemical composition, fatty acid (FA), total polyphenols and nitrogen fractions of by-products

Item[1]	By-products[2]		
	EMB	TP	GM
Chemical composition (g/100g of DM unless otherwise noted)			
DM (g/100g of as fed)	94.30	92.57	93.36
NDF	64.8	61.55	52.74
NFC	18.26	12.1	20.59
ADF	51.73	50.66	38.85
ADL	30.76	31.28	25.96
CP	7.76	15.69	11.08
Ash	3.75	4.43	8.68
Etherextract	5.43	6.23	6.91
Major FA (g / 100 g of total FA)			
C12:0	0.07	0.02	0.24
C16:0	8.4	15.55	12.07
C18:0	3.76	4.88	5.44
C18:1*cis*-9	7.9	17.6	17.64
C18:1*cis*-11	0.38	1.26	0.82
C18:2*n*-6	72.01	52.19	50.63
C20:0	0.69	0.44	0.67
C18:3*n*-3	0.68	3.22	1.56
Total polyphenols (g GAE/100g of DM)	4.09	0.23	1.48
Protein fractions (% of CP) [2]			
A	6.15	25.51	10.98
B_1	3.58	11.22	1.12
B_2	41.92	7.72	60.34
B_3	7.22	42	7.49
C	41.13	13.55	20.07

[1] By-products: EMB = exhausted myrtle berries; TP = tomato pomace; GM = grape marc.
[2] Protein fractions: A = NPN; B_1 = buffer-soluble true protein; B_2 = buffer-insoluble protein – neutral detergent soluble protein; B_3 = neutral detergent insoluble protein – acid detergent insoluble protein; C = acid detergent insoluble protein.

1212 **Table 3.** Levels of phenols measured in exhausted myrtle berries
1213

Polyphenol, mg/kg of DM	Means	± SD
Gallic acid [a]	386.35	11.84
Hydrolysabletannins[a, 1]	3723.86	160.89
Quercetin 3-galactoside[b]	77.11	0.91
Ellagic acid[b]	494.95	26.59
Quercetin 3-rhamnoside[b]	56.72	0.74
Delfidin-3-*O*-glucoside[c]	8.01	0.03
Cyanidin-3-*O*-galattoside[c]	6.40	0.38
Petunidin-3-*O*-glucoside[c]	25.84	1.64
Peonidin-3-*O*-glucoside[c]	12.33	1.09
Malvidin-3-*O*-glucoside[c]	22.24	0.37

1214 [1]Measured as gallic acid
1215 [a] 280 λmax Uv-Vis (nm); [b]360 λmax Uv-Vis (nm); [c] 520 λmax
1216 Uv-Vis (nm)
1217
1218

Table 4. Levels of phenols measured in grape marc

Polyphenol, mg/kg of DM	Means	± SD
Gallic acid[a]	112.21	16.48
Catechin [a]	122.05	15.86
Vanillic acid [a]	17.33	0.16
Syringic acid [a]	42.59	4.06
Quercetin[b]	70.89	0.73
Isorhamnetin[b]	13.86	1.82
Kampferol[b]	9.97	0.91
Petunidin-3-*O*-glucoside[c]	28.84	3.50
Peonidin-3-*O*-glucoside[c]	21.02	4.53
Malvidin-3-*O*-glucoside[c]	49.62	11.49
Delfidin-3-*O*-acetilglucoside[c]	4.64	0.74
Malvidin-3-*O*-acetiglucoside[c]	4.36	1.42
Cyanidin-3-p-coumaroylglucoside[c]	4.58	0.54
Petunidin-3-p-coumaroylglucoside[c]	55.90	16.91
Peonidin-3-p-coumaroylglucoside[c]	201.91	32.82
Malvidin-3-p-coumaroylglucoside[c]	52.90	18.69

[a] 280 λmax Uv-Vis (nm); [b]360 λmax Uv-Vis (nm); [c] 520 λmax Uv-Vis (nm)

Table 5. Levels of phenols measured in dried tomato pomace

Polyphenol, mg/kg of DM	Means	± SD
Naringenin[a]	191.84	5.61
Rutin[b]	119.10	1.68
Quercetin[b]	30.27	0.44
Kaempferol[b]	6.17	0.10

[a] 280 λmax Uv-Vis (nm); [b] 360 λmax Uv-Vis (nm)

1231 **Table 6.** Intake, milk yields, milk composition and milk coagulation properties from ewes on each treatment
1232

	Diet[2]					*P-value*[3]		
Item[1]	CON	EMB	TP	GM	SEM	D	S	D × S
BCS change	0.049	0.069	0.063	0.049	0.028	NS	NS	NS
BW change, kg	0.94	1.15	1.12	1.01	0.172	NS	***	NS
DMI, k g/day	1.88 ^{a}	1.71 ^{c}	1.71 ^{c}	1.79 ^{b}	0.009	***	***	NS
Yield, g/day								
Milk	1220 ^{b}	1050^{c}	1193^{b}	1421^{a}	19.74	***	***	NS
F.P.C.M.	1245 ^{b}	1098^{c}	1176^{bc}	1373^{a}	16.82	***	***	NS
Fat	80.92^{ab}	71.98^{c}	75.80^{bc}	86.36^{a}	1.04	***	***	NS
Protein	71.46^{bc}	63.55^{b}	65.99^{c}	79.89^{a}	1.02	***	***	NS
Casein	55.35^{b}	49.16^{c}	50.67^{bc}	61.21^{a}	0.78	***	***	NS
Lactose	57.84^{b}	48.89^{c}	56.54^{b}	66.63^{a}	1.01	***	***	NS
Urea	0.61^{ab}	0.53^{c}	0.55^{bc}	0.665^{a}	0.01	***	***	NS
Milk composition								
Fat, %	6.79 ^{a}	6.96^{a}	6.37^{b}	5.92^{c}	0.05	***	***	NS
Protein, %	6.04 ^{a}	6.21^{a}	5.65^{b}	5.63^{b}	0.03	***	*	NS
Casein, %	4.71 ^{a}	4.83^{a}	4.37^{b}	4.35^{b}	0.03	***	***	NS
Lactose, %	4.78 ^{a}	4.69^{b}	4.81^{a}	4.82^{a}	0.01	***	**	NS
Urea, mg/dL	48.43^{ab}	50.45^{a}	45.20^{b}	46.67^{b}	0.51	***	***	NS

Table 6. (Continued)

Item[1]	Diet[2]				SEM	*P-value*[3]		
	CON	EMB	TP	GM		D	S	D × S
Log SCC, × 1,000 cell/mL	2.01	2.09	2.19	2.11	0.03	†	NS	NS
Log CBT, x 1,000 UFC/mL	2.80 ^{a}	2.59 ^{b}	2.93 ^{a}	2.43 ^{b}	0.031	***	***	NS
Clotting parameters								
RCT, min	8.08^{b}	8.83^{ab}	9.42^{a}	9.28^{ab}	0.19	*	**	NS
K_{20}, min	2.94	3.46	3.18	3.03	0.16	NS	*	NS
A_{30}, mm	51.91^{ab}	54.45^{a}	51.09^{b}	49.90^{b}	0.58	**	***	NS

$^{a–c}$Means within a row with different superscripts are different ($P < 0.05$).

[1] BCS = body condition score; BW = body weight; DMI, k g/day = variation of intake referred to the diet offer; the residuals of by-product mix were zero during all the experiment; SCC = somatic cell count; CBT = Total bacterial counts; RCT = rennet coagulation time; K_{20} = curd firming time; A_{30} = curd firmness

[2]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.

[3]D = effect of diet; S = effect of sampling time; NS indicates $P > 0.10$. †$P< 0.10$, *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$.

1242 **Table 7.** Effect of dietary supplementation with by-products, sampling date, and their interaction, on blood hematological parameters in dairy
1243 ewes
1244

		Diet[2]					*P-value*[3]		
	Reference values	CON	EMB	TP	GM	SEM	D	S	D × S
Item[1]									
WBC(10^9/L)	5.10 – 15.80	9.93	9.31	9.22	9.41	0.175	NS	NS	NS
Neu (10^9/L)	1.32 – 8.96	2.74	2.73	3.49	2.88	0.090	†	**	NS
Lym (10^9/L)	2.01 – 7.80	5.71	5.48	4.57	5.41	0.130	NS	**	†
Mon (10^9/L)	0.00 – 1.52	0.44	0.43	0.42	0.52	0.016	NS	*	NS
Eos (10^9/L)	0.00 – 1.08	0.95^{a}	0.59^{ab}	0.68^{ab}	0.49^{b}	0.039	**	*	**
Bas (10^9/L)	0.00 – 0.17	0.09^{ab}	0.09^{ab}	0.06^{b}	0.12^{a}	0.005	*	***	NS
Neu (%)	0.215 – 0.680	0.28^{b}	0.30^{ab}	0.38^{a}	0.30^{ab}	0.008	**	**	*
Lym (%)	0.280 – 0.645	0.57^{ab}	0.58^{a}	0.50^{b}	0.58^{ab}	0.008	*	*	NS
Mon (%)	0.000 – 0.143	0.04	0.05	0.05	0.05	0.002	NS	*	†
Eos (%)	0.000 – 0.080	0.10^{a}	0.06^{ab}	0.07^{ab}	0.05^{b}	0.004	*	*	*
Bas (%)	0.000 – 0.015	0.0086^{ab}	0.0094^{ab}	0.0070^{b}	0.0123^{a}	0.0005	**	***	NS
RBC (10^{12}/L)	5.50 – 14.20	8.78	9.16	8.95	8.56	0.078	NS	NS	*

1245

1246 **Table 7. (Continued)**
1247

		Diet[2]					*P-value*[3]		
	Reference values	CON	EMB	TP	GM	SEM	D	S	D × S
Item[1]									
HGB (g/L)	63 – 132	95.78	100.59	99.48	98.59	0.714	NS	NS	*
HCT	0.200 – 0.390	0.29	0.31	0.30	0.30	0.002	NS	*	†
MCV (fL)	25.0 – 41.0	33.23	33.70	33.71	35.21	0.220	NS	***	NS
MCH (pg)	8.0 – 12.3	10.92	11.01	11.17	11.59	0.071	NS	NS	NS
MCHC (g/L)	290 – 360	329.04	326.67	331.63	329.15	1.081	NS	***	†
RDW_CV	0.165 – 0.262	0.20	0.19	0.20	0.19	0.002	†	**	NS
RDW_SD (fL)	20.0 – 35.0	28.73	27.17	28.36	27.89	0.301	NS	NS	NS
PLT (10^9/L)	100 - 800	355.07	473.44	495.33	396.96	18.653	NS	†	NS
MPV (fL)	3.5 – 6.0	4.88	4.81	4.82	4.90	0.059	NS	NS	NS
PDW	12.0 – 17.5	15.46	15.77	15.32	15.64	0.107	NS	NS	NS
PCT (ml/L)	0.50 – 4.20	1.74	2.37	2.39	1.91	0.090	NS	†	NS

1248 [a-b]Means values with different superscript within a row were statistically different (P< 0.05).
1249 [1]Item: WBC= white blood cell count; RBC = red blood cell; HGB = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular
1250 hemoglobin; MCHC = mean corpuscular hemoglobin concentration; PLT = platelet; NEU = neutrophil granulocytes; LYM = lymphocytes; MON = monocytes; EOS =
1251 eosinophils granulocytes; BAS = basophiles granulocytes; MPV =mean platelet volume; PDV = platelet distribution width; PCT = plateletcrit.

1252 [2]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing
1253 100g/d per head of grape marc.
1254 [3]D = effect of diet; S = effect of sampling; NS indicates P > 0.10. †P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.

CHAPTER 3

Effects of agro-industrial byproducts on milk fatty acid profile and antioxidant status of dairy ewes

This study was conducted in collaboration with **Eleni Tziplakou** and **Christine Mitsiopoulou**
of the "*Department of Nutritional Physiology and Feeding - Agricultural University of Athens*"
where analyseson the oxidation status of milk and blood samples were carried out.

ABSTRACT

The aim of this study was to evaluate the effect of diets containing different dried by-products on both milk and blood plasma antioxidant capacity of dairy ewes. Thirty-six Sarda dairy sheep, were assigned to one of the following four treatments: control diet (CON; no by-product) and the control diet supplemented with 100 g/d of dry grape marc (GM), or 100 g/d dry tomato pomace (TP), or 75 g/d of exhausted myrtle berries (EMB). The activities of superoxide dismutase (SOD), glutathione reductase (GR) glutathione transferase (GST) and glutathione peroxidase (GSH-Px) in blood plasmaand the activities of SOD, GR andlactoperoxidase lactoperoxidase (LPO) in ewesmilk were determined. Moreover, in both blood plasma and milk thetotal antioxidant capacity [by using the Ferric Reducing Ability of Plasma (FRAP) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays] as well as the oxidative stress biomarkers (malondialdehyde [MDA] and protein carbonyls [PCs]) were also measured. Finally, ewemilk fatty acid profile of ewes was investigated as well, by gas chromatography. The results showed a significantly higher antioxidant capacity measured either by FRAP or ABTS assays in the blood plasma of GM ewes in comparison with the control group. Moreover, a significant improvement in the oxidative status of ewe's milk supplemented with GM was observed, as was verify by the significant decline on the MDA and PCs contents which were found on it. The EMB supplementation increased the ABTS in ewe

blood plasma and decrease the PC in their milk compared with CON. In the blood plasma of TP ewes a significant increase in the MDA content, which was accompanied by an increase in the GPx activity, in comparison with the CON was found. Regarding fatty acid profile of milk, GM supplementation decreased significantly saturated FA and increased PUFA n-6 due to increase of linoleic acid; C18:2n-6, the main component of GM, with a consequent increase in n-6/n-3 ratio compared with CON. All by-products improve the nutritional indexes of milk fat by reduction of atherogenic and hypercholesterolemic indices. In conclusion, the results suggested that dietary GM supplementation may have beneficial effects against oxidative stress, whereas TP need further research to define the optimum inclusion level in ewediet.

1. INTRODUCTION

In the world, developed and underdeveloped countries want to adapt on the political and social importance of reducing pollution resulting from industrial activities. In fact the result is that many industries consider their waste as raw material for other industries (Mirzaei-Aghsaghali and Maheri-Sis, 2008). Tomato pomace (**TP**) is the by-product derived from process of tomato juice (Peiretti et al., 2013); grape marc (**GM**) derived from winemaking (Manso et al., 2016) and exhausted myrtle berries (**EMB**) derived from production of liqueur called "Mirto rosso" (red myrtle) (Nudda et al., 2017). These by-products can be utilized in ruminants diet and could provide extra income for the industries and, at the same time, reduce the problem of waste disposal.

Furthermore GM, TP and EMB contain natural antioxidant compounds such as polyphenols (Guerra-Rivas et al., 2016; Abbeddou et al., 2011; Nudda et al., 2017) and TP are rich also in Lycopene (Shao et al., 2013). These functional could improve the antioxidant defence system of animal.

Oxidative stress is an important field of research in veterinary medicine and this process has been implicated in sepsis, mastitis, enteritis, pneumonia, respiratory and joint diseases (Lykkesfeldt, and Svendsen, 2007).

In ruminants there are several factors (environmental, physiological, and dietary) that can cause an imbalance between reactive oxygen species (**ROS**).Normallycells produce ROS for their functions but the over production, responsable of oxidative stress, can cause cell and tissue injury (Sordillo and Aitken, 2009) and have effects on milk and meat production (Castillo et al., 2005;

Castillo et al., 2006). However, the physiological processes in body can face the oxidative stress with antioxidant mechanisms. The antioxidant mechanism can be divided into two classes: enzymatic and non-enzymatic mechanism (Ye et al., 2015). Superoxide dismutase (**SOD**), glutathione reductase (**GR**), catalase (**CAT**), glutathione transferase (**GST**) and glutathione peroxidase (**GPX**) (Board and Menon, 2013; Miller et al., 1993), endogenous enzymes are the main form of the intracellular antioxidant defence system which regulates ROS accumulation within tissues and are present in blood and milk (Celi, 2010; Sordillo, 2013). Lactoperoxidase (**LPO**), amilk enzyme, is related to the oxidation of milk lipids (O'Connor and O'Brien, 2006). One possible approach to reduce oxidative stress is given by antioxidant supplementation. The administration of polyphenol to ruminant could decrease oxidation process (Celi and Gabai, 2015). Some agro-industrial byproducts, are reported to be a suitable source of bioactive compounds. Therefore, the inclusion of by-products rich in polyphenol in dairy sheep diet could contribute to reduce oxidative stress (**OS**) in animals and improve the oxidative stability of dairy products. Moreover, dietary supplementation with antioxidant compounds could help to protect animals against the lipoperoxidation and potentially ensuring antioxidant potential for PUFA enriched dairy products (Kotsampasi et al., 2017; Tsiplakou et al., 2017). The aim of this study was to evaluate if TP, GM and EMB could be appropriate supplements to counteract oxidative stress and improve the oxidative stability of milk from Sarda sheep.

2. MATERIALS AND METHODS

2.1 Animals and Diets

The animals were handled following the European Union Guidelines on animal care (European Union, 2010). The experimental design has been described previously in chapter 1. In brief, thirty-six Sarda lactating ewes were allocated into 4 equal groups in a randomized block designeach with 3 replicates of 3 ewes housed in the same pen. Animals were assigned to four experimental groups (9 animal per group), consisting of: a control diet (**CON**), a basal diet supplemented with 100 g/d per animal of grape marc (**GM**), a basal diet supplemented with 100 g/d per animal of tomato pomace (**TP**) and a basal diet supplemented with 75 g/d per animal of exhausted myrtle berries (**EMB**). The experimental groups were balanced for milk yield, body weight, body condition score, parity and DIM. Animals were fed a basal diet consisted of TMR. Ewes were milked twice daily at 07:30 and 16:30, and the by-product mixed with beef pulp was offered during the morning milking.

2.2 Sampling and Analysis

Feed sampling and analysis. Daily amount of feed offered and orts was weighed and recorded to calculate individual average daily intake. All by-products combined in the diet were supplied by agro-industrial factories.

The dry matter (DM) of feed and by-products was determined by oven-drying at 105 °C for 24 h. The NDF, ADF and ADL content were analyzed by the sequential procedure described by Van Soest. (2015), using the filter bag

equipment of Ankom (Ankom Technology Corp., Fairport, NY). Ash, protein and ether extract contents were determined following the analytical procedures described by AOAC (2000; 2005; methods 942.05, 988.05 and 920.39, respectively). Organic matter (% DM) and NFC were calculated as follows: 100 – ash; NFC (% DM) = 100 − (NDF + CP + ash + EE). These parameters were expressed as percentage of DM. Regarding the FA of feeds were extracted and analyzed as described by Correddu et al. (2016), with the gas chromatograph Agilent and a CP-Sil88-fused silica capillary column SP™-2560 (100 m × 0.25 mm ID, 0.20-μm film, Supelco, Bellefonte, PA, USA). The concentration of total polyphenols in by-products was determined by the Folin-Ciocalteu method as described by Kim et al. (2003), here modified on purpose as detailed by Nudda et al. (2017). The phenol composition of by-products was detailed in chapter 1. The chemical composition of the diets and of the by-products, the FA and phenol composition of by-products used in this experiment are reported in Table 1. Regarding the concentration of CP in tomato pomace was higher than that in GM whereas EMB had the lowest value. The lipid content was similar among all by-products and were characterized by high proportion of linoleic acid (18:2 *cis*-9,*cis*-12; *n*-6), being the highest in EMB where it exceeded 70% of the total FA. Oleic acid (18:1 *cis*-9; *n*-9), in TP and GM, and palmitic acid (16:0) were in a similar proportion. The GM had the lower quantity of NDF and ADF, whereas the value of NFC was similar to that of EMB. The total phenol content was the lowest in TP samples.

Milk and Blood sampling.Individual milk and blood samples were collected in the 3th, 6th and 8th week.The samples of milk morning were stored at -80 °C to analyzed antioxidant capacity; another aliquot of milk sample stored at -20°C to analyze milk FA composition.Blood samples were taken from the jugular vein of each ewe into Heparin-containg tubes and then centrifuged at 3000g, 4°C for 10 min and collected supernatant. Blood samples were stored at -80° C.

Milk and Blood analysis. Enzyme assays, antioxidant and free radical scavenging activities, lipid peroxidation activity and protein carbonyl determination in blood plasma and milkweremeasuredin according to Tsiplakou et al. (2017).Briefly, the enzyme assays, in both blood plasma and milk, was monitored through glutathione transferase (GST) activity according to Labrou et al. (2001) was measured in blood plasma by using as substrate the 1-chloro-2,4-dinitrobenzene (97%, Sigma-Aldrich, USA) to observing the formation of the conjugate of each substrate and reduced glutathione (G-SH). For the glutathione reductase (GR) activity, measured in blood plasma and milk, was used the Mavis and Stellwagen's (1968) assay. According to Keesey (1987), Pütter and Becker (1983), the lactoperoxidase (LPO) activity, monitored only in milk, was registered by using ABTS (2, 2'-azino-bis (3 ethylbenzthiazoline-6- sulfonic acid) as a substrate. The superoxide of dismutase (SOD) activity,registered in blood plasma and milk, was mesured with modified method of McCord and Fridovich (1969): with the utilization of xanthine and xanthine oxidase at pH 7.8, One unit will inhibit the rate of reduction of cytochrome-c by 50% in a coupled system. In this

way the concentration of xanthine oxidase should produce an initial (uninhibited) ΔA550 nm of 0.025+/−0.005 per minute.

To evaluatethe antioxidant and free radical scavenging activities in both blood plasma and milk, Ferric Reducing Antioxidant Power (FRAP) assay was used to monitor total antioxidant potential in blood plasmaaccording to Benzie and Strain (1996). In addition, the he ABTS radical scavenging capacity assay was measured (Li et al., 2011; Pellegrini et al., 2003). The same assays, with modifications, were used to measure FRAP and ABTS in milk: was used as extraction solvent one normal solution of HC1 (1 N)/ 95% ethanol (v/v, 15/85); regarding the extraction procedure, it involved the added of 1mL of the fresh milk to 10 mL solvent separately in 12 mL tubes and shaking for 1 h at 30 °C in a rotary shaker set at 300 rpm. After that the mixture of solvent and samples centrifuged at 7800× g at 5 °C for 15 min. The supernatant fluids were stored at −20 °C in the dark until FRAP and ABTS analysis.

In according with Heath and Packer (1968) the lipid peroxidation activity, measured in both blood plasma and milk, was assayed by measuring malondialdehyde (**MDA**). Protein carbonyls (**PC**) in blood plasma were determined based on method of Patsoukis et al. (2004); furthermore the same protocol was used in milk after skimming.

Milk fatty acids determination.The preparation of fatty acids methyl esters (FAME) was carried out on fat obtained from the direct extraction described by Feng et al., (2004*)*with some modification. Briefly, milk sample was centrifuged at 12,000 rpm for 15 min at 7°C to separate of the fat at the surface. Then the fat at the surface was transferred in a new tube and it was centrifuged at 12,000 rpm for 25 min at room temperature. An aliquot of 11-14 mg of fat was weighed and collected in an amber vial and methylated by the addiction of 500 µL of sodium methoxide (0.5 N). The solution was mixed for 2 min by vortex and 1 mL of methylated C5:0 and C13:0 as internal standards (0.4 mg/ml in hexane) was added. After vigorous mixing (vortex,1 min) and 10 minutes of incubation, the upper layer was transferred into a vial for GC analysis.

FAME were determined using a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), equipped with 7693 Autosampler (Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector (FID). To perform FAME separations was used a CP-Sil 88 capillary column (100 m × 0.250 µm i.d., 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA).

Regarding the oven temperature was used: the initial temperature was set at 45°C for 4 min, increased at 13°C/min to 175°C, and held for 27 min; then it was increased at 4°C/min to 215°C, and held for 35 min. As carrier gas was used Helium (1 mL / min flow rate) a pressure of 28 psi and 1µl of sample was injected. The split ratio was 1:80. Temperatures of the injector and detecto were set at 250°C.

To calculate retention time and area of each individual FAME was used OpenLAB CDS GC ChemStation Upgrade software data system (Revision C.01.04, Agilent Technologies Inc., Santa Clara, CA, USA). The individual FAME was identified by comparing their retention times with those of methyl ester standards and published isomeric profiles, as detailed in a study of Nudda et al. (2005).

FA were reported as g/100 g of total FAME and groups of FA were calculated as follows:

saturated fatty acids (**SFA**): ∑individual saturated fatty acids;

unsaturated fatty acids(**UFA**):∑ individual unsaturated fatty acids;

monounsaturated fatty acids (**MUFA**):∑ individual monounsaturated fatty acids;

polyunsaturated fatty acids (**PUFA**): ∑ individual polyunsaturated fatty acids;

trans fatty acids (**TFA**):∑ sum of individual trans fatty acids;

odd- and branched-chain fatty acids(**OBCFA**): ∑ individual odd- and branched-chain fatty acids;

short-chain fatty acids (**SCFA**): ∑ individual fatty acids from C4:0 to C10:0;

medium-chain fatty acids (**MCFA**): ∑ individual fatty acids from C11:0 to C17:0;

long-chain fatty acids (**LCFA**): ∑ individual fatty acids from C18:0 to C22:6 (**DHA**);

PUFA ***n*-3**: ∑ individual *n*-3 fatty acids;

PUFA ***n*-6**, ∑ individual *n*-6 fatty acids;

Total CLA: ∑ individual conjugated linoleic acids;

The nutritional properties of milk fat were estimated by the n-6 to n-3 ratio and three indices, such as the atherogenic index (**AI**) and trombogenic index (**TI**) were calculated according to Ulbricht and Southgate (1991) except for the substitution of C18:0 with C12:0, as suggested by Nudda et al. (2013a)

AI = [12:0 + (4 × 14:0) + 16:0]/[(PUFA) + (MUFA)],

TI = (14:0 + 16:0)/[(0.5 × MUFA) + (0.5 × *n*-6) + (3 × *n*-3) + (*n*-3:*n*-6)];

the hypocholesterolemic to hypercholesterolemic ratio (**h:H**), calculated according to Fernández et al. (2007):

h:H = [(sum of 18:1*cis*-9, 18:1*cis*-11, 18:2 *n*-6, 18:3 *n*-6,18:3 *n*-3, 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:4 *n*-6, 22:5 *n*-3 and 22:6 *n*-3)/(14:0 + 16:0)].

To study the effect of the different diets on the capacity of desaturating SFA to Δ^9- UFA, the Δ^9-desaturase indices (**DI**) were calculated according to Schennink et al. (2008)as follows:

C10 index = [C10:1/(C10:0 + C10:1)] × 100;

C14 index = [C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)] × 100;

C16 index = [C16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)] × 100;

C18 index = [C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)] × 100;

CLA index = [CLA *cis*-9,*trans*-11/(C18:1 *trans*-11 + CLA *cis*-9,*trans*-11)] × 100;

Total index = [(C10:1+ C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA *cis*-9,*trans*-11)/(C10:0 + C14:0 + C16:0 + C18:0 + C18:1 *trans*-11+ C10:1 + C14:1 *cis*-9 + C16:1 *cis*-9 + C18:1 *cis*-9 + CLA *cis*-9,*trans*-11)] × 100.

2.3 Statistical analysis

Data were analyzed as a completely randomized design with repeated measures using the PROC MIXED procedure of SAS version 9.2 (SAS Institute, 2008). The model included the fixed effects of treatment, sampling date and the treatment × sampling date interaction plus the random effects of pen, as experimental unit.

3. RESULTS AND DISCUSSION

3.1 Milk yield and chemical composition

Effects of diets on milk yield and composition are detailed in chapter 1 and summarized in Table 6 of the chapter 1. The milk yield was increased by GM supplementation ($P<0.05$), whereas the EMB and TP dietary inclusion resulted lower milk production in ewes compared with those normally fed. Fat, protein and casein concentration was reduced in GM group compared to CON group ($P<0.001$), as probable consequence of dilution effect.

3.2 Blood plasma antioxidant status

Total antioxidant capacity, enzyme activities, and protein carbonyls content in blood of experimental group are reported in Table 2.

The EMB dietary supplementation did not affect the total antioxidant and free radical scavenging activity of ewes blood plasma,measured by both FRAP and ABTS assays, compared with those normally fed ($P > 0.05$); TP group showed a higher ABTS compared to CON group ($P < 0.05$), while the GM ewes evidenced higher both FRAP and ABTS content in their blood plasma than CON group ($P < 0.05$). According to the results of this study the higher antioxidant capacity,

measured either by FRAP, or ABTS assay, in the plasma of TP and GM sheep compared with the control ones, reflects the high content of these by-products in a variety of antioxidants compounds such as the phenols or others bioactive molecules (Tables4 and 5 – chapter 1). The higher value of both FRAP and ABTS assays compared to CON, suggest a clear antioxidant capacity of GM, at least in the dose included in the diet. The antioxidant capacity of grape marc included in the diet has been previously observed in ruminants (Kafantaris et al., 2017) and monogastric (Brennes et al., 2008; Kafantaris et al., 2018).

However, an increase in the blood plasma MDA content of TP treated sheep in comparison with the CON, was observed ($P < 0.01$). At the same time, the higher MDA content in the blood plasma of sheep fed with TP, compared with the control ones, was accompanied by a significant rise in the GPx activity ($P < 0.01$). MDA is a specific biomarker of lipid peroxidation (Ayala et al., 2014) and GPx participates in the scavenging system of animal's organism to prevent oxidative damage (Weydert and Cullen, 2010). Thus, the results of this study suggest that the dietary supplementation of TP at level used in this experiment exposed the sheep to an oxidative stress. It is likely that the dose of TP included in the diet may affected the balance between pro- and antioxidant activities. In the same line with our findings Botsoglou et al. (2004) observed significantly higher MDA content in the Japanese quail meat fed with the high supplementation level of dried tomato pomace (10%) compared with the low (5%) and the control diet. Moreover, it should be pointed out here, that although TP had the lowest phenols content, compared with the other by-products (Table 1) may also contain other

antioxidant compounds such as carotenoids and specifically lycopene (Ayed and Hussein al., 2013), which have synergetic and powerful effects as well (Palozza et al., 2011). Thus, it was assumed in this study that the total antioxidant compounds of TP may work as pro-oxidants at high supplementation levels and as potential anti-oxidants at low level, since it is known that the balance between pro-oxidants and anti-oxidants behaviour is very delicate. Since other studies have showed a decrease in the concentration of MDA in the serum (Sahin et al., 2004), meat (Sahin et al., 2008) and liver (Sahin et al., 2011) ofquails, more research is needed in order to by clarified which is the optimum supplementation level of TP in sheep diets.

The experimental diets did not effects on GST, GR, SOD and PC ($P > 0.05$). The time of sampling have an effect on ABTS and FRAP ($P < 0.05$). FRAP content in plasma blood increase during the time (Figure 1) while ABTS decrease in the 3th sampling (Figure 2). Variation with time in parameters measuring oxidative status has been previously observed in sheep (Kotsampasi et al., 2018) and goats (Mavrommatis et al., 2018). For Gpx a significant diet × sampling interaction occurred ($P < 0.05$), with TP that in the beginning having a higher amount of this enzyme, but in the 3th sampling it converged to the same value of all others diets (Figure 3), suggesting that scavenging system of GPx in TP group exert greater activity against oxidative stress with sampling time.

3.3 Milk antioxidant status

The milk antioxidant status is reported in Table 3. Total antioxidant activity and free radical scavenging activity measured by FRAP and ABTS were not influenced by by-products supplementation. Furthermore, by-products in diets did not influenced the enzymes LPO and SOD ($P > 0.05$) compared to CON group, whereas a higher LPO activity of ewes fed with the GM than EMB diet, was observed (Table 3). LPO protects mammary gland by pathogenic microorganisms (Naidu, 2000), plays an important role in preservation of raw milk during storage and transportation and it can be used to extent the shelf life of pasteurized milk (Martinez et al., 1988; Baratta et al., 1998).

Significantly higher GR activity in ewes milk, fed with the TP supplemented diet, in comparison with the CONdiet, was also found (Table 3) ($P < 0.001$). GR has an important role in the antioxidant defence system since catalyses the conversion of oxidized glutathione disulfide to the reduced form of glutathione, which is a critical molecule in resting oxidative stress (Celi, 2010; Sordillo, 2013),

A significant decline in the MDA content of ewes milk, fed with the GM supplemented diet, compared to CON, was found (Table 3) ($P < 0.01$). Since oxidative stress is often measured by the formation of lipid hydroxides (i.e. MDA), the reduction of MDA content may show lower oxidative damage in the milk of ewes fed with GM. Moreover, the inclusion of all by-products (EMB, TP and GM) in ewesdiets resulted in a significant decline of PC content in their milk (Table 3). Proteins are the more susceptible to oxidation molecules (Dalle-Donne at al., 2005; Cheah et al., 2008) and the formation of PC in milk is indicative of its

oxidation (Fenaille et al., 2006). Therefore, the dietary supplementation with by-products such as EMB, TP and GM could represent a feeding strategy to improve ewes' milk antioxidant status.

The sampling time had an effect on almost all parameters ($P < 0.05$), except for GR and PC in milk. FRAP content in milk was decrease between the second and the third sampling time (Figure 4), whereas ABTS content in milk increasedwith sampling time (Figure 5). The LPO (Figure 6) and SOD (Figure 7)decreased from the first and the third sampling, whereas MDA increased (Figure 8). These patterns could be related to the progress of lactation. A decrease in SOD as the lactation period proceeded has been documented in goat (Mavrommatis et al., 2018) and human (Yuksel et al., 2015) milk.

3.4 Milk fatty acids profile

The FA composition of milk from the ewes of the four experimental treatments is shown in Table 4.

The EMB supplementation did not change the FA profile of milk, except for C15:1, C16:0 and C18:3 *n*-3(linolenic acid, **LNA**)which were significantly lower than CON group while C20:2 *n*-9 where significantly higher than CON group ($P < 0.05$).

The TP supplementation increase the concentration of C6:0 and C8:0 ($P < 0.05$) contrary to other study about tomato pomace in ewes diet by Razzaghi et al. (2015). The content of C16:0 decreased in TP group compared with CON group ($P < 0.001$), as shown by Romano et al. (2010).Total *trans* FA specifically,

C16:1*trans*-9, C18:1 *trans*-4-9, C18:1 *trans*-11(vaccenic acid, **VA**) and the concentration of CLA *cis*-9, *trans*-11(rumenic acid, **RA**) increased compared with CON ($P< 0.05$). An increase in RA with the inclusion of tomato by-product, has been observed also in dairy cows Cassinerio et al. (2015).

On the others hand, the TP supplementation decreases the concentration of LNA compared to CON even this by-product contains higher amounts of this FA compared with CON diet ($P< 0.01$). This suggests a higher rumen biohydrogenation of LNA in TP group compared to CON, as supported by a higher concentration of VA in milk. LNA, is a precursor of VA, produced by the ruminal metabolism, in accordance with other studies by Nudda et al. (2006, 2013b) and Mughetti et al. (2012) which observed an increase of concentration of VA in milk of dairy goats and sheep fed linseed.VA is the main precursor of RA, formed by the Δ9-desaturase in mammary gland (Griinari et al., 1999).

The GM supplementation decrease the concentration of C11, C12, *anteiso*C13, C14 and C14:1*cis*-9, C16:1 *cis*-9, C17, C17:1 *cis*-9 and an increase of C18:1*trans*-9, C18:1 *trans*-13+*trans*-14, C18:2 *n*-6 (linoleic acid, **LA**) and C20:2n-9 compared to CON group. Regarding the FA groups, the inclusion of GM, reduced the concentration of SFA and increased the unsaturated form, both MUFA and PUFA. The pattern of FA groups was in line with previous observation in the same breed by using grape seed (Correddu et al., 2016), even if the extent of the increase in PUFA, due to the dietary inclusion of GM, compared with CON (+ 13.3%) was very low. Among PUFA, the GM reduced the concentration of

thePUFA*n*-3 compared to CON group and increased noticeably the concentration of PUFA *n*-6, with a consequent increase of the *n*-6:*n*-3 ratio.

The increase of PUFA *n*-6 in milk of GM, mainly related to the increase of LA ($P < 0.05$), is likely due to the high amount of linoleic acid in this by-products. This is in agreement with the findings of Correddu et al. (2016) in sheep and Moate et al. (2014) and Santos et al. (2014), who showed increased levels of LA in milk fed residues from grapes. The concentration of PUFA *n*-3, which was lower in GM group compared to CON is likely due to the lack of effect of grape marc in reducing the extent of biohydrogenation of LNA, as suggested by the similarly low levels of LNA in CON and GM groups.

4. CONCLUSIONS

The study evidenced that dietary supplementation with EMB TP and GM improves milk antioxidant capacity. However, dietary inclusion of TP, in the dose of 100 g/head/day evidenced an oxidative stress in sheep organism as indicated by the significant higher values in the MDA content and GPX activity in their blood. The higher values in the MDA content which were also found in the blood plasma of sheep fed the two other by-products (EMB and GM) indicated that more research is needed in order to define the optimum inclusion level in sheep diet. The addition of all by-products increase the MUFA content in milk, and GM decrease the saturated FA and increase polyunsaturated FA without evident effects of individual.

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6. TABLES

Table 1. Ingredients and chemical composition of all diets and chemical composition, fatty acid (FA), total polyphenols of by-product

	By-products			Diet[1]			
Item	Exhausted myrtle berries	Tomato pomace	Grape marc	CON	EMB	TP	GM
Ingredient (kg/d per animal, as fed)							
TMR[2]				2.210	2.210	2.210	2.210
By-product mix							
Soybean[3]				0.050	0.050	0.050	0.050
Beet pulp[4]				0.200	0.200	0.200	0.200
Exhausted myrtle berries					0.075		
Tomato Pomace						0.100	
Grape Pomace							0.100
Total DM supplied				2.460	2.535	2.560	2.560
Chemical composition (% of DM unless otherwise noted)							
DM (%)	94.30	92.57	93.36	88.33	88.51	88.50	88.53
NDF	64.8	61.55	52.74	33.47	34.46	34.62	34.27
NFC	18.26	12.10	20.59	39.73	39.06	38.60	38.94
ADL	30.76	31.28	25.96	3.97	4.82	5.09	4.88
CP	7.76	15.69	11.08	17.42	17.12	17.35	17.16
Ash	3.75	4.43	8.68	7.55	7.43	7.43	7.60
Ether extract	5.43	6.23	6.91	1.82	1.93	2.00	2.02
Major FA (g / 100 g of total FA)							
C12:0	0.07	0.02	0.24				
C16:0	8.4	15.55	12.07				
C18:0	3.76	4.88	5.44				
C18:1c9	7.90	17.6	17.64				
C18:1c11	0.38	1.26	0.82				
C18:2n6	72.01	52.19	50.63				
C20:0	0.69	0.44	0.67				
C18:3n3	0.68	3.22	1.56				
Total polyphenols (g GAE/100g of DM)	4.09	0.23	1.48				

[1]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc

[2]TMR composition: pellet hay = 19.67%; soybean meal = 14.21%; flaked corn = 12.53%; medium chop straw = 11.80%; meal corn = 5.83%; Beet pulp = 4.44%; molasse = 3.64%; premix = 0.73%; medium chop hay = 9.05%; commercial pellet = 18.10%. Chemical composition: DM = 88.05% ; NDF = 32.39% of DM; NFC = 40.74% of DM; ADL =3.86% of DM; CP = 17.38% of DM; Ash = 7.54% of DM; Ether extract = 1.94% of DM.

[3]Soybean, chemical composition: DM = 89.56%; NDF = 18.59 % of DM; NFC = 21.11% of DM; ADL = 0.44% of DM; CP = 52.14% of DM; Ash = 7.06% of DM; Ether extract = 1.10% of DM. [4]Beet pulp, chemical composition: DM = 90.59%; NDF = 48.79% of DM; NFC = 33.48% of DM; ADL =6.09% of DM; CP = 9.32% of DM; Ash = 7.82% of DM; Ether extract = 0.60% of DM.

Table 2. Total antioxidant capacity, enzyme activities, malondialdehyde, and protein carbonylis content from ewes blood plasma on each treatment

	Diet[1]					*P-value*[2]		
	CON	EMB	TP	GM	SEM	D	S	D x S
FRAP[3], µmol ascorbic acid	0.860 [b]	0.946 [ab]	0.956 [ab]	1.038 [a]	0.024	*	**	NS
ABTS[4], % inhibition	31.381 [b]	32.867 [ab]	33.694 [a]	33.642 [a]	0.381	**	***	NS
GST[5], Units/ml	0.234	0.252	0.229	0.236	0.005	NS	NS	NS
GR[6], Units/ml	0.050	0.054	0.053	0.051	0.001	NS	NS	NS
GPx[7], Units/ml	0.061 [b]	0.063 [b]	0.077 [a]	0.061 [b]	0.002	**	NS	*
SOD[8], Units/ml	13.339	13.745	13.312	12.408	0.215	NS	NS	NS
MDA[9], µM	0.476 [b]	0.502 [b]	0.594 [a]	0.536 [ab]	0.012	**	NS	NS
PC[10], nmol/ml	7.403	7.577	7.449	7.444	0.065	NS	NS	NS

[a–b]Means within a row with different superscripts are different (P < 0.05).
[1] CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.
[2] D = effect of diet; S = effect of sampling; NS indicates P > 0.10.
†P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.
[3] FRAP: Ferric Reducing Ability of Plasma
[4] ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulforic acid)
[5] GST: Glutathione transferase
[6] GR: Glutathione reductase
[7] GPx: Glutathione peroxidase
[8] SOD: Superoxide Dismutase
[9] MDA: Malondialdehyde
[10] PC: Protein carbonyls

Table 3. Total antioxidant capacity, enzyme activities, malondialdehyde, and protein carbonylis content from ewes milk on each treatment

	Diet[1]					*P-value*[2]		
	CON	EMB	TP	GM	SEM	D	S	D x S
FRAP[3], µmol ascorbic acid	2.750	2.929	2.830	2.778	0.08	NS	***	NS
ABTS[4], % inhibition	50.719	52.319	51.087	47.912	1.12	NS	***	NS
LPO[5], Units/ml	0.410 ^{ab}	0.317 ^{b}	0.465 ^{ab}	0.600 ^{a}	0.03	*	**	NS
GR[6], Units/ml	0.089 ^{b}	0.081 ^{b}	0.154 ^{a}	0.075 ^{b}	0.01	***	NS	NS
SOD[7], Units/ml	89.070	85.678	82.176	82.606	1.92	NS	**	NS
MDA[8], µM	0.513 ^{a}	0.471 ^{ab}	0.467 ^{ab}	0.432 ^{b}	0.01	**	***	NS
PC[9], nmol/ml	6.474 ^{a}	5.851 ^{b}	5.674 ^{b}	5.289 ^{b}	0.09	***	NS	NS

$^{a–b}$Means within a row with different superscripts are different (P < 0.05).
[1] CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.
[2] D = effect of diet; S = effect of sampling; NS indicates P > 0.10.
†P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.
[3] FRAP: Ferric Reducing Ability of Plasma
[4] ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulforic acid)
[5] LPO: Lactoperoxidase
[6] GR: Glutathione reductase
[7] SOD: Superoxide Dismutase
[8] MDA: Malondialdehyde
[9] PC: Protein carbonyls

Table 4. Fatty acid profile of milk from ewes fed with experimental diets

Fatty acid (g/100 g of FAME)[1]	Diet[2]				SEM	*P-value*[3]		
	CON	EMB	TP	GM		D	S	D x S
C4:0	1.269	1.296	1.389	1.393	0.0190	NS	**	NS
C6:0	1.615 ^{b}	1.618 ^{b}	1.739 ^{a}	1.636 ^{ab}	0.0181	*	***	NS
C7:0	0.019	0.021	0.022	0.019	0.0006	†	***	NS
C8:0	2.025 ^{b}	2.016 ^{b}	2.206 ^{a}	1.957 ^{b}	0.0259	***	***	NS
C9:0	0.085	0.089	0.077	0.071	0.0032	†	***	NS
C10:0	7.952	7.963	8.479	7.415	0.1366	†	**	NS
C10:1	0.041	0.035	0.037	0.038	0.0013	NS	NS	NS
C11:0	0.480 ^{ab}	0.518 ^{a}	0.438 ^{bc}	0.427 ^{c}	0.0075	***	NS	NS
C12:0	5.619 ^{a}	5.449 ^{ab}	5.531 ^{ab}	4.815 ^{b}	0.0998	*	NS	NS
iso C13:0	0.014	0.017	0.014	0.014	0.0006	NS	*	NS
anteiso C13:0	0.111 ^{ab}	0.116 ^{a}	0.096 ^{bc}	0.089 ^{c}	0.0025	***	NS	NS
iso C14:0	0.103	0.113	0.100	0.112	0.0025	NS	NS	NS
C14:0	13.806 ^{a}	13.627 ^{ab}	13.678 ^{ab}	13.105 ^{b}	0.0955	*	NS	NS
C14:1*cis*-9	0.535 ^{a}	0.564 ^{a}	0.444 ^{b}	0.452 ^{b}	0.0120	***	***	NS
iso C15:0	0.238	0.255	0.231	0.232	0.0060	NS	NS	NS
anteiso C15:0	0.412	0.457	0.434	0.425	0.0072	NS	NS	NS
C15:0	1.091 ^{ab}	1.141 ^{a}	1.000 ^{b}	1.036 ^{ab}	0.0179	*	NS	NS
C15:1	0.125 ^{ab}	0.104 ^{c}	0.122 ^{b}	0.137 ^{a}	0.0022	***	NS	NS
iso C16:0	0.329	0.321	0.317	0.346	0.0053	NS	NS	NS
C16:0	29.043 ^{a}	27.673 ^{bc}	26.534 ^{c}	28.388 ^{ab}	0.1881	***	NS	NS
C16:1 *trans*-6 + *trans*-7	0.074 ^{ab}	0.065 ^{b}	0.080 ^{a}	0.078 ^{a}	0.0012	***	NS	NS
C16:1 *trans*-9	0.143 ^{b}	0.124 ^{b}	0.180 ^{a}	0.153 ^{b}	0.0048	***	NS	NS
C16:1 trans-10	0.022	0.020	0.023	0.022	0.0004	NS	NS	NS
C16:1 *cis* 7	0.266	0.262	0.265	0.250	0.0033	NS	NS	NS
C16:1 *cis* 9	1.715 ^{a}	1.724 ^{a}	1.281 ^{b}	1.447 ^{b}	0.0375	***	NS	NS
C16:1 *cis* 10	0.038	0.032	0.034	0.037	0.0009	NS	NS	NS
iso C17:0	0.409	0.425	0.431	0.427	0.0060	NS	NS	NS
anteiso C17:0	0.448	0.482	0.495	0.457	0.0075	NS	NS	NS
C17:0	0.792 ^{a}	0.810 ^{a}	0.718 ^{b}	0.718 ^{b}	0.0092	***	NS	NS
C17:1 *cis*-9	0.249 ^{ab}	0.267 ^{a}	0.216 ^{c}	0.231 ^{bc}	0.0046	***	NS	NS
C18:0 (SA)	4.809	5.198	5.477	5.273	0.1021	NS	NS	NS
C18:1 *trans*-4	0.017 ^{c}	0.017 ^{bc}	0.020 ^{ab}	0.020 ^{a}	0.0004	**	NS	NS
C18:1 *trans*-6+ *trans*-8	0.347 ^{bc}	0.314 ^{c}	0.439 ^{a}	0.409 ^{ab}	0.0103	***	NS	NS

Table 4. (Continued)

Fatty acid (g/100 g of FAME)[1]	Diet[2]					*P-value*[3]		
	CON	EMB	TP	GM	SEM	D	S	D x S
C18:1 *trans*-9	0.317 ^{b}	0.294 ^{b}	0.387 ^{a}	0.371 ^{a}	0.0075	***	NS	NS
C18:1 *trans*-10	1.022	1.095	1.321	1.109	0.0769	NS	NS	NS
C18:1 *trans*-11 (VA)	1.518 ^{bc}	1.304 ^{c}	1.932 ^{a}	1.702 ^{ab}	0.0503	***	NS	NS
C18:1 *trans*-13 + *trans*-14	0.451 ^{ab}	0.429 ^{b}	0.496 ^{a}	0.495 ^{a}	0.0074	**	NS	NS
C18:1 *cis*-9	13.766	15.048	14.439	14.995	0.2016	†	NS	NS
C18:1 *cis*-11	0.445	0.434	0.444	0.447	0.0059	NS	NS	NS
C18:1 *cis*-12	0.430 ^{ab}	0.385 ^{b}	0.363 ^{b}	0.482 ^{a}	0.0102	***	NS	NS
C18:1 *cis*- 13	0.074	0.070	0.075	0.079	0.0010	†	**	NS
C18:1 *trans*-16 + *cis*-14	0.179	0.203	0.184	0.208	0.0052	NS	NS	NS
C18:2 *trans*-9, *trans*-12	0.019	0.018	0.017	0.017	0.0005	NS	NS	NS
C18:2 *trans*-8, *cis*-13	0.110 ^{ab}	0.125 ^{a}	0.107 ^{b}	0.125 ^{a}	0.0024	**	*	NS
C18:2 *cis*-9, *trans*-12	0.105 ^{b}	0.112 ^{ab}	0.106 ^{ab}	0.116 ^{a}	0.0015	*	NS	NS
C18:2 *trans*-9, *cis*-12	0.032 ^{ab}	0.035 ^{ab}	0.032 ^{b}	0.036 ^{a}	0.0006	*	**	NS
C18:2 n-6 (LA)	3.001 ^{b}	3.120 ^{b}	3.071 ^{b}	3.621 ^{a}	0.0503	***	NS	NS
C18:3 n-6	0.115	0.126	0.109	0.119	0.0029	NS	NS	NS
C18:3 n-3 (LNA)	0.304 ^{a}	0.254 ^{b}	0.260 ^{b}	0.301 ^{a}	0.0046	***	NS	NS
CLA *cis*-9, *trans*-11 (RA)	1.147 ^{b}	1.075 ^{b}	1.346 ^{a}	1.312 ^{ab}	0.0305	*	NS	NS
C18:4 n-3	0.008	0.008	0.008	0.008	0.0002	NS	***	NS
C20:0	0.171	0.183	0.177	0.189	0.0026	†	NS	NS
CLA *trans*-9, *cis*-11 +C21:0	0.080 ^{ab}	0.074 ^{b}	0.078 ^{ab}	0.084 ^{a}	0.0010	**	**	NS
CLA *trans*-10, *cis*-12	0.016	0.015	0.017	0.016	0.0004	NS	**	NS
CLA *trans*-11, *trans*-13	0.016	0.017	0.014	0.013	0.0007	†	NS	NS
CLA *trans*-9, *trans*-11	0.026	0.024	0.022	0.025	0.0006	NS	***	NS
CLA *trans*-12, *trans*-14	0.010	0.011	0.011	0.010	0.0003	NS	†	NS
C20:2 n-9	0.022 ^{b}	0.025 ^{a}	0.026 ^{a}	0.026 ^{a}	0.0004	***	NS	NS
C20:2 n-6	0.045	0.045	0.050	0.048	0.0007	NS	**	NS
C20:3 n-9	0.040 ^{ab}	0.038 ^{b}	0.043 ^{a}	0.036 ^{b}	0.0009	**	NS	NS
C20:3n-6	0.039	0.040	0.041	0.039	0.0006	NS	NS	NS
C20:4 n-6	0.205 ^{ab}	0.207 ^{a}	0.206 ^{ab}	0.189 ^{b}	0.0030	*	*	NS
C20:3 n-3	0.008	0.007	0.008	0.008	0.0002	NS	NS	NS
C22:0	0.103	0.098	0.099	0.109	0.0015	†	NS	NS
C20:4 n-3	0.009	0.010	0.010	0.009	0.0003	NS	NS	NS
C22:1n-9	0.020	0.018	0.019	0.019	0.0003	NS	NS	NS
C22:5 n-3 (EPA)	0.028	0.027	0.028	0.026	0.0004	NS	NS	NS

Table 4. (Continued)

Fatty acid (g/100 g of FAME)[1]	Diet[2]					*P-value*[3]		
	CON	EMB	TP	GM	SEM	D	S	D x S
C22:2 n-6	0.037	0.035	0.037	0.038	0.0007	NS	*	NS
C22:4 n-6	0.035	0.034	0.036	0.032	0.0007	NS	***	NS
C24:0	0.035	0.032	0.033	0.035	0.0007	NS	NS	NS
C22:5 n-3 (DPA)	0.060 ^{ab}	0.061 ^{ab}	0.061 ^{a}	0.055 ^{b}	0.0008	*	*	NS
C22:6 n-3 (DHA)	0.022 ^{ab}	0.023 ^{a}	0.018 ^{c}	0.018 ^{bc}	0.0005	***	***	NS
Groups								
SFA	71.239 ^{a}	70.190 ^{ab}	69.991 ^{ab}	68.964 ^{b}	0.2382	**	NS	NS
UFA	28.856 ^{b}	29.918 ^{ab}	30.099 ^{ab}	31.136 ^{a}	0.2380	**	NS	NS
MUFA	22.721 ^{b}	23.734 ^{a}	23.750 ^{a}	24.184 ^{a}	0.1999	*	†	NS
PUFA	6.135 ^{b}	6.184 ^{b}	6.349 ^{b}	6.951 ^{a}	0.0721	***	NS	NS
TFA	5.234 ^{b}	5.042 ^{b}	6.218 ^{a}	5.810 ^{ab}	0.1217	**	NS	NS
OBCFA	4.585 ^{ab}	4.831 ^{a}	4.444 ^{b}	4.432 ^{b}	0.0432	**	NS	NS
SCFA	13.007 ^{ab}	13.039 ^{ab}	13.949 ^{a}	12.531 ^{b}	0.1627	**	***	NS
MCFA	56.403 ^{a}	54.909 ^{ab}	53.003 ^{c}	53.734 ^{bc}	0.2556	***	NS	NS
LCFA	30.590 ^{b}	32.052 ^{ab}	33.048 ^{a}	33.735 ^{a}	0.2856	***	NS	NS
PUFA n-3	0.4379 ^{a}	0.391 ^{b}	0.393 ^{b}	0.427 ^{b}	0.0049	***	*	NS
PUFA n-6	3.487 ^{b}	3.617 ^{b}	3.559 ^{b}	4.097 ^{a}	0.0524	***	NS	NS
n6:n3	8.014 ^{b}	9.277 ^{a}	9.099 ^{a}	9.677 ^{a}	0.1276	***	**	NS
Total CLA	1.295 ^{ab}	1.217 ^{b}	1.487 ^{a}	1.459 ^{ab}	0.0308	*	NS	NS

$^{a–c}$Means within a row with different superscripts are different ($P < 0.05$).

[1] SA = stearic acid; VA = vaccenic acid; LA = linoleic acid; LNA = linolenic acid; RA = rumenic acid; EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid; SFA = sum of the individual saturated fatty acids reported in this table; UFA = sum of the individual unsaturated fatty acids reported in this table; MUFA = sum of the individual monounsaturated fatty acids reported in this table; PUFA = sum of the individual polyunsaturated fatty acids reported in this table; TFA = trans fatty acids, sum of the individual trans fatty acids reported in this table, except CLA isomers; BCFA = branched-chain fatty acids, sum of iso- and anteiso-FA reported in this table; OBCFA = odd- and branched-chain fatty acids, sum of odd-, iso-, and anteiso-FA reported in this table; SCFA = short-chain fatty acids, sum of the individual fatty acids from C4:0 to C10:0 reported in this table; MCFA = medium-chain fatty acids, sum of the individual fatty acids from C11:0 to C17:0 reported in this table; LCFA = long-chain fatty acids, sum of the individual fatty acids from C18:0 to DHA reported in this table; PUFA n-3 and PUFA n-6 = sum of individual n-3 and n-6 fatty acids, respectively, reported in this table; Total CLA = sum of individual conjugated linoleic acids reported in this table.

[2] Diet: CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.

[3] D = effect of diet; S = effect of sampling; NS indicates $P > 0.10$.

†$P< 0.10$, *$P< 0.05$, **$P< 0.01$, ***$P< 0.001$.

77 **Table 5.**Nutritionalindices of Fatty Acids
78

	Diet[2]				SEM	*P-value*[3]		
	CON	EMB	TP	GM		D	S	D x S
Item[1]								
AI	3.13 [a]	2.97 [ab]	2.91 [ab]	2.77 [b]	0.037	**	NS	NS
TI	2.96 [a]	2.79 [b]	2.71 [b]	2.69 [b]	0.030	**	**	NS
h:H	0.42 [b]	0.47 [a]	0.47 [a]	0.48 [a]	0.006	**	NS	NS
Δ^9 - desaturase indices								
C10 index	0.49	0.44	0.43	0.52	0.016	NS	NS	*
C14 index	3.72 [ab]	3.96 [a]	3.13 [c]	3.33 [bc]	0.075	***	***	NS
C16 index	5.54 [a]	5.86 [a]	4.61 [b]	4.82 [b]	0.109	***	***	NS
C18 index	74.30 [ab]	74.65 [a]	72.43 [b]	73.99 [ab]	0.309	*	†	NS
CLA cis-9,trans-11 index	43.37 [ab]	45.70 [a]	41.45 [b]	43.75 [ab]	0.417	**	**	NS
Total index	23.13 [b]	24.80 [a]	23.80 [ab]	24.60 [ab]	0.248	*	*	NS

79 [1] AI = atherogenic index; TI = thrombogenic index; h:H = hypocholesterolemic to
80 hypercholesterolemic ratio;
81 [2] Diet: CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP
82 = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of
83 grape marc.
84 [3] D = effect of diet; S = effect of sampling; NS indicates P > 0.10.
85 †P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.
86

7. FIGURE

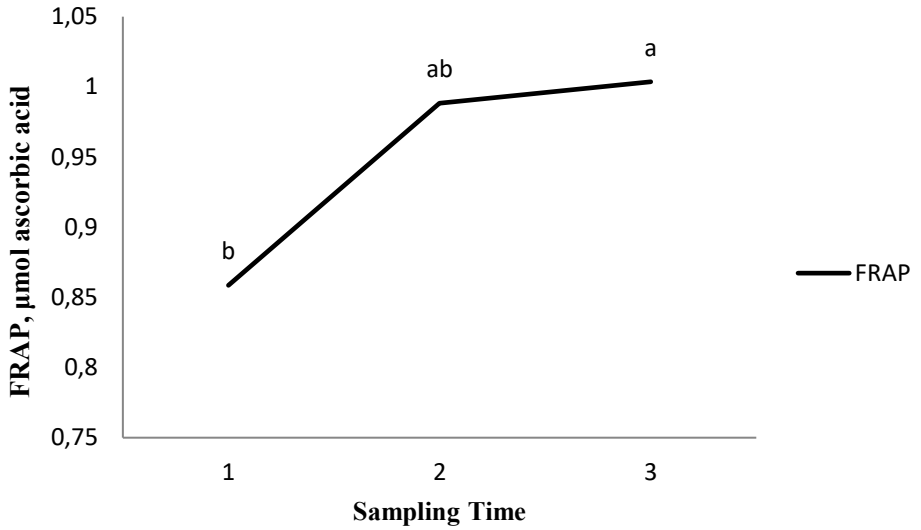


FIGURE 1. Temporal evolution of FRAP (Ferric Reducing Ability of Plasma) in blood plasma during the trial. Different letters (a, b, c) show statistical differences ($P < 0.05$).

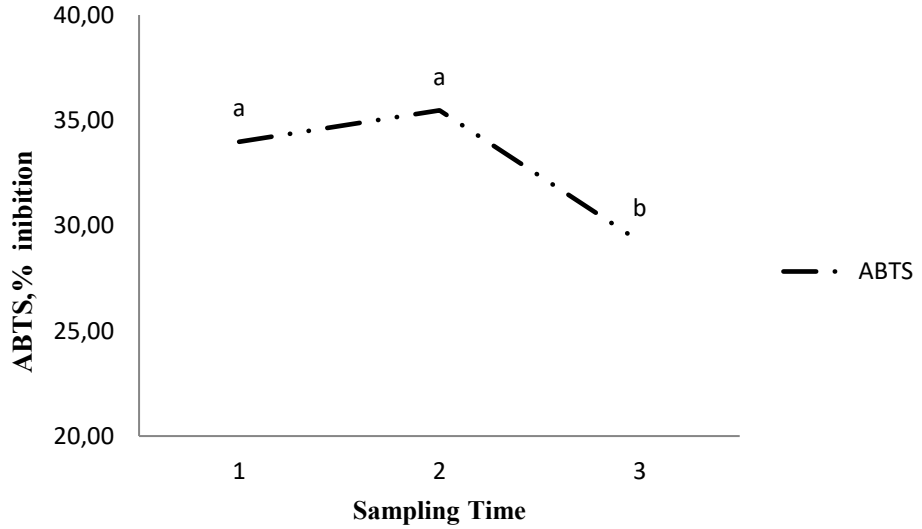


FIGURE 2. Temporal evolution of ABTS: 2,2'-azino-di (3-ethylbenzthiazoline-6-sulforic acid) in blood plasma during the trial. Different letters (a, b, c) show statistical differences ($P < 0.05$).

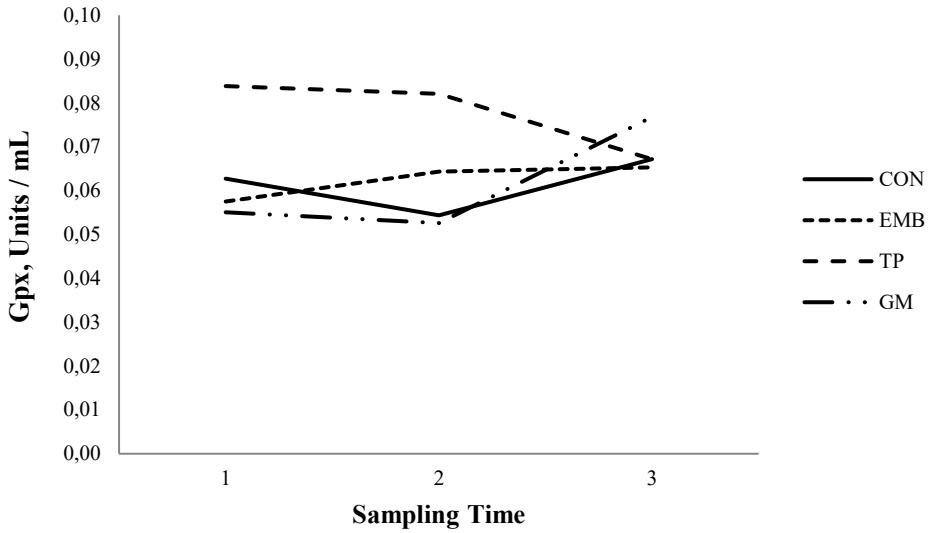
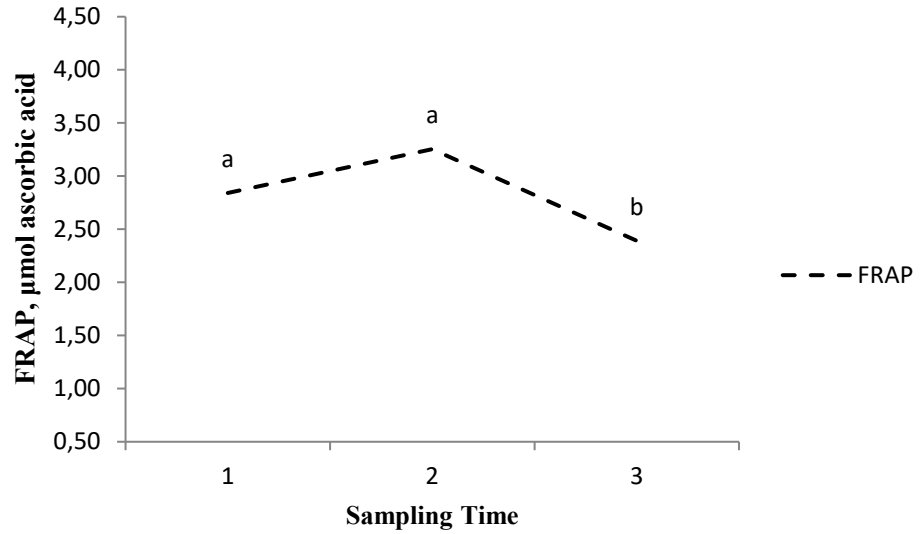
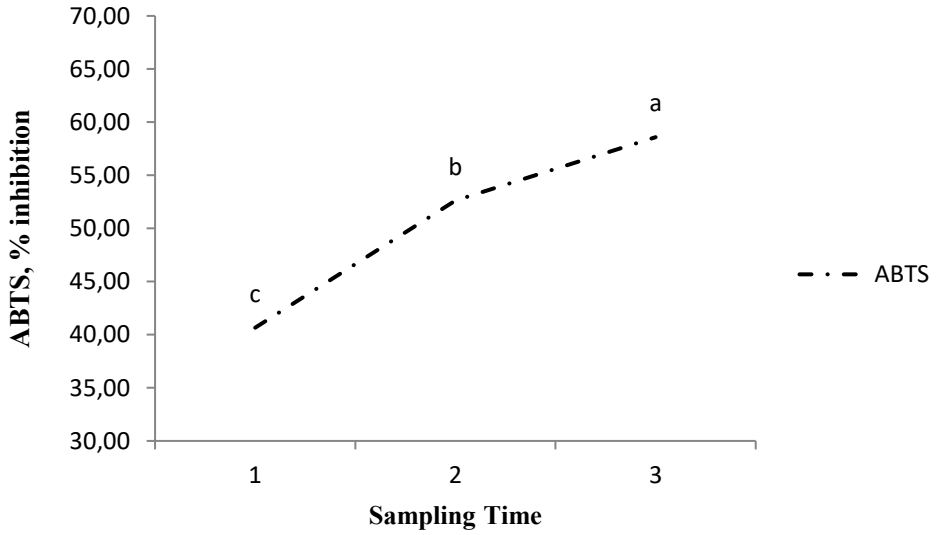


FIGURE 3. Temporal evolution of the GPx (Glutathione peroxidase) in blood plasma on three sampling.

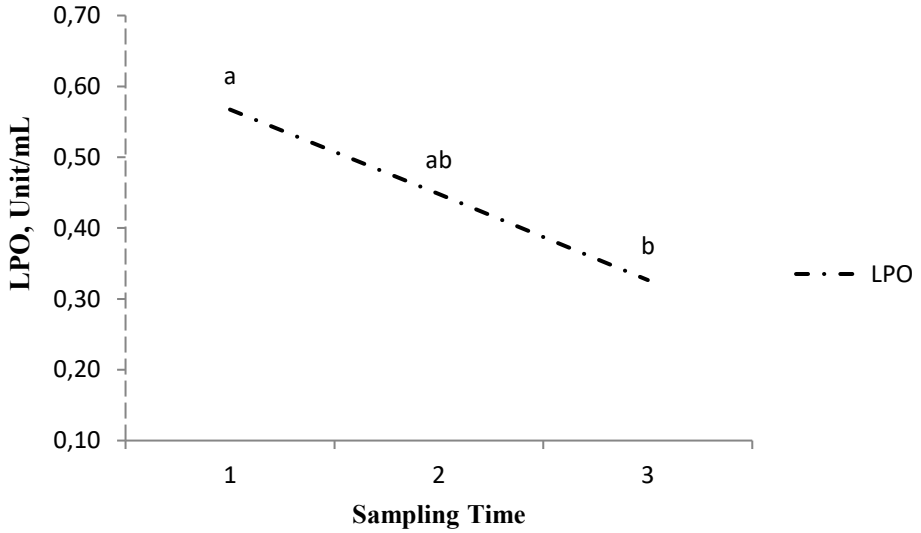


104
105 **FIGURE 4.**Temporal evolution of FRAP(Ferric Reducing Ability of Plasma) in
106 milk during the trial. Different letters (a, b, c) show statistical differences (P <
107 0.05).
108
109



110
111 **FIGURE 5.**Temporal evolution of ABTS: 2,2'-azino-di (3-ethylbenzthiazoline-6-
112 sulforic acid) in milk during the trial. Different letters (a, b, c) show statistical
113 differences (P < 0.05).
114

115



116
117 **FIGURE 6.**Temporal evolution of LPO (lactoperoxidase) in milk during the trial.
118 Different letters (a, b, c) show statistical differences (P < 0.05).
119
120
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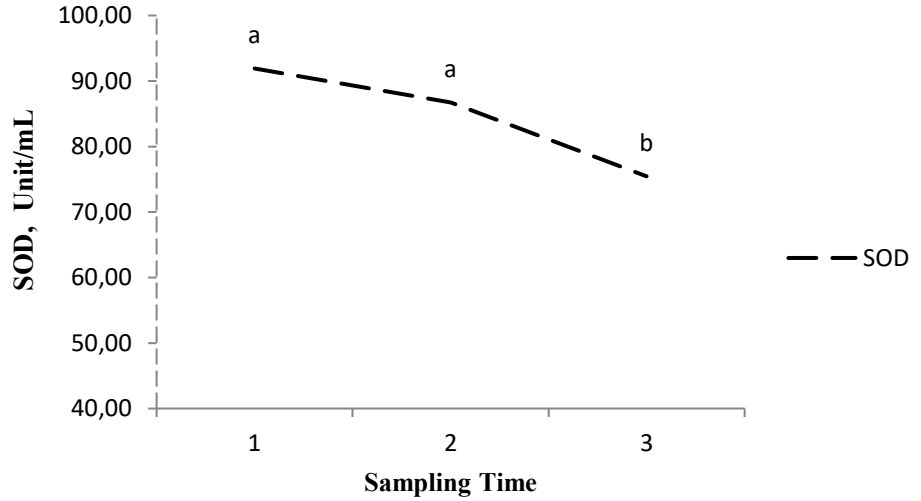
122



123
124 **FIGURE 7.**Temporal evolution of SOD (superoxide dismutase) in milk during
125 the trial. Different letters (a, b, c) show statistical differences (P < 0.05).
126

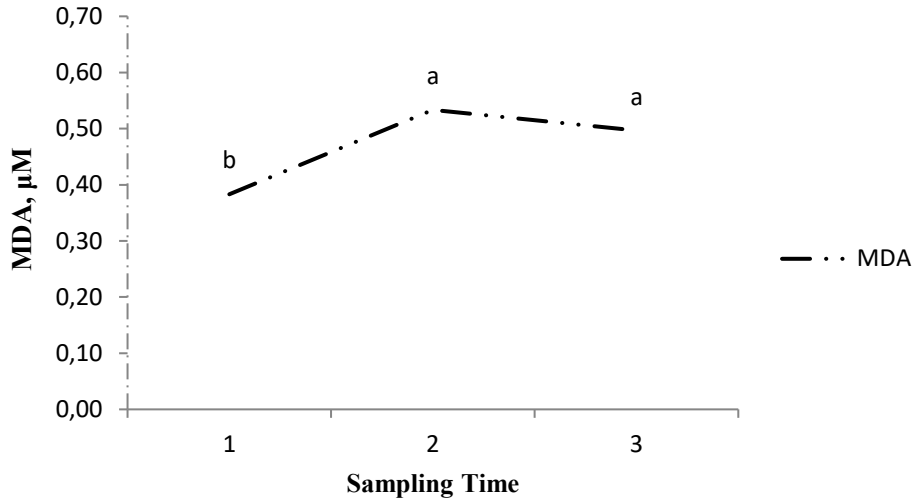


FIGURE 8. Temporal evolution of MDA (malondialdehyde) in milk during the trial. Different letters (a, b, c) show statistical differences ($P < 0.05$).

CHAPTER 4

Effects of supplementation with agro-industrial byproducts from tomato, myrtus and grape on rumen function of dairy sheep

ABSTRACT

Aim of the study was to evaluate if the use ofby-products containing bioactive compound, in particular polyphenols, in ruminant diet could modulate rumen fermentation parameters and rumen microbiota in dairy ewes. Thirtysix eweswere assigned to four dietary treatments consisting of: a control diet (CON), a basal diet supplemented with 75 g/d per animal of exhausted myrtle berries (EMB), a basal diet supplemented with 100 g/d per animal of tomato pomace (TP) and a basal diet supplemented with 100 g/d per animal of grape marc (GM). The supplementation with these by products did not affectthe rumen pH content compared to CON group. The interaction diet x sampling time was significative for NH_3 content. The total volatile fatty acids (VFA), the molar proportions of acetate, propionate, butyrate and the ratio acetate:propionate were not affected by the diet. Diets contain EMB, TP and GM did not influence the estimated production of methane compared to CON group.Sampling time affected almost all the rumen fermentation parameters, except acetate concentration. The abundances of the ruminal bacterial population were affected by sampling time.

Supplementation of EMB resulted in higher abudance of *Succinivibrionaceae* and the differences were also detected for the *Veillonellaceae* family. Furthermore, the *Paraprevotellaceae* and *Prevotellaceae* families dominated the phylum of *Bacteroidetes* in EMB group.Not differences in the proportion of any phylum have been detected in GM group compared to CON group.Results of TP compared to CON group, evidenced the higher abundance of *Proteobacteria* phylum and this phylum was dominated by *Acetobacteraceae* family with the genus *Acetobacter*.The estimated methane emission and rumen fermentation parameters did not evidenced any effects of by-produts.In conclusion, the supplementation of by-products in the diets of dairy sheep in doses used in this trial did not cause consistent variations in the structure of rumen microbiota and in rumen fermentation parameters.

1. INTRODUCTION

The industry of food production generatesa large quantity of food waste (Kasapidou et al., 2015). Some of the more common agro-industrial byproducts available in large quantity are the by-product of winemaking (grape marc, **GM**) composed of skin, pulp and seeds (Spanghero et al., 2009) and by-product of tomato industry (tomato pomace, **TP**) composed of peels and seed (Fondevila et al., 1994).Another by-product, considered a special waste with costly disposal is a by-products of liqueur Myrtle process (Exhausted myrtle berries, **EMB**) (Nudda et al., 2017). The most known and easiest system to recycle the agro-industrial wastes is to use them in animal nutrition(Laufenberg et al. 2003;Wyman, 2003). These food waste are interesting for their content of bioactive compound, in fact GM and EMB are rich in polyphenols (Chedea et al., 2017 ; Nudda et al., 2017), while TP is rich in lycopene and polyphenols (Kalogeropoulos et al., 2012). In ruminant diets polyphenols may affect the ruminal microbial populations, inducing shifts in the bacteria, fungi and protozoa, (Patra and Saxena, 2009), causing relevant changes in ruminal metabolism of nutrients (Patra and Saxena, 2011; Buccioni et al., 2012). Polyphenols, may decrease of methanogenesis (Dschaak et al., 2011; Liu et al., 2011) and protein degradation in rumen and an increase of protein production and protein flow to the duodenum targeting specific groups of rumen microbial populations. (Patra and Saxena, 2011). Furthermorethe polyphenols in ruminant diets have effects on reduction of ammonia production (Bhatta et al., 2009; Theodoridou et al., 2010; Ishida et al., 2015).

Tannins are able to interfere with membrane functions of rumen bacteria, binding enzymes or by the privation of iron (Patra and Saxena, 2011). Vasta et al., (2010) showed that *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* could be the most sensitive bacterial species involved in biohydrogenation process of polyunsatured fatty acids (**PUFA**). Studies about *in vivo* and *in vitro* experiments of the tannineffects on the biohydrogenation showed confliting results; the causes of these results could be the differences between tannins species, the quantity of tannins in association with other ingredients in the diet (Buccioni et al., 2015). In fact, the condensed tannins are not usually toxic to ruminants since they are not absorbed (Reed, 1995), but they may bind several nutrients irreversibly, making them unavailable to the animal. Also the condensed tannins can bind to gastrointestinal tract, causing adverse effects (Makkar et al., 2007). While the hydrolysable tannins may cause toxicity responses to ruminants when consumed at excessive amounts, by they provide beneficial effects when used at low to moderate concentrations (Reed, 1995).

For ruminant nutrition, developing feeding strategies to minimize methane emissions and N excretion is desirable both for conserving the environment as well as for increasing the efficiency of energy and protein utilization. For these reason we carried out an *in vivo*study to evaluate if supplementation to lactating ewes of GM, TP and EMB, by-products rich in bioactive compound, could affects rumen fermentation parameters and ruminal microbiota population.

2. MATERIAL AND METHODS

2.1 Animals and Diets

Thirty-six Sarda dairy sheep were assigned to four experimental groups: nine animals per group. The groups were homogenous for milk yield (1720 ±430 g/d), body weight (45.5 ±4.83 kg), body condition score (BCS, 2.77±0.11), parity (4.2±0.25) and DIM (120±10 days).One group was fed TMR only, control group (CON); the second group was fed TMR with a supplement of ground exhausted myrtle berries (EMB group), whereas the third group was fed a supplement of ground dry tomato pomace to the TMR (TP group); and the fourth group was fed a supplement of ground grape marc to the TMR (GM group). Chemical composition of the three by-products EMB, TP and GM, respectively, was DM 94.30, 92.57 and 93.36%; NDF 64.8, 61.55 and 52.74% of DM, ADF: 51.73, 50.66 and 38.85 % of DM, CP: 7.76, 15.69 and 11.08% of DM, Ash: 3.75, 4.43 and 8.68% of DM and Ether extrac was 5.43, 6.23 and 6.91% of DM.

The TMR formulated to meet energy and protein requirements of the ewes calculated by the Small Ruminant Nutrition Model (Tedeschi et al., 2010).

The chemical composition of the diets and of the by-products, the FA and phenol composition of by-products used in this experiment are reported in chapter 1 (Table 1 and 2).

2.2 Sampling and Analysis

Rumen liquid sample collection. Individual sample of rumen was collected and day30 and 45 of the experiment. Rumen content was sampled 2 hours after morning feeding (by-product mix), for the sampling was used a stomach tube and an evacuation pump. To reduce saliva contamination the first 30 mL of the rumen fluid was discarded. The sample of the rumen fluid was immediately filtered through a sterile dressing. After sampling the rumen liquid pH was immediately recorded with a pH meter (Orion 250A, Orion Research Inc., Boston, MA, USA) and using a glass electrode (model 238405, Hamilton Company, Reno, NV, USA).Individual rumen liquid was divided into 3 tubes: 2 tubes (10 mL each), with 0.2 H_2SO_4 (50%) to acidifies the sample, were used to ammonia and volatile fatty acids (VFA) analysis. Another aliquot (about 250 μL) of the rumen liquid was collectedinto a stool stabilizer tube includedin the extraction Kit (PSP® Spin Stool DNA Kit) for subsequent DNA analysis.

Ammonia content and volatile fatty acids determination in rumen liquor. $NH_3$and VFA content in rumen liquor was determined in according to Correddu et al., (2015). Briefly, ammonia content was determined with colorimetric method, according to Chaney and Marbach (1962) with one modification: the use of salicylate instead of phenol and using a UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA, USA).

For the analysis of VFA content in rumen fluid was used a high-performance liquid chromatography (HPLC) method. An aliquot of sample (about 2 mL) was defrozen and then centrifuged at 4°C for 10 min x 15,000 g. The surnatant was it drawn by syringe and, after filtration (PTFE 0.45 m, 13 mm), injected into a HPLC system (Varian Inc., Palo Alto, California, USA). The HPLC was provided with an auto sampler (Varian 9300), a degasser (Varian 9012 Q), a UV detector (Varian 906P Polychrom) and an Aminex HPX 87H column (Biorad Laboratories, Hercules, CA, USA). The column was operated at 55 °C with 0.008N H_2SO_4 at 0.6 mL/min as eluent.Concentrations of VFA were estimated by comparison with a calibration curve obtained by injecting 5 L of five standard solutions: for the acetic acid 5.6, 11.25, 22.5, 45 and 90 mmol/L; for the propionic (C3:0) and butyric (C4:0) acid 5, 10, 20, 40 and 80 mmol/L. The standard solution obtained by appropriate dilutions of a standard mixture of VFA (5.40, 5.76 and 7.02 mg/mL of acetic, propionic and butyric acids, respectively, in H_2SO_4 0.1N).

Estimation of methane emission. The methane production was estimated according to the equation of Moss et al. (2000). CH_4production (mol/mol of VFA) = $0.45 \times C_2 - 0.275 \times C_3 + 0.4 \times C_4$.

Where C_2, C_3 and C_4 are acetate, proprionate and butyrate concentration, respectively, expressed as mol/100mol of VFA.

2.3 Microbial analysis

DNA extraction. Total DNA was extracted from approximately 250 µL of rumen liquid with PSP® Spin Stool DNA kit, with some modification. Briefly, 1.4 mL of stabilized rumen sample was transferred into a 2-ml safe look tube, incubated for 10 min at 95°C on a thermomixer at 900 rpm; 5 zirconia beads were added to the homogenate, and then the samples were centrifuged at 11,000 x g for 1 min at room temperature. The supernatant was transferred into InviAdsorb-Tube and vortex vigorously (15 sec.). The suspension was incubated for 1 min at room temperature, and centrifuged at 14,000 x g, for 3 min at room temperature. The supernatant was transferred completely into a new tube and discarded the pellet, after centrifuged the sample at 14,000 x g for 3 min at room temperature. The supernatant was transferred in a new tube contain proteinase K (25 µL) and incubated at 70° C for 10 min in the thermomixer under continuous shaking at 900 rpm. The binding of the DNA was obtained by adding 200 µL of binding buffer to the lysate and immediately vortex. The whole mixture was transferred to the RTA-spin filter and, after incubation for 1 min at room temperature it was centrifuged at 11,000 x g for 2 min. The filtrate and the tube was discarded and the RTA Spin Filter was put in a new tube and washed two time with the buffer solutions prepared previously with kit tools. To eliminate any traces of ethanol the tube was centrifuged again and then the tube was discarded. The RTA spin filter was placed in a new tube and added with elution buffer (preheated to 70°C) and

centrifuged (11,000 x g for 1 min) for two times. The tube with filter (RTA-Spin filter) DNA was discarded and the total DNA was stored at -20° C.

Identification and Quantification of microbial population. The analyses were carried out at CBM S.c.r.l. laboratory (Trieste, Italy). The 16S rRNA Metagenomic Sequencing Library was prepared following the manufacturer's protocol (Illumina Inc., San Diego, CA, USA). Briefly, samples were amplified in the V3 and V4 regions using denaturated primers (Klindworth et al. 2013) in a limited cycle PCR, followed by an AMPure XP bead clean-up (A63880l; Beckman Coulter Inc., Brea, CA, USA). A second PCR reaction was then performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index Kit; followed by a final AMPure XP bead clean-up. Final library concentration was measured by fluorimetric quantification using Qubit 2 (Invitrogen Inc., Carlsbad, CA, USA) and each library was validated using a 1:50 dilution of the library on a Bioanalyzer DNA 1000 chip to verify sample size. Calculate DNA concentration in nM, based on the size of DNA amplicons, was used to pool the obtained 72 libraries in equimolar concentration. Pooled library was sequenced using the Illumina Miseq technology in 2x300bp run with 5% of PhiX library as control. The sequences of raw data were filtered out and the reads were trimmed to a consistent length. Then the data was denoised, chimera filtered, and taxonomically assigned using DADA2 v1.1.5 (Callahan et al. 2016). For the taxonomic analysis, the sequencing reads were clustered into operational taxonomic units (OTUs) defined as groups of sequencing reads that differ by less

than a fixed dissimilarity threshold (97%) generated in DADA2 using the Greengeenes database v13.5 (McDonald et al. 2012).

2.4 Statistical analysis

Rumen parameters.Data of rumen parameters (AGV, pH, NH3 and estimated methane production) were analyzed with the PROC MIXED procedure of SAS (2002). The model included: dietary treatment (CON, TP, EMB and GM), sampling date (S1 and S2) and theinteraction treatment × sampling,as fixed effects, and the animal nested within the treatment as random effect. Means were separated using Tukey test and significance was declared at $P < 0.05$, whereas $0.05 < P < 0.10$ was considered as a tendency.

Microbial analysis. Microbial data were analyzedusing the software packages from the open-source Bioconductor project (Callahan et al., 2016).All data was collected into a phyloseq (McMurdie and Holmes 2013) object and used for the further exploratory analysis. Briefly alpha diversity (within-sample diversity) was assessed using Simpson and Shannon metrics on inverse Simpson diversity index (Simpson, 1949; Hill, 1973) and Shannon diversity index (Shannon and Weaver, 1949).

Beta diversity (between sample diversity comparison) was assessed with Non-metric multidimensional scaling (NMDS) chosen to represent, the dissimilarity between samples in a low-dimensional space based on Bray-Curtis dissimilarity matrix. Variance stabilizing transformation (McMurdie and Holmes, 2014)was

used for 16S rRNA generated count data using DESeq2 package (Love et al., 2014). We proceeded with a test statistic for each bacteria individually, measuring its association with sample diet, and then jointly adjust p-values to ensure a False Discovery Rate through the Benjamini-Hochberg procedure.

3. RESULTS AND DISCUSSION

3.1 Rumen pH, VFA, methane and ammonia

The results of the effects of EMB, TP and GM on feed intake, milk production and composition and haematological parameters are shown in Chapter 1.

The concentration of total VFA, molar proportions of individual VFA, acetate:propionate ratio, NH3 and estimated methane production are presented in Table 1.

Rumen pH was not affected by the diets contain the by-products compared to the CON diet. The ruminal pH values were between 6.30 and 6.44 and were within the normal range for rumen liquid pH in sheep (Dziuk, 1984). The pH of groups supplemented with by-products did not change compared to the unsuplemmented group and this result could be an indicator that the individual TMR intake in each experimental group was similar to the CON group.

The treatments did not influence the rumen ammonia content ($P > 0.05$), but a significant interaction sampling time x treatment ($P < 0.01$) for NH3 was observed (Figure 1), due to an increase of NH3 in CON and EMB in the second sampling and a stable concentration in the TP and GM groups. The effects of pure polyphenol or inclusion of by-products containing polyphenol did not give

univocal results in different studies, the authors observed that the supplementation of polyphenols in the ruminant diet was usually associated with a decrease in protein degradation (Abarghuei et al., 2010; Dschaak et al., 2011). This process is caused by the ability of polyphenols to bind protein, therefore reduce the microbial enzymes activity and decreasing the growth of proteolytic bacteria (Molan et al., 2001). In our study the rumen ammonia in groups fed with supplementation of by-products containing polyphenols (GM and EMB) did not decreased, this different between other studies and the present work could be caused by level of polyphenols in the ewes diet was low to influence the activity of some strains proteolitic bacteria. In TP group the rumen ammonia was not affect; in contrast with another study on cattle fed with TP, in fact the authors showed that the rumen ammonia increase with the replacement of soybean meal by dried tomato (Yuangklang et al., 2010).

Acetate, propionate and butyrate, which are the main compositions of volatile fatty acids, were unaffected by the experimental factors, as well as the concentration of isobutyrate and the estimated methane production ($P > 0.05$).

Sampling time affected almost all the rumen fermentation parameters, except acetate concentration that did not change during the experimental period.

3.2 Rumen Microbiological Population

Effects of the dietary by-products on the rumen microorganisms are shown in Table 2. The Shannon- index and Simpson's index of bacteria were influenced by diet ($P< 0.05$). The Shannon index of bacteria did not change between sampling

time for any treated group. At the sampling 1, Shannon index was significantly different between EMB and GM, with lowest value for GM, but any differed from CON group.

The Simpson's index was significantly higher for EMB compared to GM at sampling 1, but any treated group differs from CON. Data of α-diversity indices are shown in Figure 2 and evidences that total microbial species richness of the sheep rumen microbiome was not significantly influenced by diets.

The structure of the microbiomes as assessed by beta diversity measures showedclearly a difference between sampling time (Figure 3). Total Abundance grouped by diet and separate for sampling time (Figure 4a) and the Log transformed abundance and rank colored by diet (Figure 4b) confirm the differences between sampling time.

The Taxa prevalence in Phylum is reported in Figure 5. Table 3 shown the top 10 of bacteria ranked by significance for DESeq2 results in EMB comparison to CON diet, whereas the remaining taxa were not listed.

The rumen microbiome for the EMB versus control diet was dominated by OTUs assigned to the *Bacteroidetes*, *Proteobacteria*, *Cyanobacteria* and Firmicutes phylum. An increase in the proportion of sequences assigned to the *Bacteroidetes*, Proteobacteria, *Cyanobacteria* phylum was observed for the EMB group with a concomitant decrease in *Firmicutes*.

In this study, supplementation of EMB resulted in higher abudance of *Succinivibrionaceae*. These bacteria have been reported to contain urease genes

and have urease activity (Patra and Aschenbach, 2018) and have been associated to low methane emissions in Tammar wallabies(Pope et al., 2011).

Differences in OTU abundances were also detected for the *Veillonellaceae* family (Table 3) that are considered involved in the production of propionate as its major fermentation product (Kishimoto et al., 2006). The *Para-Prevotellaceae* and *Prevotellaceae* families dominated the phylum of *Bacteroidetes*.

Table 4 shown the top 10 of bacteria results in GM compared to CON diet, whereas the remaining taxa were not listed.Not differences in the proportion of any phylum have been detected between the two groups.

Results of TP compared to CON group, evidenced the higher abundance of *Proteobacteria* phylum (Table 5). This phylum was dominated by *Acetobacteraceae* family with the genus *Acetobacter*, which has been found positively correlated with CH4 emissions in dairy heifers (Cunha et al., 2018**).**This could explain the tendency for highest CH4 emission per kg of DMI and per kg of milk observed in TP group, even if did not reach the level of significance compared to CON.

4. CONCLUSIONS

The inclusion of by-products in the diets of dairy sheep did not cause consistent variations in the structure of rumen microbiota in comparison to control diet. The structure of the microbiomes showed clearly a difference between sampling time, but was not well defined among diets. The rumen microbiome for the EMB versus

control diet was dominated by OTUs assigned to phylum different from that observed in GM and TP groups. The estimated methane emission did not evidenced any effects of by-prodcuts in the dose used in the trial, in mitigation of methane in dairy sheep.

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6. TABLES

Table 1. Effects of dietary treatments on rumen fermentation characteristics.

	Diet[1]				SEM	*P-value*[2]		
	CON	EMB	TP	GM		D	S	D x S
pH	6.36 ^{ab}	6.40 ^{ab}	6.26 ^{b}	6.44 ^{a}	0.025	*	**	NS
Ammonia, mg/dL	8.44	10.65	9.19	7.52	0.522	NS	***	**
VFA[3], mol/100 mol								
Acetate	62.54^{f}	60.72^{f}	66.23^{e}	59.20^{f}	1.044	†	NS	NS
Propionate	17.28	17.51	18.19	16.60	0.459	NS	***	NS
Butyrate	14.55	13.05	14.54	13.54	0.443	NS	***	NS
Iso valerianic	0.29	0.30	0.29	0.24	0.014	NS	**	NS
Valerianic	8.32	8.29	5.88	5.33	0.707	NS	**	NS
Acetate:Propionate	3.75	3.61	3.68	3.60	0.064	NS	**	NS
Acetate, %	66.56	66.68	67.01	66.42	0.358	NS	***	NS
Propionate, %	18.28	19.14	18.39	18.65	0.342	NS	†	NS
Butyrate, %	15.15	14.18	14.60	14.93	0.260	NS	***	NS
CH_4emission[4,5]	29.21	27.73	30.62	27.49	0.570	NS	†	NS
CH_4/kg of DM[5]	17.36^{f}	18.23^{ef}	20.28^{e}	17.40^{f}	0.476	†	**	NS
CH4 /kg of milk[5]	35.00^{ef}	34.95^{ef}	37.77^{e}	27.77^{f}	1.565	†	*	NS

[a–b]Means within a row with different superscripts are different (P < 0.05).
[e–f]Means within a row with different superscripts are different (P < 0.10).
[1]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.
[2]D = effect of diet; S = effect of sampling time; NS indicates P > 0.10.
†P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.
[3] VFA: volatile fatty acids.
[4]Estimated according to Moss et al. (2000);
[5]CH_4 =Methane, mol/mol of VFA

Table 2. Diet's effect on biodiversity indices of rumen microbial population

Index	Sampling	Diet[1]				SEM[2]	AOV P-value[3]		
		CON	EMB	TP	GM		D	S	D × S
Shannon	1	2.48ab	3.02a	2.38ab	2.23b	0.06	**	NS	NS
	2	2.48	2.65	2.59	2.23	0.05			
Simpson	1	0.76ab	0.86a	0.74ab	0.71b	0.01	*	NS	NS
	2	0.76	0.79	0.80	0.70	0.01			

[a–b]Means within a row with different superscripts are different (P < 0.05).
[1]CON = control diet; EMB = diet containing 75g/d per head of exhausted myrtle berries; TP = diet containing 100g/d per head of tomato pomace; GM = diet containing 100g/d per head of grape marc.
[2]SEM: standard error of the mean.
[3]D = effect of diet; S = effect of sampling time; NS indicates P > 0.10.
†P< 0.10, *P< 0.05, **P< 0.01, ***P< 0.001.

152 **Table 3.**Taxonomic identity of the rejected hypotheses in EMB vs CON Diet. Top10bacteria ranked by significance for DESeq2 results in EMB vs
153 CON comparison
154

Counts[1]	LogFC[2]	padj[3]	Kingdom	Phylum	Class	Order	Family	Genus
57.06	1.10	0.04	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Paraprevotellaceae	n.a
15.82	1.49	0.05	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	n.a
278.96	1.56	0.05	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	n.a
5.59	2.02	0.05	Bacteria	Cyanobacteria	Chloroplast	Streptophyta	n.a	n.a
12.65	1.80	0.05	Bacteria	SR1	n.a	n.a	n.a	n.a
6.68	-1.14	0.05	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Schwartzia
132.02	1.36	0.14	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
23.71	-0.88	0.14	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Asteroleplasma
1.38	2.61	0.14	Bacteria	Spirochaetes	Spirochaetes	Sphaerochaetales	Sphaerochaetaceae	Sphaerochaeta
1.86	2.26	0.14	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	n.a

155 [1] Counts:
156 [2] FC = Fold change
157 [3] padj: p-value adjusted
158 n.a. = not available

159 **Table 4.**Taxonomic identity of the rejected hypotheses in GM vs CON Diet. Top 10 bacteria ranked by significance for total DESeq2 results in GM
160 vs CON comparison
161

Counts[1]	LogFC[2]	padj[3]	Kingdom	Phylum	Class	Order	Family	Genus
14.59	2.28	0.17	Bacteria	SR1	n.a	n.a	n.a	n.a
9.04	2.50	0.41	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	n.a
56.92	-1.81	0.44	Bacteria	Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae	n.a
2.47	2.38	0.60	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	n.a
12.55	-0.97	0.66	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
76.95	0.74	0.79	Bacteria	Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	Fibrobacter
123.65	-0.39	0.91	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Succiniclasticum
135.37	-0.46	0.99	Bacteria	Firmicutes	Clostridia	Clostridiales		
25.45	-1.31	0.99	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
16.09	0.88	0.99	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium

162 [1] Counts:
163 [2] FC = Fold change
164 [3] padj: p-value adjusted
165 n.a. = not available

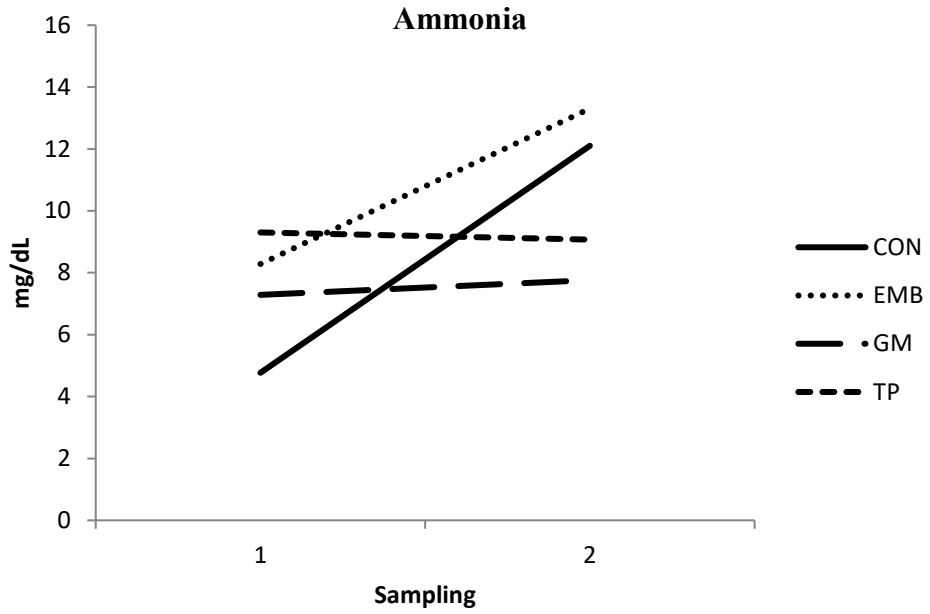
Table 5. Taxonomic identity of the rejected hypotheses in TP vs CON Diet. Top 10 bacteria ranked by significance for total DESeq2 results in TP vs CON comparison

Counts [1]	LogFC [2]	padj [3]	Kingdom	Phylum	Class	Order	Family	Genus
4.86	5.61	0.00	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter
56.03	-1.72	0.13	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma
63.51	1.05	0.55	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
43.28	-0.82	0.55	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
27.11	-1.49	0.55	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
17.16	1.73	0.55	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Paraprevotellaceae	Prevotella
4.75	-1.08	0.55	Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Pyramidobacter
4.57	-4.26	0.55	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
38.46	-1.38	0.86	Bacteria	Cyanobacteria	4C0d-2	YS2	n.a	n.a
22.00	0.99	0.86	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium

[1] Counts:
[2] FC = Fold change
[3] padj: p-value adjusted
n.a. = not available

173 **7. FIGURE**

174
175
176



177

178 **FIGURE1.**Temporal evolution of rumen NH3 production during the trial

179

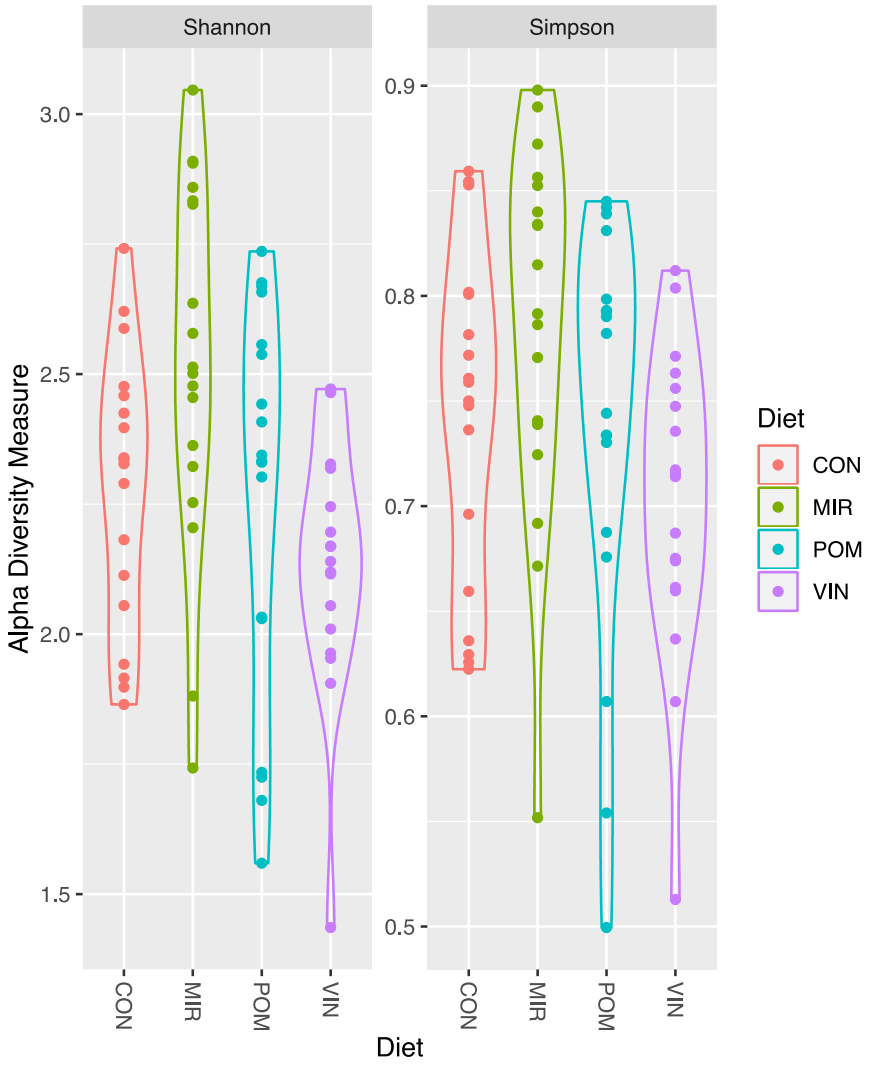
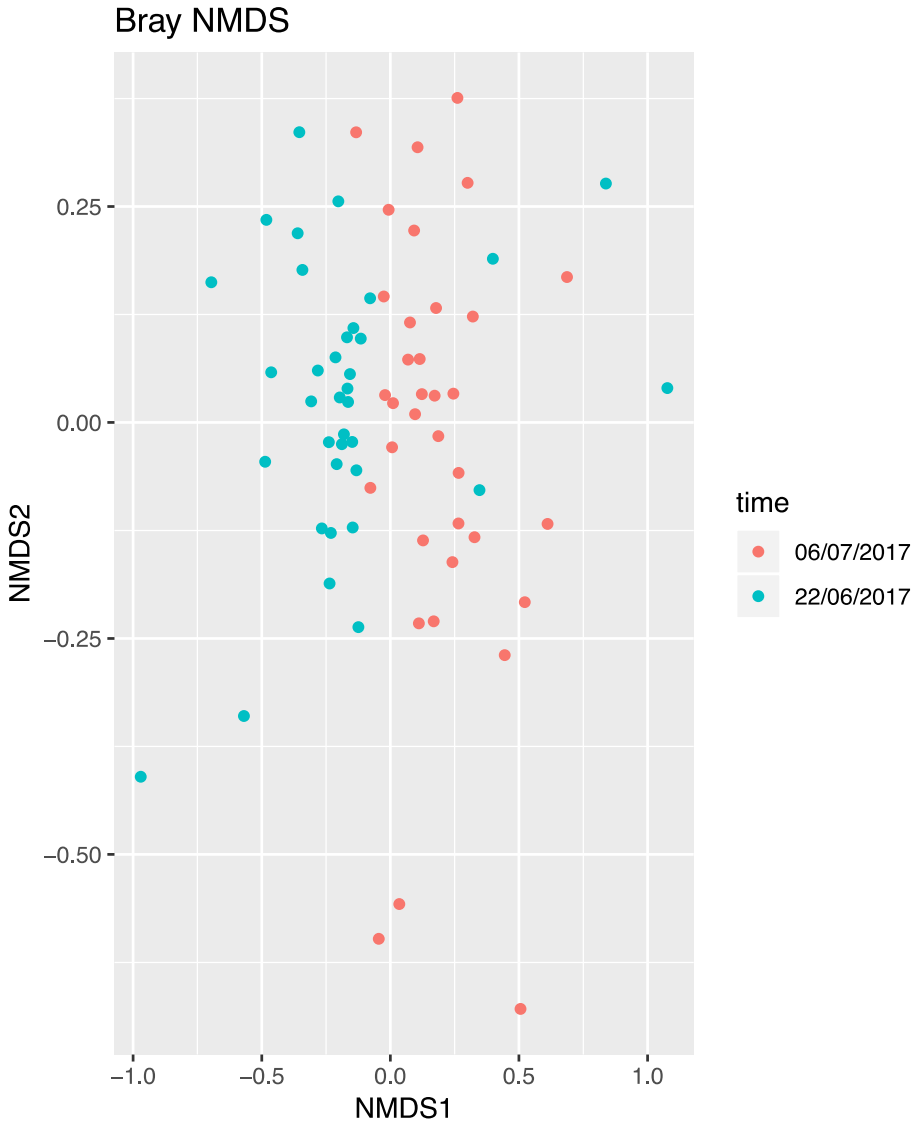


FIGURE2. Violin Plot of Alpha Diversity Measures. Different colours indicate different Diet treatments, violin shapes represents all possible results, with thickness indicating density.



186

187 **FIGURE 3.** Beta diversity.Non-metric multidimensional scaling (NMDS) based
188 on Bray-Curtis dissimilarity matrix shows time as possible element of diversity
189 between sample.

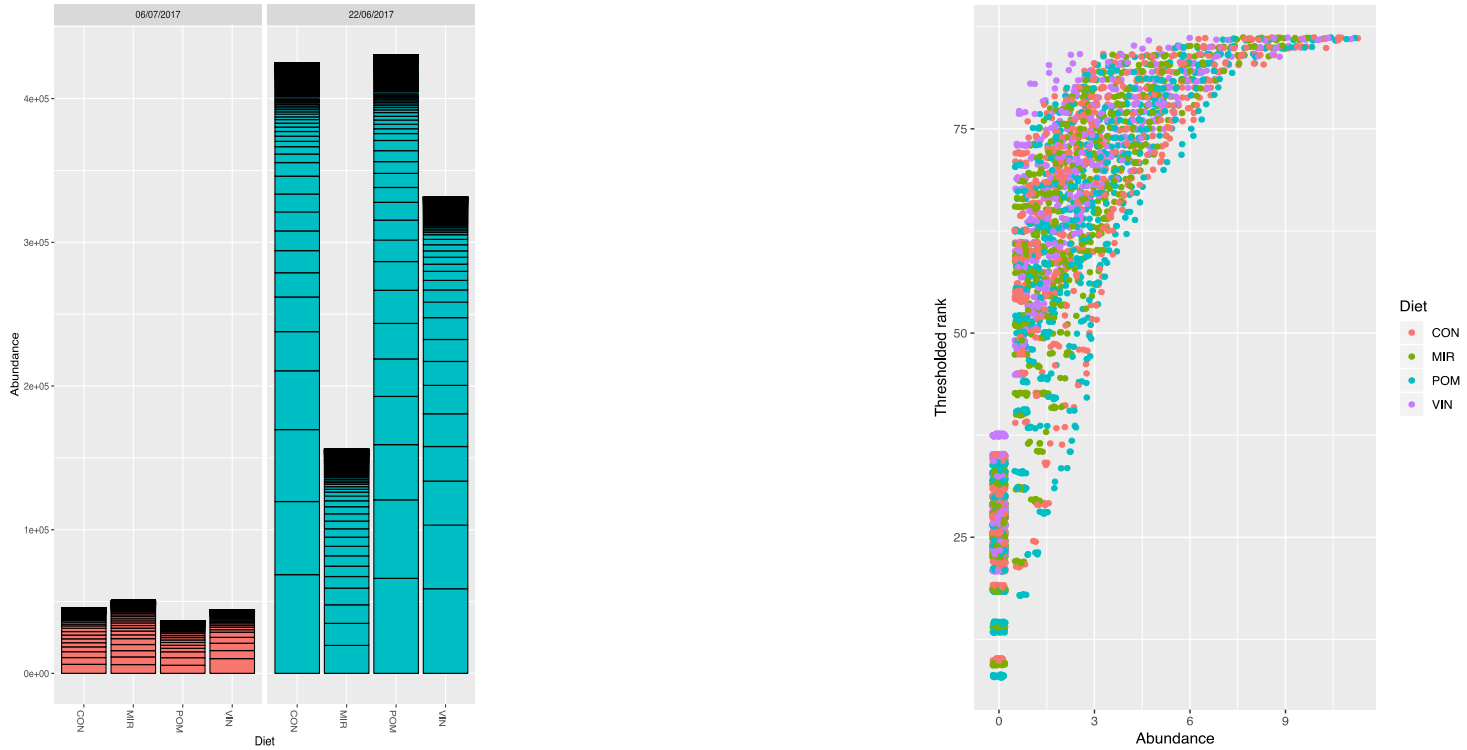


FIGURE4. a) Total Abundance. Total Abundance grouped by diet and separate for time (collection date).
b) Log transformed abundance and rank colored by diet.

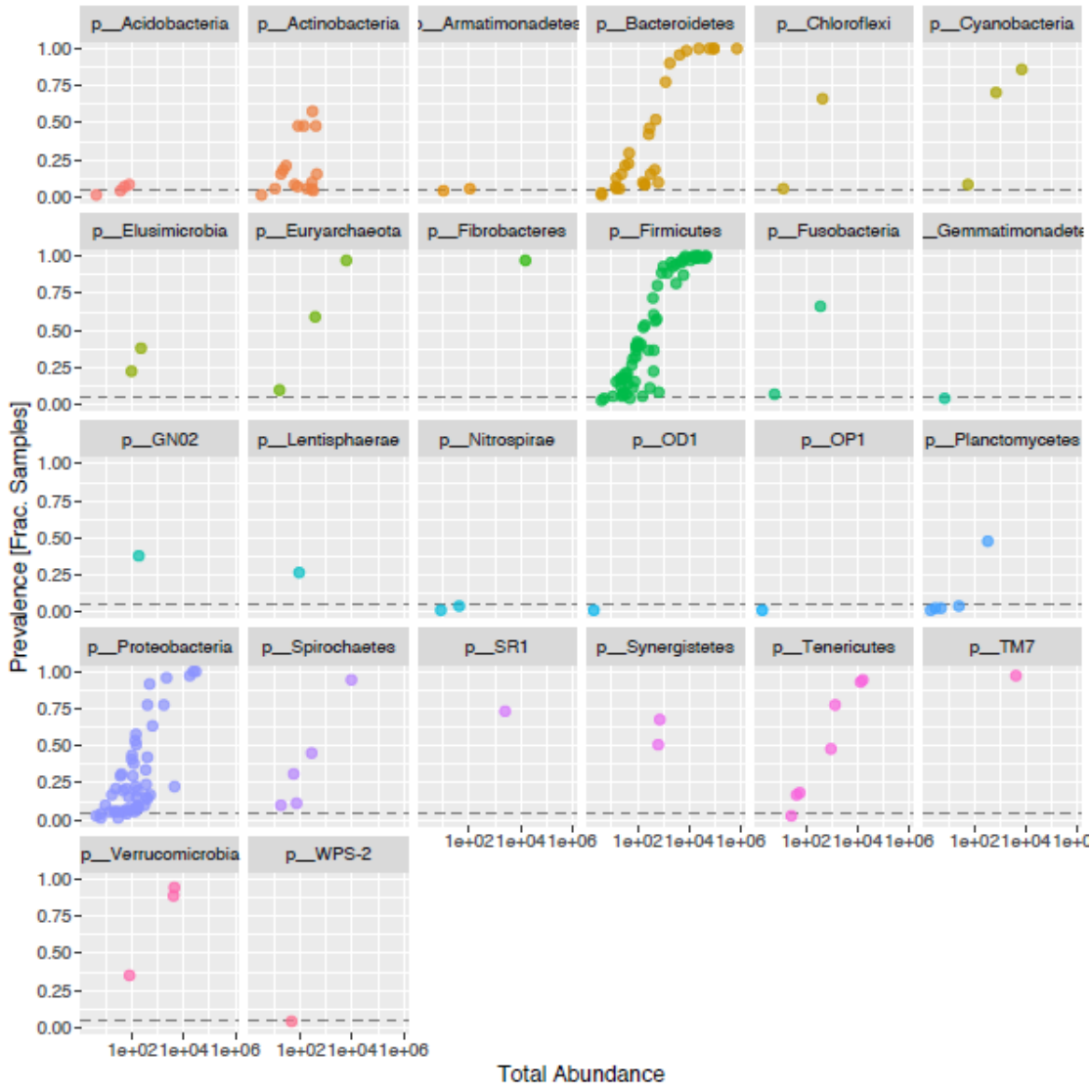


FIGURE 5. Taxa prevalence in Phylum. Multiple Plot of different Phylum. Inside each plot x axis indicate total abundance y axis prevalence.Each point is a different taxa.