Dipartimento di Scienze Agrarie, degli
Alimenti e dell'Ambiente

Ph.D Course in

MANAGEMENT OF INNOVATION IN THE AGRICULTURAL AND FOOD SYSTEMS OF THE MEDITERRANEAN REGION (XXX CYCLE)

Exploitation of winery by-products as immune modulators in sheep

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ABSTRACT

This thesis focused on the potential reuse of winery by-products as immunomodulants in sheep.

Recently, EU Parliament introduced the "Waste Framework Directive" (Directive 2008/98/EC) with the intent to promote the recycling and recovery of waste and by-products in order to obtain a secondary raw material. As describe in this directive a by-product is: a substance or object, resulting from a production process, the primary aim of which is not the production of that item. In this scenario is integrated the "zero waste economy" which is based on the circular economy concept. In this point of view waste can be used as new material to generate products.

Nowadays, consumers are attentive to the healthiness and safety of animal products moreover, worldwide is generally accepted that industries generate a large amount of waste leading to a huge environmental impact.

It is generally known that winery products contain considerable number of bioactive compounds mainly phenols with strong antioxidant activity, antimicrobial and anticancer activity, modulation of detoxification enzymes, activity on the immune system and modulation of hormone metabolism.

Starting from this consideration this thesis is oriented to characterize two different oenological by-products, wine lees and grape pomace, with the objective of extracting bioactive substances which can be used as supplements in sheep diet. This dissertation provides an in vitro overview of the immunomodulants properties of winery by-products extracts, so further researchers are required to evaluate their impact in vivo.

The thesis is divided into three different trials. Winery by-products where collected at two local wineries: Cantine La Marchesa (Lucera, FG) and Cantine Pirro (Troia, FG), in order to support our economy, moreover the choice was made taking into account the vines processed in order to valorizate the Apulia Region production. The winery by-products chosen belong to local cultivar of Vitis Vinifera: Bombino and Trebbiano d'Abruzzo for white vinification (Cantine La Marchesa) and Nero di Troia for rosè and red vinification (Cantine Pirro). To operate in an "enviromentaly friendly" approach solvent and procedures were carefully chosen.

In the first trial wine lees, classified as the residues remain at the bottom of recipients after wine production, were collected and submitted to extraction procedures in order to isolate bioactive compounds. The extraction of bioactive substances was conducted using a microwave assisted extraction (MAE) technique with low impact solvents such as water, ethanol and their mixture 1:1 and catalyser/no catalyser to increase the extraction yeald.

In this experiment, three different wine lees from Bombino/Trebbiano d'Abruzzo in white vinifiaction and Nero di Troia in rosè and red vinification were submitted to MAE extraction operating at four different temperatures 50°C, 100°C, 150°C and 200°C then total phenols, antocians, flavonoids content and antioxidant capacity were assessed. MAE extracts were tested at 0.4 mg/mL and 0.8 mg/mL on in vitro PBMC proliferation and cytokines' production. In addition, an apoptosis ELISA assay was done to measure the presence of pro-apoptotic and anti-apoptotic proteins in cells supernatants. Wine lees extracts were submitted to a GC-MS/MS to investigate the presence of further compounds such as 5-hydroxymetylfurfural. An enzymatic determination of sulphites and organic acids was done to excludes the impact of these substances on wine lees' antioxidant capacity. Results from this in vitro trial demonstrates that wine lees extracts contain a different total phenols content depends on the type of extraction solvent: white wine lees contains more flavonoids while Rosè lees contains more antocians and $ABTS^+$ ability was higher in Red lees in water extraction. Tests on PBMCs confirm the hypothesis that wine lees are able to affect sheep immune system, reporting a reduction of their proliferation with all wine lees extracts. Even though no significative variation of pro-inflammatory cytokines were found, anti-inflammatory IFN-γ and IL-10 result augmented when Nero di Troia red wine lees in water (ReW) were added to PBMC, demonstrating an immunostimulatory effect of this wine lees extract which can be associated to the high scavenging activity of this extract. From GC-MS/MS analysis in Nero di Troia red wine lees extracted in water results the precense of 5-hydroxymethylfurfural (5-HMF) to whose is connected the high $ABTS^+$ capacity. Moreover, 5-HMF affected the apoptotic pathway through the BCl2 protein family resulting in an increment of the level of pro-apoptotic Bax proteins.

In the second trial, MAE' wine lees extracts in water at 200°C from previous trial were further extracted in separating funnel then purified by flash liquid chromatography (FLC) and submitted to a GC-MS/MS analysis. Results from gas chromatography report the presence of a family of diketopiperazines in these wine lees fractions with a different isomers distribution in different fractions. Based on these results fractions were merged and then two fractions (F1 and F2) of each wine lees where chosen and tested on in vitro PBMCs at two concentration 0.4 mg/mL and 0.8 mg/mL. This trial consist of two experiments conducted at two different temperatures simulating condition of thermal stress (43°C) for 24 h and condition of normothermia (37°C) for 48 hours; proliferation assay and cytokines determination were done for both experiment and, in addition, a mitochondrial health assay was conducted on cells cultivated in heat stress condition with EVOS FL Cell Imaging System (Thermo Fisher) using the HCS Mitochondrial Health Kit (Invitrogen). Recent studies revealed that diketopiperazines have antioxidant, antiviral, antimicrobial and immunstimulants properties. Results from this second trial report a marked decrement of lymphomonocytes proliferation and cells viability in condition of normothermia even more accentuated in thermal stress conditions. As concern the cytokines pattern, in condition of normothermia a decrement of IL-6 was observed in supernatants of cells harvested with WhF1, RoF1 0.8, ReF1 0.8 and ReF2 while an increment of IL-10 was observed in presence of White wine lees F2. Differently, in condition of thermal stress at 43°C, IL-6 undergone a reduction when cells were stimulated with F2 of each wine lees and with ReF1 0.8 while at 39°C a decrement was registered with RoF1 0.8. IFN-γ level increase in presence of ReF2 0.8 when administered in condition of thermal stress demonstrating the capacity of this wine lees fraction to increase anti-inflammatory cytokines at the expense of pro-antiflammatory cytokines.

Third trial focused on grape pomace which consist mainly in grape skin and seeds. Nero di Troia grape pomace were collected and submitted to MAE extraction with water, ethanol and water/ethanol 1:1 and catalyser/no catalyser at 50°C, 100°C, 150°C and 200°C and then total phenols, antocians and flavonoids content and antioxidant capacity were assessed. Grape pomace

extracts were tested on in vitro PBMC of sheep during transition period. Blood where collected 15 days (t1), 7 days (t2) before lambing, at lambing (t3) and 7 days (t4) after lambing.

Results demonstrate a different impact on PBMC proliferation depending on the types of extracts; PBMCs proliferation increase respect to CS at t3 and t4 except in presence of 200°C extracts in water at both concentrations and in ethanol at 0.8 mg/mL that undergone to a decrement. This result could be associated with an immunosuppressive role of these extracts. The level of IL-6 resulted higher at all time of experiment when cells were stimulated with grape pomace extracted in water/ethanol or in ethanol at 150 and 200°C respectively. At day of lambing and seven days after, it was registered an increment of the level of IL-10. This cytokine and the IL-6 resulted both higher in some extracts and this effect can be linked to the activation of the innate immune response. Lastly, in this trial the level of the anti-inflammatory IFN-ɣ was higher in cells harvested with ethanol and catalyser and ethanol extracts seven days after lambing.

From the above consideration is possible to assert that the reuse of winery by-products is possible to obtain bioactive compounds useful in animal nutrition thanks to their immunomodulants capacity.

To sum up, wine lees extracts can be used as immunostimolants and antioxidants and their purified fractions, as obtained in the second trial, are useful as chemotherapeutic and anti-inflammatory compounds. Lastely, grape pomace can be used as anti-inflammatory and immunomodulants even in condition of stress linked to the transition period.

Further studies can be directed to in vivo experiment in order to better understand the immune impact of these extracts and fractions with the last aim of sustaining animal immune response and meanwhile reducing the environmental impact of oenological by-products.

Keywords: sheep welfare; immune system; antioxidants; winery by-products

CHAPTER 1

GENERAL INTRODUCTION

1.1 OVERVIEW ON WINE SECTOR AND THE INSERSECTION WITH ANIMAL WELFARE

The Wine sector is one of the most important compartments of the Italian economy. In the last few decades the demand and supply of wine has increased and Italy is now the largest exporter in the world with 21% of the world market. Wine represents the 7.2% of the income of the agro-alimentary industry. In 2016 wine production stood at 51.6 million hectoliters, 6% more than in 2015 (ISTAT 2017), and in 2017 it was 39.3 million of hectoliters confirming Italy as the first producer in the world (OIV 2017).

Recently, concerns have been raised about the high amount of by-products originating from the wine industry, in which some of them could have an impact on environment due to their chemical composition and seasonal character. In this regard, it is estimated that annually the winery industry in Europe produces 14.5 million tons of grape by-products (Chouchouli et al., 2013). Fiori et al., (2009) reported that the winery industry processes 35,000 billion kilograms of grapes with a by-product production of 7000 billion kilos.

Approximately one ton of processed grape produces 0.13 t of pomace, 0.06 t lees, 0.03 t of stalks and 1.65 m^3 of wastewater (Oliveira and Duarte, 2016).

The European Parliament with the "Waste Framework Directive", (2008/98/EC) of the Council of 19 November 2008 put the accent on the possibility to re-use waste and by-products as they have added value.

Various by-products originate from wine production e.g. grape pomace, grape seed, grape skin and wine lees. The latter and the grape pomace must be sent to distilleries as imposed by (EC) 479/2008 of the European Council Regulation to produce exhausted grape pomace and wastewater with the aim of having products with genuine and merchantable quality. For this reason, this regulation

prohibits over-pressing and re-fermentation of these by-products to obtain wine or any other beverage for direct human consumption.

Each year, wine making industry produces a substantial amount of grape by-products with a significant environmental impact due to high content of phenolic compounds and considerable chemical and biochemical oxygen demands (COD and BOD).

The type of waste and their composition depends on winemaking procedures, some of them can have not only vegetable components but also technological adjuvants used during the vinification like bentonite clay, and albumin which have impact on the antioxidant content (Villano et al., 2006).

A majority of these wastes are valuable to the pharmaceutical and cosmetic industry as they contain polyphenols and anti-oxidants that can be extracted and sold as supplements; moreover, the presence of bioactive compounds like polyphenols make them very useful in animal feeding.

Grape composition influences not only the wine quality, but also the composition of wastes recovered. Figure 1.1 reports the composition of grape berry, while Table 1.1 shows the composition of berry juice and wine. Grape berries contain water 65 - 85%, sugars, organic acids, aromatic substances and phenolics. Simple sugars are represented mainly by glucose and fructose and in lesser extent sucrose and raffinose; these compounds are converted into alcohol during the alcoholic fermentation. Organic acids are related to the freshness and sapidity perception and the major are tartaric acid and malic acid. Other acids include citric, ascorbic, oxalic, succinic, lactic, glutaric, alpha-ketoglutaric, pyruvic, oxalacetic and galacturonic acid. Grapes contain a large variety of vitamins such as vitamin C, B2, B5, B6 and PP and nitrogenous compounds such amminoacids which are important for yeasts growth and activity. It is crucial to underline the presence in grape and wine of bioactive compounds such phenolics which will be exhaustively describe in the next paragraph. Nowadays there is a widely concern on animal welfare as well as on the potential harm derived by consuming animal products in regard of their safety and healthiness. This is coming out more and more after some epidemics such as BSE, dioxin and antibiotic

resistance. On this topic, the European Union took an important initiative in 1998 with the Council Directive 98/58/EC on the protection of animals kept for farming purposes which gave general rules for the protection of animals of all species kept for the production of food, wool, skin or for other farming purposes, including fish, reptiles or amphibians. This directive is based on the "Five freedoms" described in the Brambell Report (1965) that are: freedom from hunger and thirst, from discomfort, from pain, injury and disease, freedom to express normal behaviour and freedom from fear and distress.

Recently, as concern the safety of the animal products, another issue is raising the attention of the scientific community: the antimicrobial resistance. This resistance is due to microorganisms with intrinsic resistance to antibiotics that can be heighten with the indiscriminate usage of them, for therapeutic and non-therapeutic purpose in animal production, even more when animals are reared in intensive production systems. It is generally known that the antibiotics used for animals belong to the same class of the ones used for humans with the results of the transmission of zoonoses to human (EFSA, 2012). It is widely demonstrated that this transition can happen via the consumption of animal-based food and already contaminated with resistant microorganisms or by the transmission of resistant genes to commensal bacteria present in humans (Smith et al., 2002; Gündoğan et al., 2006).

In recent years, scientists and pharmaceutical industries have focused their attention on developing alternative types of antimicrobial drugs with the intent of novel therapeutic approaches to this worldwide problem.

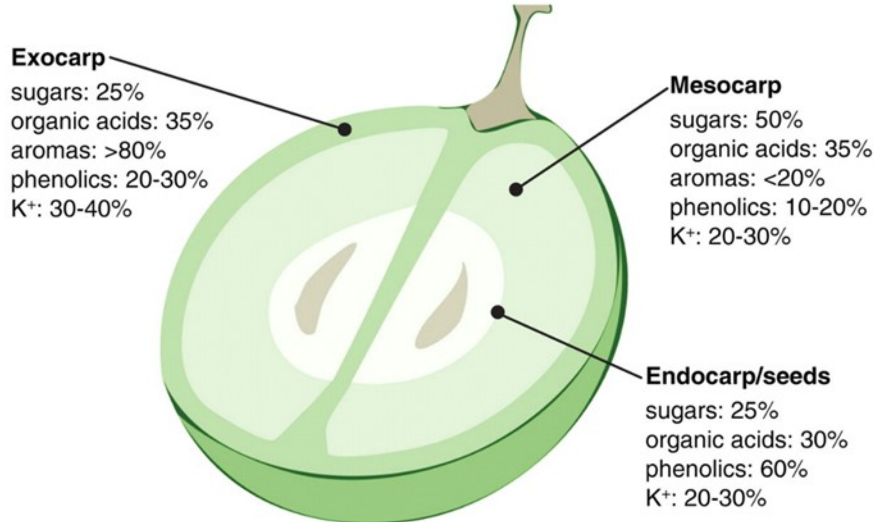


Figure 1.1 Fontes et al., 2011

	Juice (mean values in %w/w)	Dry Wines (mean values in %w/w)
Water	79	85
Carbohydrates (total)	21	0.2
Fructose	11	0.07
Glucose	10	0.06
Pectin	0.06	0.2 (as galacturonic acid)
Pentoses	0.1	0.1
Alcohols		
Ethanol	trace	12.5 (v/v)
Glycerol	0	0.6 - 1.0
Higher alcohols	0	0.02 - 0.04
Methanol	0	0.01
Aldehyde	trace	0.01
Organic acids	0.8	0.7
Acetic	0.01	0.03 - 0.07
Amino acids (total)	0.04	0.1 - 0.25
Citric	0.02	0.02
Lactic	0	0.03 - 0.5
Malic	0.1 - 0.8	0.0 - 0.6
Succinic	0	0.1
Sulphurous	0	0.02
Tartaric	0.6 - 1.2	0.5 - 1.0
Phenolics		100 - 2500 mg L^{-1}
Simple		6 - 150 mg L^{-1}
Hydrolysable tannins		T (red and chardonnay)
Condensed tannins		50 - 800 mg L^{-1}
Anthocyanins		0 - 1000 mg L^{-1}
Nitrogenous compounds	0.12	0.03
Amino	0.07	0.1
Ammonium	0.006	0.03
Protein	0.005	0.01
Resdiual	0.015	0.01
Minerals (ash)	0.4	0.3
Calcium	0.015	0.004 - 0.01
Chloride	0.01	0.005 - 0.02
Magnesium	0.015	0.004 - 0.012
Phosphate	0.03	0.0025 - 0.085
Potassium	0.2	0.06 - 0.12
Sodium	trace	0.004
Sulphate	0.02	0.07 - 0.3

Table 1.1 Grape juice and wine composition: Residual

1.2. PHENOLIC COMPOUNDS

The potential of the use of some winery by-products is linked to the natural presence of bioactive phytochemicals such as phenolic compounds which are the most widely distributed plant secondary metabolites found in many plants used as foods and feeds (García-Marino et al., 2006). These metabolites are primarily synthesized through the pentose phosphate pathway (PPP), shikimate and phenyl-propanoid pathways (Randhir et al., 2004). They are produced in response to environmental

stress, wounds or infection and possess strong antioxidant activity as free radical scavengers (Bors and Michel, 2002), electron or hydrogen donors, and possess strong metal chelator activity (Andjelković et al., 2006). In addition, the antimicrobial and anticancer activity, modulation of detoxification enzymes, activity on the immune system and modulation of hormone metabolism have been widely investigated (Daglia, 2012, Saxena et al., 2013).

A briefly overview on these pathways is reported in Fig. 1.2.1.

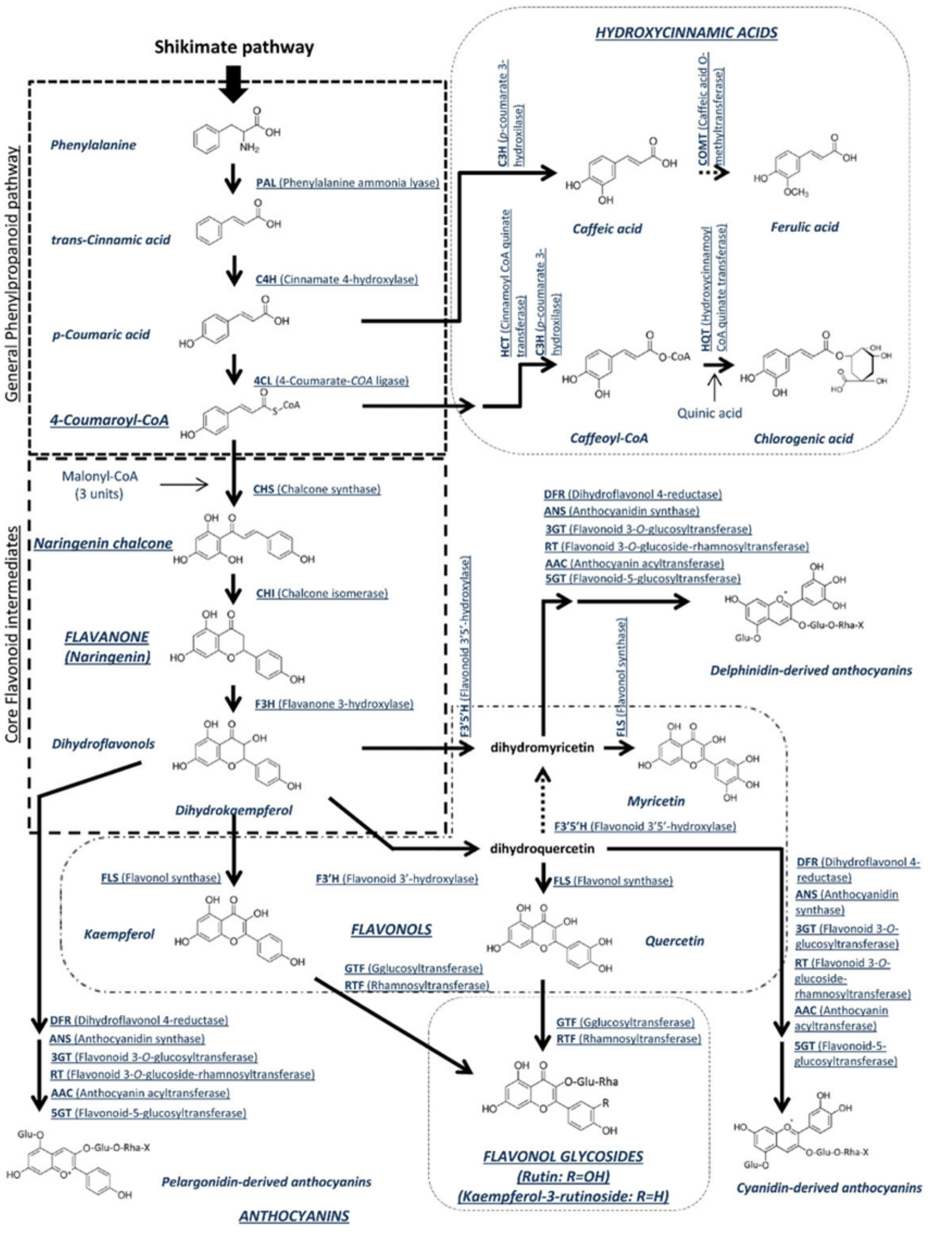


Figure 1.2.1 Martí, et al., (2016).

There are two classes of phenolic compounds: flavonoids with C6-C3-C6 skeleton and non-flavonoids both further divided into several families (figure 1.2.2). Flavonoids can be divided into 6

subclasses based on the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins) (Manach et al., 2004).

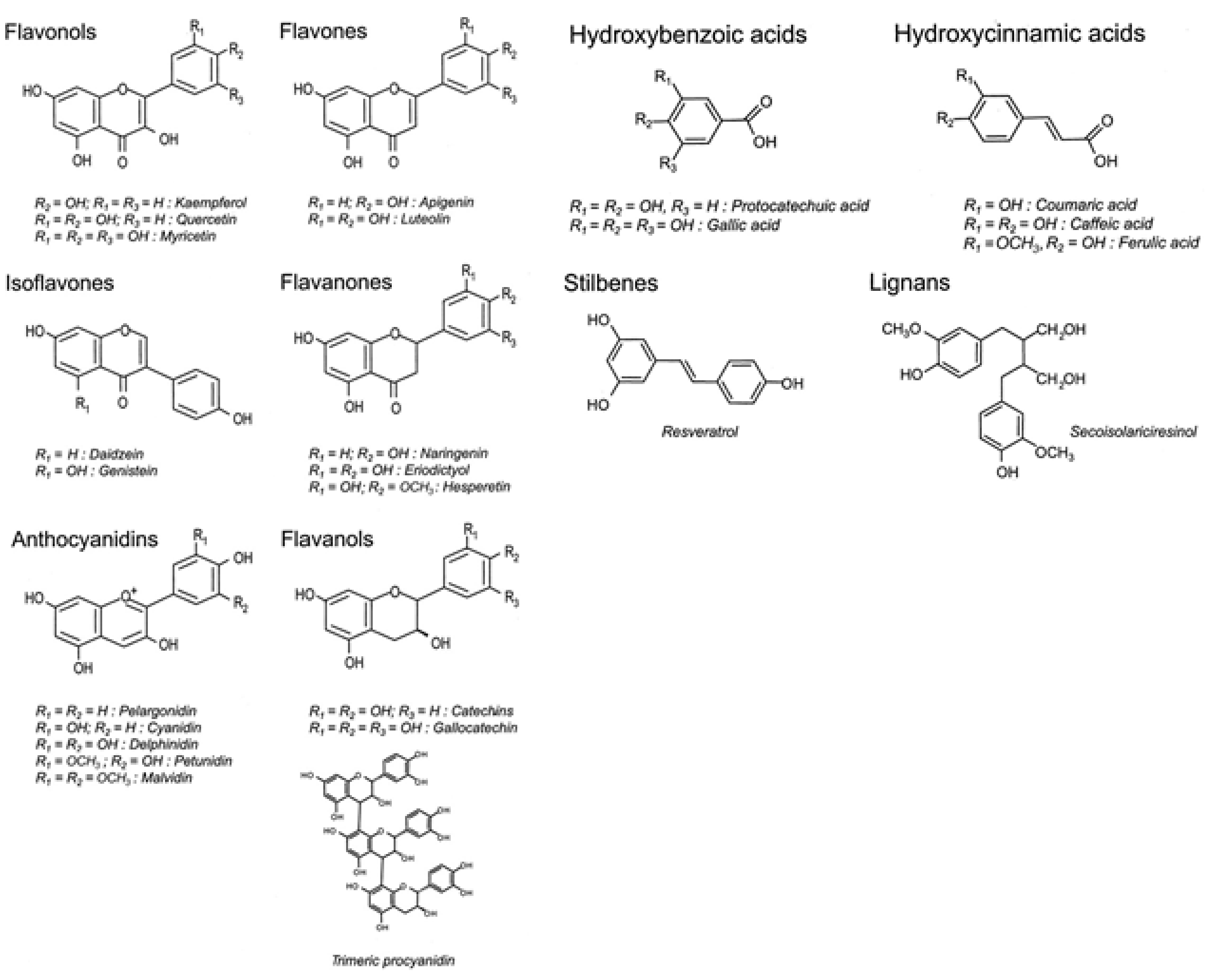


Figure 1.2.2 Phenolics compounds

These compounds differ for their chemical structures and activity.

Flavonols (e.g. kaempferol, quercetin and myricetin) have a hydroxyl group on position 3 of the C ring which can be glycosylated (Tsao and McCallum, 2009), and a double bound C=C between C2 and C3 of the C ring.

Flavones are characterized by the presence of a double bond between C-2 and C-3, and the attachment of the B ring to C-2 while in isoflavones the B-ring is attached to the C3 (Halake et al., 2016).

The family of flavanones comprise compounds with a benzopyranone unit in C2 position; flavanols have a hydroxyl group in position C3 of the C ring, but dissimilar to flavonol they do not have any double bound between C2 and C3 of C-ring (Panche et al., 2016). Complex structure of flavanols monomers, such as oligomers and polymers, form proanthocyanidins or condensed tannins with features different for variable length of chains, kind of monomer and kind of link among them (Cheynier et al., 2006, Fraga et al., 2010). Further condensations of monomers lead to polymers of two to more than fifty blocks (Khanbabaee and van Ree, 2001). For their particular structure, condensed tannins can react with metals and macromolecules (proteins, polysaccharides and lipids) present in food. The interaction of tannins and salivary proline-rich proteins lead to astringency and bitterness of food and beverages (Hofmann et al., 2006, El Gharras, 2009) while, the reaction with enzymes such as lipase, protease and glucosidases can modify the nutritional value and digestibility of food and feed (Gonçalves et al., 2011).

Sikwese and Duodu (2007), reported the ability of condensed tannins from sorghum, to act as antioxidants by chelating metal ions which made them very useful, also to remove metal pollution from water. The ability to interact with macromolecules is attracting attention due to the possibility to use this polyphenols in functional food. For example, they can act as emulsifier protecting oil from oxidative reaction. Aside that, the interaction with lipids can reduce the fat absorption while fats can protect polyphenols during their transit in the gastrointestinal tract (Jakobek, 2015). As concern the reaction with carbohydrates they can delay the retrogradation of starch amylose by acting as plasticizer (Bordenave et al., 2014).

Apart from condensed tannins, hydrolysable tannins are multiple esters of gallic acid and ellagic acid with glucose and products of their oxidative reactions (Arapitsas, 2012). As their name suggest, hydrolysable tannins are susceptible of acids and bases hydrolysis leading to carbohydrate and phenolic acid (Bele et al., 2010). Okuda and Ito (2011), reported different bioactivities of these phenolics such as radical scavenger, antitumor effects and antimicrobial effects by acting synergistically with antibiotics.

The non-flavonoids group includes non-colored compounds represented by hydroxycinnamic acids, hydroxybenzoic acids and stilbenes (Rentzsch et al., 2009). Hydroxicynnamic acid are mainly represented by p-coumaric acid, caffeic acid, sinapic acid, and ferulic acid, while hydoxybenzoic acids are gallic acid, gentisic acid, protocatechuic acid, and p-hydroxybenzoic acid. Their concentration depends on grape variety, climate and soil condition (Ali et al., 2010).

Stilbens are known as health promoting, in particular resveratrol is widely investigated since the "French paradox". It is a phytoalexine formed by 2 benzene rings linked via an isopropylene group ring structure separated by a double bond with several biological effects (Kasiotis et al., 2013).

Many studies showed that resveratrol possesses anticancer activity (Schaafsma et al., 2016), anti-diabetic effects by controlling glycaemic levels (Bhatt et al., 2012), anti-ageing (Baxter, 2008) and antinflammatory effects (Tung et al., 2015).

1.3 CLASSIFICATION OF WINERY BY-PRODUCTS

Winery industry produce different types of by-products as reported in figure 1.3. The main solid and liquid by-products and wastes produced during winemaking are grape stalk, grape pomace or marc, wine lees and, winery wastewater (Bustamante et al., 2008); the latter can be used as soil amendment after aerobic treatment (Nerantzis and Tataridis, 2006). Some authors even reported plant germination problems when these by-products were utilized as fertilizer, because of their high phenolic content (Kammerer et al., 2004).

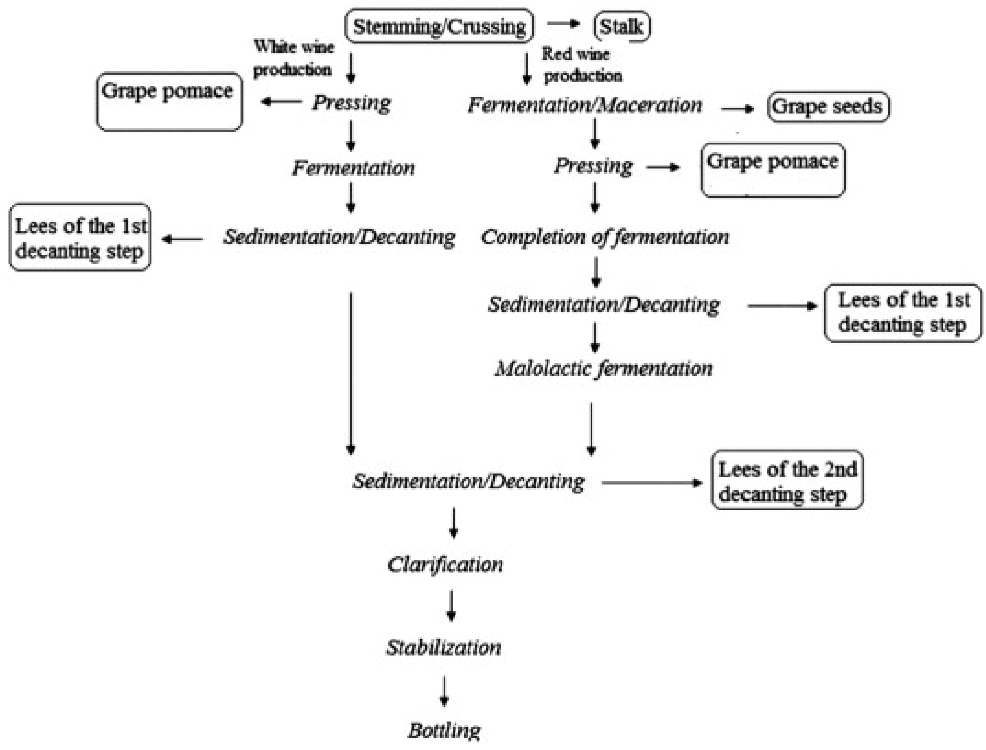


Figure 1.3 Naziri et al., (2014)

1.3.1. Grape pomace

Grape pomace (or marc) consists of fruit pulp residues, stem chips and for the major amount skin and seeds. (González-Centeno et al., 2010). Boucque and Fiems (1988) reported that the dried grape pomace consists of 40% seeds and 60% pulp; the seeds can be dried and extracted with hexane to obtain 85% of grape-seed oil meal. Grape pomace contains between 60% to 90% of dietary fiber of total dry matter, pectic substances ranging between 40-54% of total cell wall polysaccharides and lignin accounting for around 20-25% of dietary fiber (González-Centeno et al., 2010). In addition, it contains significant amounts of lipid, proteins and minerals (Yu and Ahmedna, 2013). Amico et al. (2004), demonstrated the high presence of flavonols and anthocyanins in grape pomace from a Sicilian cultivar which can exert a strong antiradical activity; moreover it was reported that polyphenols in grape pomace have a higher antioxidant capacity than in wine (Su and Silva, 2006).

Grape seeds are one of the most abundant residues after the wine making process, and contain lipid, protein, carbohydrates, and 5-8% polyphenols, depending on the variety (Shi et al., 2003). Their

importance is attribute to the high content in (+)-catechins, (-)-catechins and (-) epicatechin- 3- O-gallate (Jayaprakasha et al., 2001) and mono, oligo and polymeric proanthocyanidins (Shrikhande, 2000) which can exert an antimicrobial, antioxidant and antiviral effects showing chemo-preventing activity in mouse skin tumorigenesis (Zhao et al., 1999). Grape seeds are an interesting source of oil: grape seed oil contains about 14–17% of oil rich in unsaturated fatty acids such as linoleic acid (72–76%, w/w) (Martinello et al., 2007), which contributes to reduce the risks of peroxidation (Cao and Ito, 2003). In addition, they also have 10 to 20% oil with high vitamin E content (Baydar and Akkurt, 2001) and β-carotene and some xanthophylls (Howitt and Pogson, 2006).

Grape skin constitutes 65% of the total material of grape pomace (Teixeira et al., 2014) and contains proanthocyanidins (Kennedy et al., 2002), tartaric esters of hydroxycinnamic acid, monomeric and dimeric flavan-3-ols and flavonols (Rodríguez Montealegre et al., 2006) such as myricetin, quercetin, laricitrin, isorhamnetin, syringitin and kaempferol (Downey and Rochfort, 2008) and resveratrol derivates (Waterhouse, 2002); the composition of polyphenols is extensively affected by climate, soil, geographical position and agronomical technique and exposure to disease (Bruno and Sparapano, 2007).

Different re- uses of grape pomace are reported in literature; for instance as pesticide to control the incidence of diseases in some crops like tobacco (Benouaret et al., 2014), and for the extraction of tannins for use as wood adhesives (Ping et al., 2011). Grape pomace was used also for the extraction of phenols for instance catechin and epicatechin (López-Miranda et al., 2016) and to obtained citric acid by fungal production (Hang and Woodams, 1986).

Recently, green methods to recover phytochemicals from vegetable by-products have been developed with the benefit of using technique at a lesser impact on environment and a higher extraction rate, as reported by Drosou et al. (2015), who used the ultrasound assisted extraction technique to retrieve anthocyanins and flavonols from Agiorgitico red grape pomace.

Özkan et al. (2004), also reported the potential of grape pomace, for food preservation purposes and suggests its use as a natural additive to prevent the deterioration of stored foods by bacteria.

1.3.2. Grape stalk

Grape stalks or stems are derived from the grape stripping step and during vinification operations like concentration of must. They contain lignin, cellulose, hemicelluloses, protein, ash and minerals, mainly nitrogen and potassium (Bertran et al., 2004, Deiana et al., 2009, Prozil et al., 2012).

As listed by Souquet et al. (2000), phenolic compounds in grape stems are represented by phenolic acids, flavonols, and flavanonols, tannin such as (-)-epicatechin and to a lesser extent (+)-catechin,(-)- epicatechin gallate, and (-)-epigallocatechin.

Some authors have demonstrated the possibility to re-use grape stalk as a sorbent of metals in aqueous solutions (Villaescusa et al., 2004, Martínez et al., 2006); besides, others have shown the possibility to use this waste in association with olive waste as compost (Alburquerque et al., 2006, Cayuela et al., 2006).

A very new sector of expertise is to use grape material such as grape stalks to obtain a new class of green nanoparticles useful in medical application, in molecular imaging, and in cancer therapy (Krishnaswamy et al., 2014).

The most investigated field is the opportunity to recover antioxidants from grape stalks by solvent extraction (Makris et al., 2007, Karvela et al., 2009). Although, other authors indicate the possibility of using non-conventional methods such as supercritical fluid extraction using carbon dioxide as the extracting solvent (Casas et al., 2010) or ultrasound assisted extraction (Garretón et al., 2010, Piñeiro et al., 2013).

1.3.3. Wine lees

Wine lees are defined (EEC No. 337/ 79) as “the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product”.

Wine lees can be distinguished in “heavy or gross” and “light or fine” depending on the decanting step and the particles size (Martín et al., 2013) and are composed for the major part of yeast, tartaric acid, inorganic compounds and phenols (Pérez-Serradilla and Luque de Castro, 2011).

Wine lees are mainly investigated because of their importance in wine aging due to the capacity to adsorb phenolic compounds and consequently modifying the phenolic fraction of wine (Pérez-Serradilla and De Castro, 2008). As consequence, during the wine-making process phenolic compounds can remain in this waste and make it useful for different uses.

Concerning the chemical composition of wine lees, they contain insoluble carbohydrates (cellulosic or hemicellulosic materials), lignin, proteins, metals, inorganic salts, organic acid salts (mainly tartrates) (Pérez-Bibbins et al., 2015) and metals especially in red lees such as Cu, Mg, Fe, Mn, Ca, Al and Zn and beta glucans (6-12 % of dry weight) (Nerantzis and Tataridis, 2006).

Some authors reported that wine lees obtained from red wine are a good source of anthocyanins (Barcia et al., 2014) and condensed tannin (Mazauric and Salmon, 2005), such as peonidin 3-O-glucoside, peonidin 3-(6-p-coumarylglucoside), malvidin 3-(6-p-coumarylglucoside), catechin, epicatechin, gallocatechin, procyanidin B2 and cinnamtannin A1 (Delgado de la Torre et al., 2015).

The same authors identified by liquid chromatography-mass spectrometry (LC–MS/MS) a wide range of the non-flavonoid phenols, including mainly phenolic aldehydes, stilbenes (trans-resveratrol) and phenolic acids, which can be classified into hydroxycinnamic (sinapic acid) and hydroxybenzoic acids (syringic, gallic, protocatechuic).

Flavonols were the main compounds identified in wine lees, including myricetin, kaempferol, isorhamnetin, quercetin (Luque de Castro et al., 2007), and a glucoside derivative of quercetin namely, quercetin 3-O-glucoside.

A wide array of alternative uses of wine lees are reported in literature but the recovery of tartaric acids from wine lees is the most studied topic. The concentration of tartrate species in wine lees was reported to be 100–150 kg in a tonnes of wine lees (Salgado et al., 2010). The same authors reported the use of these salts as a natural acid useful in food industry as an alternative of citric and phosphoric acid. As reported by Kontogiannopoulos et al. (2016), the larger amount of this salts in wine lees is in the form of rarely soluble potassium and a minor part is bound to dead yeast, particulate solids and other organic substances. New green-extraction methods are being developed to increase the purity of these salts and to reduce the recovery costs especially of evaporation.

Besides that, the retrieval of squalene from wine lees is under attention because it is a precursor of sterols with protective and chemopreventive activity and it attracts attention as an excipient in pharmaceutical formulations (Reddy and Couvreur, 2009). Methods such as ultrasound assisted extraction (Naziri et al., 2012) and supercritical fluid extraction (Naziri et al., 2016) are widely used for this purpose.

Nonetheless, a very new prospective use for this waste are the possibility to generate electricity with a new biotechnology called "Microbial fuel cell" (MFC) (Sciarria et al., 2015), or their application for the production of biogas and high quality digestate through anaerobic co-digestion. This technology has some advantages in process management, environmental impact and economic sustainability because the effluent produced could be used as soil amendment, with lesser pollutants load (Da Ros et al., 2014).

1.3.4. Wastewater

Winery wastewater derived from different steps during the winemaking process such as washing of tanks, barrel and floor and during cleaning of bottling line, filtration unit and transfer lines. The characteristics of wastewater are linked to the specific type of wine produced (rosè, white or red) and they change in depending on winery size and activity (Ioannou et al., 2015).

As regard the composition of this by-product it contains ethanol, sugar and organic acid, nitrates and phosphates (Bustamante et al., 2005, Mosse et al., 2010) which led to high COD level (Da Ros et al., 2014). These characteristics make this waste harmful for water surface because of eutrophication for which reason a prior treatment is necessary (Melamane et al., 2007).

1.4 WINERY BY-PRODUCTS USE IN ANIMAL FEEDING

Recently, there is a high pressure by the Italian government to reduce the impact of industry wastes as well as the increase in funding to enhance the environmentally friendly production and improve sustainable agricultural practices. Besides that, nowadays farm animals are blamed to contribute to the greenhouse effect, soil, surface, and subsoil water pollution due to their gas emissions by respiration, feces, and urine. In this direction, it is raising the possibility to re-use industrial by-products as aforementioned. Of course, the chosen by-product must be safety for animals, for animal-based food products, and humans.

After a lack of studies on the impact of polyphenols in ruminant nutrition, recently some scientists addressed this topic, and controversial effects of these compounds are coming over their researches. Studies focused mainly on the detrimental effects of tannins on digestive process in ruminants due to their ability to bind enzymes and endogenous proteins (Lorenz et al., 2014, Gómez et al., 2015).

1.4.1 Overview on polyphenol digestion in ruminants

The widely distribution of polyphenols in plants make them very easily to found in herbivores diet. Due to their antioxidant properties, grape by-products are interesting in using in animal nutrition, despite studies on their metabolism in ruminant gut are very poor.

The estimation of the biological effects of polyphenols on animal health is very important to define the proper level of intake, their bioavailability and absorption and their disposition in the target tissues and cells. Most naturally occurring phenolics are present as free aglycone form or

conjugated with glycosidic moiety. Catechins and oligomeric proanthocyanidin exist in unglycosilated form while phenolics acids, phenylpropanoids, flavonoids and isoflavonoids are linked with sugar units such as glucorhamnose, galactose, arabinose, and rhamnose. (Heim et al., 2002). The fate of the flavonoids, including their dietary glycoside forms, is highly complex, dependent on a large number of processes such as technological treatments or fermentation which affect the content, the stability and furthermore the bioavailability of phenol compounds.

The absorption of flavonoids is also affected by the vehicle in which they are presented to the body i.e. food matrix, dissolution, and nature of solvent. The presence of alcohol in red wine did not affect the plasma concentrations in humans of catechin, an unglycosylated flavonoids, although it had some effect on the urinary concentrations.

The digestion in ruminants, as in human, begins in the mouth with the food chewed and mixed with saliva, which is the most important fluid for diet adjustment. During the forage passage into the mouth, phenol compounds, like tannins, link to saliva proteins leading to TBSPs (tannin-binding salivary proteins); therefore, the saliva is the first line of defense against tannins ingestion (Lamy et al., 2011). The expression of TBSPs is induced in animals after tannins ingestion and this complex passes through the gut to the feces to be excreted (Butler, 1992).

The vast majority of phenols digestion occurs in rumen. As reported by Patra and Saxena (2011), phenolics glycoside and flavonoids are hydrolysed in rumen with acetate, butyrate, monohydroxyphenolics and phloroglucinol production. The last two products originated from the B ring and A ring, respectively (McSweeney et al., 2001).

Rumen microbiota and endogenous enzymes lead the degradation of polyphenols in ruminants. Furthermore, some phenols act as a substrate for enzymes in small intestine, such as hydrolyzing and conjugating enzymes (Landete, 2012). It is established that rumen ecology is modified by a phenolic-rich diet and simple phenols are more toxic on bacteria than high weight tannins. Some microorganisms such as *Selenomonas ruminantium* and *Streptococcus spp*. are able to act on ester and depside bonds (which are typical ester linkage in gallotannin) producing esterase, tannin

acylhydrolase to form gallic acid and ellagic acid (Skene and Brooker, 1995; Goel et al., 2005). After that, gallic acid is decarboxylated in the rumen to pyrogallol and converted to resorcinol and phloroglucinol, then the phloroglucinol ring is converted to acetate and butyrate by rumen microorganisms (Murdiati et al., 1992, Bhat et al., 1998).

The intestinal absorption of phenols is not completely understood, and only 5-10 % of the intake is absorbed. It is postulated that the chemical structure of phenolics influences their metabolism and the fate of phenolics is not common for all of them.

Monomeric and dimeric features as aglycones are rapidly absorbed through the small intestine and reach the colon unchanged (Manach et al., 2004, Brenes et al., 2016). Glycosidic unit resists hydrolysis by pancreatic enzymes, so it had long been assumed that intestinal microbiota were responsible for beta-hydrolysis of sugar moieties.

Flavonoids are rapidly absorbed from gastro-intestinal tract and some of them interact like vitamins in the cytosolic phase of cells.

González-Barrio et al. (2012), reported that the microbial metabolism of ellagiotannins in cattle rumen produced urolithin which can be converted to isourolithin A and urolithin B by the ruminal and fecal microbiota. Urolithin can be detectable in ruminal feces as urolithin aglycons and as sulfate derivaties in urine, and plasma. As concern the intestinal metabolism, the gut bacteria can degrade catechin and gallocatechin to velerolactone and phenylpropionic acid that are excrete through urine and faeces as glucuronic acid or sulphate conjugates of velerolactone (Hollman, 2001, Mueller-Harvey, 2006). Moreover, Halake et al. (2016), reported that flavanone glycosides typically reach the colon, where bacteria are able to hydrolyze them to the corresponding aglycones by the enzyme glycosidases. After absorption complex, polyphenols are hydrolyzed and biotrasformed inside enterocytes and hepatocytes leading to water soluble derivates which enter first the systemic circulation and then the duodenum to be hydrolyzed by bacterial enzymes such as β-glucoronidase in large intestine. Some conjugated phenols, excreted in intestinal lumen by bile, react with phenols in the enteropatic cycle and are subjected to enzymatic activities of gut microflora. Microbic

derivatives are excreted in feces or after their absorption, they reach the liver and enter in systemic circulation, get to target organs and are expelled through the urine.

1.4.2. Impact of winery by-products on animal performances and production

Several studies have been conducted on the exploitation of dietary bioactive compounds on ruminants' performances.

A major limitation in using grape by-products in animal supplementation is related to their low palatability (astringency). Opinions on the effect of tannins ingestion in ruminants are controversial. Some authors reported positive effects associated with the alteration of ruminal fermentation and microbial protein synthesis leading to an increase of amino-acids in the small intestine (Aerts et al., 1999; Abarghuei et al., 2010), whereas others reported a detrimental impact on ruminal parameters and a dramatic decrease of food intake (Landau et al., 2000; Silanikove et al., 2001). Moreover, studies on nitrogen metabolism in lactating cows showed the supplementation of grape pomace is able to decrease the nitrogen urinary excretion due to the capability of condensed tannins to bind proteins and make them unavailable for ruminal degradation (Greenwood et al., 2012).

In vitro studies revealed the capacity of bioactive compounds to limit the methanogenesis, inhibit the deamination of amino-acids and shift fermentation towards propionate and butyrate reducing the environmental impact (Jayanegara et al., 2009; Jayanegara et al., 2013; Oh and Hristov, 2016).

In addition, studies conducted in vivo reported a higher daily gain with the addition of tannins from oak leaves compared to the normal diet (Raju et al., 2015).

The interaction of polyphenols with enzymes and microbial population cause variations in carbohydrates, proteins, and lipids metabolisms.

The ability of plant extracts, including phenols compounds, to modify the utilization of nitrogen depends on the amount of proteins that reach the abomasum, and therefore affect the animal's performance. Rumen microorganisms are able to degrade soluble proteins in order to obtain amino-acids useful for their protein synthesis, but when the rate of degradation is too high the excess of

amino-acids undergoes an oxidative process leading to NH_3 which is absorbed from rumen and excreted through urine. It is clearly understandable that increasing the flow rate of proteins to abomasum together with decreasing of microbial proteolysis can augment the microbial protein synthesis.

In a study conducted by Alipour and Rouzbehan (2010), the ability of grape pomace to reduce rumen degradation of soybean meal proteins in rumen cannulated sheep was evaluated. Results demonstrated a decrease of gas production and an increase of in vitro digestibility of proteins. Moreover, a work of Abarghuei et al. (2010), when replacing dietary alfalfa with grape pomace confirmed its ability of reducing proteins degradation and NH_3 production.

Information on the effect of phenolics compounds on carbohydrate digestion are controversial. Flavonoids are known to interfere with starch digestion through their reaction with gut digestive enzymes and glucose transporter in intestinal brush border. This phenomenon affects the starch digestibility (Bordenave et al., 2014).

It is clear that tannins can complex lignocellulose or directly inhibits cellulolytic bacteria reducing fiber digestion (McSweeney et al., 2001). The previous statement can be confirmed by a study conducted by Al-Dobaib (2009), where the administration of 22,5 g of condensed querbacho tannins in sheep feed reduced the digestion of lucerne hay fiber.

In the study of Abarghuei et al. (2010), it was also reported the reduction of organic matter and neutral detergent fiber digestibility when feeding sheep with grape pomace, probably depending of its high content of lignin. On the contrary, an in vivo study reported the capacity of resveratrol to enhance the digestibility of neutral and acid detergent fiber by an increase of cellulolytic bacteria (Ma et al., 2015).

It is established that diet composition can affect, also, the lipid metabolism. In poligastric animals, fatty acids are metabolized in the rumen with severe modification and then they reach the duodenum as free fatty acids. In rumen, lipids go through enzymatic modification by rumen microorganisms. First, they are subjected to lipolysis which convert esterified fatty acids in free

fatty acids, then they are isomerized and hydrogenated by microbial isomerases and reductases (Doreau et al., 2016).

The extent of biohydrogenation can be modified by the replacement or the addition of polyphenols to the ruminant diet. An in vivo study of Vasta et al., (2010), reported that the inclusion of tannins into lambs' diet caused a shift in microbial population leading to an increment of cellulolytic bacteria such as *Butyrivibrio fibrisolvens* at the expense of *Butyrivibrio proteoclasticus*. Therefore, the last step of biohydrogenation is inhibited. This effect is also confirmed by an in vitro study of Vasta et al., (2009) were cow buffered ruminal fluid was incubated for 12 h with tannins. In this experiment, the inclusion of tannins did not affect the CLA isomers but led to an increase of vaccenic acid. Furthermore, is evident that the effect is greater if tannins are associated with concentrate than with forages. Phenols can also interfere into the synthesis of odd branched fatty acids leading to their reduction as reported by Fievez et al. (2012).

As reported before, winery by-products are known for their antioxidant capacity and recently several studies demonstrated this activity in ruminants.

Flavones can act as scavenger of reactive oxygen species (ROS). By regulating non coding RNAs and toll like receptors, flavones demonstrated also anti-inflammatory and antimicrobial activities; moreover, they can act as anti –cancer substances by leading cancer cells to apoptosis (Jiang et al., 2016).

The inclusion of grape pomace and winery sediment in wethers diet, due to their high level of polyphenols with radical scavenging activity, demonstrated a sudden decrease of urinary 8-hydroxy-2'-deoxyguanosine, an index of oxidative damage (Ishida et al., 2015). As well as, the scavenging ability of grape waste like seeds and peel have been demonstrated in sheep exposed to continuous linseed oil infusion in duodenum (Gladine et al., 2007). In addition, this study revealed that in poligastrics, proanthocyanidin could be hydrolyzed by rumen microorganisms in

bioavailable monomers such as epicatechin because this bioactive compound was found in collected plasma.

The immune system provides a biological marker to evaluate the potential health benefits of dietary supplements in food animal production. Several studies suggested the detrimental role of oxidative stress on immune function even because the redox homeostasis in immune cells produces reactive oxygen species necessarily for their functions (Álvarez et al., 2006; Sordillo and Aitken, 2009). As previously mentioned, the metabolism of polyphenols can affect their bioavailability and this could lead to a discrepancy of results between in vitro and in vivo studies. An in vitro study on the role of phenols on the immune competence revealed that polyphenols from pomegranate are able to increase the secondary humoral response and cytokine synthesis, while these compounds had no effects on neutrophil function and mononuclear cytokine production (Oliveira et al., 2010). Zhong et al. (2014), reported the ability of tannic acid to modulate the immune response of white blood cells in vitro by inhibiting Th1 response in favor of Th2 cytokines expression.

It has been reported that tannins inhibit cells proliferation, cytokines production and modulate T cells differentiation (Gao et al., 2001) while, by regulating the apoptosis, they can keep on balance the homeostasis in physio-pathological condition (Yadav et al., 2009). Studies conducted on Negramaro cultivar highlighted the attitude of polyphenols from grape pomace to up-regulate the expression of Foxp-3 molecules and the release of IL-10 (Marzulli et al., 2014). Among flavonoids, genistein regulated the activity of enzymes involved in inflammatory response such as proliferation of T cells and cells activation (Mustelin et al., 2002), while quercetin reduced the production of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α in LPS stimulated cells (Cho et al., 2003).

Studies conducted by Jiang et al. (2016) on macrophages underlined the ability of apigenin to reduce the phosphorylation of NRkB p65, and therefore the inflammatory cytokine pathway. In addition, to this flavone is attributed the attitude to arrest the cell cycle progression at G1/S and the induction of DNA damage in a PKCδ and p38 dependent pathway, but independent of ROS production (Arango et al., 2012).

Recently, several efforts have been done to enhance the nutritional quality of animal-based food products. As previously reported, polyphenols have the ability to shift the microbial balance in the rumen and this could be reflected on the quality of milk and meat.

Several factors can influence the level of phenolic compounds in milk and dairy products such as pasture, animal metabolism, amino acids catabolism or microbial activity (O'connell and Fox, 2001). Several studies have demonstrated that the level and the type of phenolic compounds found in milk and dairy products are related to the crops administrated to ruminants (King et al., 1998; Besle et al., 2010). As reported by Kuhnen et al. (2014), this association could be used as a fingerprint to trace the animal diet.

Table 1.4.3 shows the average and the relative concentrations of some phenols recover from milk of different ruminant species. These values are related to the basal diet and their concentration can be enhanced by supplementing winery by-products in animal feed. Several studies demonstrated that phenolic compounds such as isoflavonoids and lignans can be transferred to the milk (King et al., 1998; Petit and Gagnon, 2009). Hilario et al., (2010) found the presence of catechin, quercetin, ferulic and chlorogenic acid in goat cheese from milk of grazing animals, whereas Jordán et al., (2010) found polyphenols in milk fed with rosemary extracts.

	Bovine	Caprine	Ovine
Average (mg GAE/L)	49±10,77	69,03±6,23	167,60±58,77
tiophenol	-	+	-
phenol	+	++	+++
o-cresol	+++	+	++
p-cresol	+	+++	++
m-cresol	+	+++	++
2-etilphenol	+	++	-
3(+/or)4-ethylphenol	-	++	-
3,4-dimethylphenol	-	++	+
2-isopropylphenol	-	+	+
3(+/or)4-isopropylphenol	-	+	-
Tymol	+		
Carvacrol	+	++	+++

Tabel 1.4.3: Average ± SEM and relative concentration of phenolics compounds in bovine, caprine and ovine milk respectively (O'connell and Fox, 2001, Vázquez et al., 2015)

The inclusion of polyphenols in ruminant diet aims to improve the milk fatty acids profile through the reduction of biohydrogenation of PUFA by inhibiting the rumen microorganisms metabolism. Results reported in literature are controversial: some authors reported the capabilityof tannins to increase the rumen accumulation of vaccenic acid in vitro (Khiaosa-Ard et al., 2009; Vasta et al., 2009a), while an in vivo experiment of Toral et al. (2013) did not show any increment of vaccenic and rumenic acids in ewes supplemented with different types of tannins. On the contrary, an in vivo study of Vasta et al. (2009b) showed the inhibition of the conversion of vaccenic acid to stearic acid when quebracho tannins are administered to sheep. These results could be related to the type of tannins and their level of administration in animal feeding (Buccioni et al., 2015). In addition, Dschaak et al., (2011) reported an increment of linolenic and total 18:1 trans FA in milk from cows supplemented with quebracho tannins. As previously mentioned, tannins can affect the synthesis of the odd branched chain fatty acids as confirmed by the study by Vasta et al., (2010) who demonstrated that the level of these fatty acids decreased in lambs fed quebracho tannins. Moreover, these fatty acids are so sensible to the action of phenolics compounds that some authors

reported the possibility to use them as predictor of microbial population balance (Vlaeminck et al., 2004).

An in vivo study of Correddu et al., (2015a) demonstrated that the inclusion of tannins from red grape seed in Comisana ewe diet increased the accumulation of rumenic acid and to a lesser extent of vaccenic acid in rumen.

Tsiplakou and Zervas (2008) conducted a study in which Alpine goats and Friesen sheep were fed with olive trees leaves and grape pomace. Results indicated a significant augmentation of PUFA in milk from sheep fed with grape pomace. Moreover, the diet increased markedly the C18:0, the cis-9, trans-11 CLA and the vaccenic acid content in sheep milk.

The addition of grape residue silage (100 g/kg D.M.) in dairy cows fed soybean oil modified the milk fatty acid composition (Santos et al., 2014). The effect was dose-dependent, so the higher the level of grape silage the higher was the increment of PUFA and the reducing power of milk, without modification of total polyphenols and flavonoids concentration. The authors related the higher concentration of PUFA to two different mechanisms. Firstly, the decrement of adipose lipoprotein lipase activity. As suggested by Moreno et al. (2003), grape seed extract can inhibit lipoprotein lipase in adipocytes cultured in vitro and therefore, adipocytes can increment the translocation of fatty acids from adipose tissue to the mammary gland (Van den Top et al., 2005). Secondly, the high level of linoleic acid limited the de novo synthesis of short and medium chain fatty acids in the mammary gland by acting on acetyl-CoA carboxylase (Piperova et al., 2000). In contrast, the addition of two different doses of grape pomace (5g/Kg DM and 10 g/Kg DM) did not interfere with the biohydrogenation in rumen and with the odd branched fatty acids synthesis as demonstrated by the results from an in vivo study of Manso et al. (2016). In addition, the authors did not found any significant differences in the concentration of cis-9,trans-11 C18:2, C18:1 trans-11, and C18:1 trans-10 in ewe's milk.

As concern the total protein content of milk, an experiment by Petacchi and Buccioni (2007) on lactating ewes showed that a pure extract of tannins from chestnut lead to an increment of the heat

coagulable protein content of milk. In discordance to them, Mokni et al. (2017) did not found any significant differences between ewes fed with or without grape skin and seed supplement as confirmed also by Nistor et al., (2014) who administered grape by-products to ruminants. The latter authors attribute these results to the reduction of ruminal protein degradation with direct effect on the protein content of milk.

Phenols are related to sensory quality of milk and dairy products such as the aroma, color and organoleptic attributes, which are quantity dependent. To support this statement several experiments have been done to evaluate the effects of the direct addition of grape by-products on sensory quality of milk and their related products (Chouchouli et al., 2013, Kamble and Kokate, 2015, Torri et al., 2016). The addition of different grape extracts in milk modulated the cheese making characteristics of milk by increasing the rennet-induced clotting time, by decreasing the clotting rate, and by decreasing the syneresis with the increase of grape extracts (da Silva et al., 2015). A study conducted by Correddu et al. (2015b) revealed that the inclusion of grape seed in sheep fed with or without linseed did not affect the level of lipid oxidation products in milk and meat, but was able to reduce the oxidation of unsaturated fatty acids in milk exposed to light. Dietary tannins improved products' flavor by reducing the ruminal biosynthesis of skatole, which is an organic indole compound with strong fecal odor, and its accumulation in milk (Vasta and Luciano, 2011).

Meat and meat products play an essential role in human diet because of their content of macronutrients (proteins and fats) and micronutrients (vitamins and minerals) which are highly bioavailable.

Recent concerns are rising over the last few years about the impact of these products on human health, therefore, meat consumption is markedly decreased. Mainly, the consumers' behavior is attributed to the meat fatty acid profile, which is characterized by the presence of saturated fatty acids that could promote cardiovascular diseases. Nowadays, the research is directed in modifying the nutritional content of meat by acting on feedstuff.

Studies conducted by Jerónimo et al. (2010) did not report any improvement of meat fatty acid profile after the addition of grape seed condensed tannins in the diet of oil-supplemented lambs. Modification of the biohydrogenation pattern are linked to the metabolic pathways and microbial equilibriums in the rumen and therefore to the intramuscular deposition of lipids. Briefly, in this study the authors reported that 25 g/kg of DM of condensed tannins had no effect on biohydrogenation pattern, except for slight changes in minor non-conjugated C18:2 and conjugated C18:3. As concern the lipid compositions of muscle, Vasta et al. (2009c) reported an increased expression of Δ9-desaturase in meat of Comisana ewes fed fresh vetch supplemented with quebracho tannin and a reduction of C18:0 and an increase of C18:1 trans-11 levels in intramuscular fat. A study by Guerra-Rivas et al. (2015a) in sheep at the beginning of lactation, showed that the inclusion of grape pomace (5% and 10% of D.M) increased the level of CLA and vaccenic acid in the intramuscular fat. Accordingly, the same authors found that the addition of 5% of DM of grape pomace in lamb diet was able to enhance the amount of polyunsaturated fatty acid content in their meat (Guerra-Rivas et al., 2013). The authors speculated that these results are related to the presence of linoleic acid in this by-product which can modulate the ruminal biohydrogenation. The inclusion in ruminant diet of different supplements rich in secondary bioactive compounds are able to modify the fatty acid profile of meat. Gravador et al. (2015) reported the ability of tannins from Carob pulp to reduce the level of saturated fatty acids, stearic acid and n-6/n-3 ratio while enhancing the amount of total PUFA, rumenic, vaccenic, and linolenic acid in ovine meat. Mapye et al. (2011) reported a major content of vaccenic acid, linoleic and n-3 PUFA in meat from cow supplemented with Acacia Karroo.

The improvement of fat healthfulness of animal meat by the addition of polyphenols can be also attributed to their antioxidant ability. In the last decade, the main ruminant diet manipulation was the addition of different source of PUFA with the aim of enhancing the level of "healthy" fatty acids in meat (Wachira et al., 2002, Kronberg et al., 2006). Unfortunately, it is well documented that this strategy could increase the oxidative deterioration of meat during storage (Gatellier et al.,

2005, Scollan et al., 2006). The oxidative reactions can lead to off-flavors compounds, which can be translated to a minor acceptance by consumers, and an impairment of nutritional value (Lund et al., 2011). The color of meat is one of the most important aspect considered by consumers and a diet supplemented with polyphenols such as condensed tannins can positively affect this aspect. The translocation of dietary phenols to meat lead to a minor oxidation of myoglobin as suggested by Luciano et al. (2011). Surely, the level of administration and the chemical structure of phenols influenced these phenomena as reported by different authors (Gladine et al. 2007a, Gladine et al. 2007b) who recovered epicatechin in the plasma of sheep that received grape extract directly in rumen. Several studies suggested that polyphenols undergo ruminal degradation and they can reach the blood stream and the tissue as simple monomers (Terrill et al., 1994, Perez-Maldonado and Norton, 1996), while others reported a direct effect on the antioxidant status of animals by acting on the intestinal tract (Halliwell et al., 2005). When grape pomace was added in ewes diet the content of malondialdehyde decreased in lamb meat after refrigeration storage (Guerra-Rivas et al., 2015b).
Studies have explored the use of grape seeds as supplements with the purpose of reducing some negative odors of meat such as faecal, barnyard, and sheepy flavor. Results are controversial; some authors reported a positive impact on meat flavor (Schreurs et al., 2007), while others did not found variation of the level of off-flavor (Jerónimo et al., 2012).

From the above discussion, the addition of grape by-products to ruminants diet it can be considered a good strategy to enhance the productivity and the quality of animal products also in an environmentally friendly approach. In this direction, further studies are required in order to implement balanced diet and novel formulations, which are able to achieve this goal while reducing the anti-nutritive effect of some supplements.

CHAPTER 2

AIM OF DISSERTATION

The aim of the trials described was to evaluate the impact of different extracts from winery by-products rich in bioactive compounds on sheep welfare. The hypothesis was that extracts from winery by-products, taking into account their potential antioxidant capacity, could be used as new functional feed ingredients in animal nutrition to sustain the immune response of sheep and, at the same time, to reduce the impact of these “wastes” on the environment. For this purpose, two different types of wastes were chosen: grape marc (or pomace) and wine lees. To promote our local winery and our regional economy the winery by-products belonged to typical cultivars of grape from our territory: Nero di Troia, Bombino, and Trebbiano d’Abruzzo.

As far as our knowledge, little has been done on the impact of winery by products on sheep immunity.

A brief overview of the trials is reported below.

Trial 1. In the first trial two different concentrations of Bombino/Trebbiano d’Abruzzo and Nero di Troia (red and rosè vinification) wine lees extracts obtained by microwave assisted extraction (MAE) were tested on in vitro ovine pheripheral blood mononuclear cells (PBMCs) and, then, their proliferation and cytokines production were evaluated. Phenolic compounds content and antioxidant capacity were also assessed.

Trial 2. In the second trial Bombino/Trebbiano d’Abruzzo and Nero di Troia (red and rosè vinification) wine lees extracts were further separate by flash liquid chromatography (FLC) and two different fractions were tested on PBMCs during in vitro thermal stress (43°C) and normothermia (37°C) for 48 hours.

Trial 3. For this experiment Nero di Troia grape pomace MAE extracted were tested on sheep PBMCs followed by proliferation assay and cytokines production. Extracted marcs were characterized in term of phenolic compounds content and antioxidant capacity.

CHAPTER 3

FIRST TRIAL

3.1 INTRODUCTION

Phenolic compounds are a class of secondary metabolites produced in plants of great interest because of their health benefits. Polyphenols are ubiquitous in human diet as well as in ruminant diet but, despite that, only recently they come to attention since their antioxidant properties and their effects in disease prevention have been assessed (Vauzour et al., 2010).

Grape is one of the most important agricultural production in Apulia Region, and it is mainly transformed in wine, which is certainly recognized as a beverage rich in phenolic compounds such as antocians, flavonoids and catechins.

As wine, by-products that originates from wine are rich in bioactive compounds (polyphenols); studies report the possibility to add grape seeds extracts to human (Abhijit et al.,2018,) and animal diet (Gladine et al.,2007). Antioxidants have a high impact in preventing the damaging effects of free radicals; moreover, antioxidant metabolites ability to stimulate animals' immune response has been demonstrated (Surai 2014; Karasawa et al., 2011)

In this study we focused our attention on wine lees defined by EEC regulation n°337/79 as "the residue that forms at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product". Wine lees represent 2-6% of total wine production, and a high concentration of polyphenols was reported in their composition (1.2- 19 g/kg) (Galanakis 2017). They contain yeast cells walls, derived from the alcoholic fermentation, bacteria, salt of tartaric acid, vegetable cells and ethanol (Bai et al., 2008, Naziri et al., 2012). Besides that, studies conducted by Barcia et al. (2014) demonstrated that wine lees contain low molecular weight phenols such flavonol, anthocyanins and aglycons with functional properties.

Recently, different studies demonstrated the possibility to extract bioactive compounds from

winery wastes with different methods; new “low impact” methods of extractions such as microwaves assisted extraction (Perez-Serradilla e Luque de Castro, 2011) or ultrasounds extraction (Tao et al., 2015) have taken an advantage on the traditional technique.

The aim of this study was to investigate the immune modulation activity of six different wine lees extracts obtained by microwave assisted extraction on sheep PBMCs proliferation and cytokines production.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Wine lees were provided by local wineries; for this experiment two different cultivars and three vinifications were chosen: Bombino/Trebbiano d’Abbruzzo for White lees and Nero di Troia for Rosè and Red lees. Bombino/Trebbiano d’Abruzzo white lees were provide from the vineyard “La Marchesa” located in Lucera (Foggia, Apulia Region, Italy). Nero di Troia lees were provided from Pirro’s winery situated in Troia (Foggia).

Samples collection was done in November 2015 referred to grape harvested in 2014.

3.2.2 Microwave assisted extraction of wine lees

Microwave-assisted extractions (MAE) were performed in MARS™ 6 (CEM) configured with a 12 positions carousel. White and Rosè lees (5 mL), and Red lees (2.5 mL) were transferred to the teflon extraction vessels. Extractions were performed testing three different solvents: water, water:ethanol 1:1, and ethanol with a total solid:solvent ratio of 1:40. For the catalyzer extraction, 2 mol/kg ss of Na_2CO_3 were added in each position. All vessels were closed and refer vessel was provided of a fibre optic probe to measure the temperature in the systems and a probe to measure the pressure. The operational parameters employed in the MAE apparatus were the following: magnetron power 100%, ramp temperature time 30 min, and maintaining temperature time 10 min. Four temperature programs were tested 50°C, 100°C, 150°C and 200°C. A total of 3 (vinification) x

3 (solvents) x 4 (temperature) x 2 (catalyzer/no catalyzer) extracts were tested. From MAE extraction 72 different extracts were obtained.

3.2.3 Antioxidant capacity assay

3.2.3.1 FRAP Assay

FRAP reagent was prepared according to Francavilla et al. (2013). Briefly, the FRAP reagent was prepared adding 20 mL of TPTZ (10mmol/L) in HCl (40 mmol/L) to 20 mL of $FeCl_3$ (20 mmmol/L) and 200 mL of aceate buffer (0.3mmol/L, pH 3.6) and then it was warmed up to 37°C. The reaction is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous-blue-coloured form in the presence of antioxidants. 100 µL sample of each extract was mixed with 3 mL of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm after incubation at 37 °C for 10 min. For the present study, the standard curve was constructed using Trolox solution (3-120 µM) and the results were expressed as µmol Trolox/g dry weight of extract. Butylated hydroxytoluene (BHT), whose concentration was 0.1 mg/mL, was used as positive control.

3.2.3.2 ABTS Assay

The scavenging activity of wine lees extract was detected by the ABTS [2-2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)] assay according to Francavilla et al. (2013) and Re et al. (1999).

Firstly, the ABTS stock solution was prepared by a reaction of the colorless ABTS 7mM with 2.45 mM of $K_2S_2O_8$. The solution was allowed to stand in the dark for 16h meanwhile the blue-green ABTS+ form was generated. Secondly, the working solution was prepared adding 1 mL of ABTS stock solution to 88 mL of PBS (5mM ph 7.4) and then 2 mL of this solution was added to 200 µl of samples in methanol. In presence of antioxidant the $ABTS^+$ could also be reduced to its colorless form. After 6 min of reaction the absorbance was measured at 645 nm and the results were expressed as mmoL Trolox g^{-1} dry weight of extract. For the present study, the standard curve was

constructed using Trolox solution (3-1200 μM). Gallic acid (GA) at the concentration of 0.1 mg mL^{-1} was used as positive control.

3.2.4 Phenolics determination

Total phenols (TP) were dermined following the Folin-Ciocalteu method according to Francavilla et al., (2013). Briefly, 200 μL of each extract was mixed with 1.5 mL of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min. A 1.5 mL sodium bicarbonate solution (60 g/L) was added to the mixture. After incubation for 90 min at room temperature, the absorbance was measured at 750 nm. For this study the standard curve was constructed using a gallic acid standard solution (25–150 μg/mL in 50% methanol). The results were calculated as gallic acid equivalent (GAE)/g dry weight of extract.

Antocians and flavonoids were determined according to Cliff et al. (2007). Briefly, 0.5 mL of each extract was diluted 1/10 with ethanol 10%, then 0.25 mL of each diluted samples was added to 0.25mL of 0.1% HCl in ethanol 95%and to 4.55 ml of 2% HCl. Each sample was vortexed and allowed to stand for 15 min; then, the absorbance was measured at 520 nm and 360 nm for antocians and flavonoids, respectively. For this study the standard curve of antocians and flavonoids were constructed using malvin standard (oenin chloride 0-250 μg/mL, Sigma-Aldrich) and quercetin (0-100 μg/ml), (Sigma-Aldrich), respectively. The results were expressed as mg/mL of malvin and mg/mL of quercetin respectively for antocians and flavonoids.

Among the 72 extracts obtained, based on the results of the above determination we choose to focus our attention on six extracts: Trebbiano white wine lees extracted in water and ethanol with catalyzer (WhEt/k), Trebbiano white wine lees extracted in ethanol and water (WhEtW), Nero di Troia rosè wine lees extracted in ethanol and catalyzer (RoEt/k) and water and catalyzer (RoW/k) and Nero di Troia red wine lees extracted in water (ReW) and water and catalyzer (ReW/k). Extracts characteristics are reported in table 3.4.1.

3.2.5 5-Hydroxymethil furfural (5-HMF) detection by GC-MS/MS:

5-HMF is a furan compounds originates from Maillard reaction and degradation of hexoses.

To assess whether this compound was contained into the extracts a calibration curve of 5-HMF (Sigma-Aldrich) was constructed measuring the absorbance of known concentrations of 5-HMF standard solutions (0,5-5 g/L in etylacetate/methanol 8:2). Extracts were characterized by gas chromatography-mass (tandem) spectrometry GC-MS/MS analysis (GC-MS/MS Ion Trap 240, Agilent Technologies, Santa Clara, CA, USA). A VF-5 ms (Agilent J&W, Santa Clara, CA, USA) capillary column (30 m × 0.25 mm × 0.25 μm film thickness) was used for the analyses. The GC carrier gas was He (1.0 mL/min). The injector temperature was 250 °C. The ion trap was held at 230 ◦C, the manifold at 80 °C, and the transfer line was 250 °C. The GC-MS was operated in electron ionization (EI) mode over a mass range of 50–800 m/z.

A library search was carried out for all peaks using the NIST 05.

3.2.6 Sulfites determination:

Sulfites determination was carried out using the enzymatic analyzer Miura One (Biogamma) in order to assess whether the antioxidant capacity was to be attributed to the presence of SO_2. The determination of total sulphurous dioxide, was made in basic environment, with the 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, EN023BM15 Biogamma) and read at 416 nm. The free sulphurous dioxide was determined in acid environment with p- fuchsine and formaldehyde to obtain a coloured compound which absorb at 575 nm (EN022BM15 Biogamma). To determine the molecular SO_2, pH of extracts was detected using a pHmeter Crison 20. Table 3.5.3 shows the molecular SO_2 linked to pH and free SO_2.

3.2.7 Organic acids

Tartaric acid, malic acid and lattic acid determination was carried out using the enzymatic analyzer Miura One (Biogamma), to assess whether the antioxidant capacity was to be attributed also to the presence of organic acids. In acid environment tartaric acid reacts with vanadic acid forming the coloured compound metapervanadyl tartrate readable at 492 nm (EN021AM15 Biogamma).

Malic acid content was carried out using the enzymatic kit of Biogamma (EN007BM15). The reaction involved firstly, the oxidation of L-malic to oxaloacetate by NAD^+ in presence of L- malate dehydrogenase (L-MDH) then the enzyme glutamic-oxaloacetic transaminase (GOT) converts the oxaloacetate to L-aspartate in presence of L-glutamate. The amount of L-malic is directly proportionate to the amount of NAD reduced to NADH read at 340 nm.

The determination of lactic acid was carried out using the enzymatic kit of Biogamma (EN00BM15). The reaction involved firstly, the oxidation of L-lactic to pyruvate by NAD+ in presence of L- lactic dehydrogenase (L-LDH), then the conversion of oxaloacetate to L-alanine in presence of L-glutamate by the enzyme glutamic-pyruvate transaminase (GPT). The amount of L-lactic is directly related to the amount of NAD reduced to NADH read at 340 nm.

3.2.8 Animals and experimental treatments

Dairy sheep used in the study were allocated at Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE). In the experimental design sixteen sheep were chosen with a random design, and an in vitro experiment was performed, with two different concentration 0.4 mg/mL and 0.8 mg/mL of each wine lees extracts. Before testing the extracts were dried out in N_2 flow and then suspended into DMSO. All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU (2010) on the protection of animals used for experimental and other scientific purposes. The sheep were healthy and their conditions were carefully examined by veterinarians throughout the trial to exclude the presence of any signs of disease. The in vitro study was performed by the split-plot component involving the isolation of sheep peripheral blood mononuclear cells (PBMC), and the evaluation of their proliferative response and cytokine production after stimulation with the mitogen Concanavalin A and lipopolysaccharide. Wine lees concentrations were chosen according to previous studies of Caroprese et al (2012) on PBMCs treated with bioactive compounds such as phytosterols from *Dunaliella tertiolecta.*

3.2.9 Isolation of PBMC

Blood samples were collected in Na heparinised vacuum tubes from the jugular vein of sheep for the isolation of PBMCs by density-gradient centrifugation according to Ciliberti et al. (2014) with some modification. Each sample was diluted 1:2 in PBS (pH 7.2 at 4°C) and centrifuged 20 minutes at 670 g at 4°C. The buffy coat recovered was suspended in Hank's buffered saline solution (HBSS) with 2% of FBS, 50 µg/ml of gentamicin and 1000U/ml heparin (Sigma Aldrich, Milan, Italy) then layered onto 10 mls of Lymphoprep (density of 1.077 g/mL) (Stemcell) and centrifuges 30 minutes at 1130g at 15°C.

3.2.10 Lymphocyte stimulation assay, in vitro treatment and cytotoxicity-trypan blue dye exclusion

PBMC stimulation assay was performed in a 96 U bottom well plate. In each well 100 µl of cells suspension for each sample was added in quadruplicate. Cells were activated by addition of 50 µl per well of ConA 5 µg/mL + LPS 1 µg/mL (Sigma-Aldrich) (SC, stimulated cells).

To perform in vitro treatment PBMC were treated with 50 µl per well of two different concentration (0.4 mg/mL and 0.,8 mg/ml) of each wine lees from white (Wh), rosè (Ro) and red wine (Re), extracted with water (W), water/ethanol 1:1 (WEt), and ethanol (Et), with or without Na_2CO_3 x 10 H_2O as catalyzer (W/k, WEt/k, Et/k).

Medium only provided negative control wells. The plates were incubated in a humidified incubator with 5% CO_2 at 37 °C for 24 h. After that, plates were centrifuge at 200 g at 37°C for 5 minutes and cell-free supernatant from each well was collected and stored at -80 °C until ELISA to measure cytokine production. All treatments were reinsert as above and to test lymphocyte proliferation 20 µL/well of Bromodeoxyuridine (BrDU) (Exalpha Biologicals, Inc., Shirley, USA) was added. After 18h of incubation, BrDU cell proliferation assay was performed. The plates were spin down at 200 g at 37°C for 1 minutes. Subsequently, 170 µL/well of supernatant were aspirated, the plates were dried out by hairdryer and 200 µl of Fix solution were added. Plates were washed with washing solution provided for 6 times. Following step were executed as reported in BrDU instruction

manual.

BrDU incorporation during DNA synthesis was measured by determining optical density with spectrophotometer (Power Wave XS, Biotek, UK) at 450 nm.

Cells viability was determined by trypan blue exclusion and cells were counted on a hemacytometer considering that the uptake of this acid dye indicated an irreversible membrane damage preceding cell death.

3.2.11 Determination of cytokines in culture supernatants by ELISA-test

IL-6 and IL-1β were detected by ELISA assay into 96-well plates according to Caroprese et al. (2006) with some modification.

All incubations were at 37 °C. Mouse monoclonal antibodies specific for IL-6 and for IL-1β (Clone 4B6 for IL- 6 and Clone 1D4, Serotec Ltd, Killington, UK) were dissolved in carbonate buffer (pH 9.6) at final concentration of 2 μg/mL and used to coat plate overnight at 4 °C.

Plates were washed up 4 times with PBST and 200 μL of blocking solution (3% BSA in PBST) was added in order to block nonspecific binding; then plates were incubated for 1h. Subsequently, 50 μL per well of supernatants were added and incubated for 1 h. 3% of BSA in PBST provided negative control wells. After washing step, 100 μL of rabbit polyclonal anti IL-6 and IL 1β were added and plates were incubated for 60 minutes. To detect the binding of IL-6 and IL-1β a sheep anti-rabbit IgG conjugated with HPR (horseradish peroxidise) was added and optical density was measured at 450 nm. Culture supernatants were read against a standard curve obtained using scalar dilution of recombinant ovine IL-6 (Cusabio Biotech Co., Wuhan, P.R. China) and recombinant bovine IL-1β (Kingfisher Biotech Inc., St Paul, USA). Data were expressed as ng/mL of IL-6 or IL-1β.

The ELISA detection of IL-10 and IFN-ɣ were done according to Kwong et al. (2002) and Caroprese et al., (2014) respectively. Plates were coated with specific monoclonal anti IL-10 (Clone CC318, Serotec; 2μg/mL) and anti-bovine IFN-ɣ (Clone MCA2112 Biorad) at final concentration of 2 μg/mL; then, plates were allowed to stay overnight at 4°C. After washing plates with PBST (0.01%Tween 20 in PBS), blocking solution (BSA 3%in PBST) was added to each well. All steps

were performed at room temperature. Supernatants (50 µL) were dispensed in each well while blocking solution provided negative control wells. After washing step 100µL of Mouse anti IL-10 with biotin (Clone CC320 for IL-10, Serotec, and mouse anti-bovine IFN-ɣ conjugates with biotin (MCA 1783B) were added respectively at 2 µg/mL and 0.5 µg/mL. After 60 minutes at room temperature plates were washed up and added of 100 µL of streptavidin conjugated to horseradish peroxidase (1/500 in PBS, Serotec). BSA 1% in PBST provided negative control for steps above. After 45 minutes 100 µL tetramethyl benzidine (TMB) was added, with 2µL of H_2O_2 and dimethylsulfoxide (DMSO) in phosphate citrate buffer pH 5.0. Plates were incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µL/well of H_2SO_4 (2M) and the optical density was measured at a wavelength of 450 nm.

3.2.12 Apoptosis detection

Sheep BCL2 Associated X protein Elisa kit and a sheep Beta Cell Leukaemia/Lymphoma 2 Elisa kit were used respectively to determine the proapoptotic protein BAX and the anti-apoptotic BCL2 in cells supernatants. All procedures were done following the kit instruction and then optical density was measured at 450 nm. Results were expressed in ng/mL.

3.3. STATISTICAL ANALYSIS

All variables were tested for normality using the Shapiro-Wilk test. Data were analyzed using ANOVA for mixed models using the MIXED procedure of SAS. Concentration, type of extract and their interaction were used as fixed effect. Where significant differences were found ($P<0.05$) Fisher's Least Significant Difference test was used to identify significant differences between means.

3.4. RESULTS

3.4.1 Extracts characteristics

Table 3.4.1 reported the amount of total phenols, antocians, flavonoids and the antioxidant capacity of the extracts in terms of reducing power (FRAP) and scavenging activity ($ABTS^+$). Wine lees extracts have a different total phenols content depends on the type of extraction solvent. White wine lees contains more TP when extracted in EtW, while TP content in Rosè and Red wine lees is higher in W/k than in other extraction solvent. WhEt/k contains the highest concentration of antocians and, at the same time, it displays high FRAP and $ABTS^+$ capacity. WhEtW was chosen thanks to its good FRAP and $ABTS^+$ capacity even though it has a very poor content in antocians and flavonoids. The lowest amount of total phenols was found in extract RoEt/k, while the highest value of antocians was registered into RoW/k. ReW extract reports the highest $ABTS^+$ capacity and lowest FRAP activity. Lastly, the extract ReW/k reports interesting results of all determinations.

3.4.2 Sulphites and organic acids content

Table 3.4.2 reports the amount of total, free and combined SO_2 in mg/L, tartaric acid, malic acid and lactic acids in g/L. The active form, the antioxidant form of sulphur dioxide is the molecular SO_2 on which the concentration of free SO_2 and the pH depended (Ribéreau-Gayon et al., 2006). As reported in table 3.5.2 in our samples the level of molecular SO_2 was negligible.

3.4.3. GC-MS/MS detection of 5-Hydroxylmetylfurfural

Figure 3.4.1 shows the chromatogram relative to extracts ReW. The concentration of 5-hydroxymetylfurfur represent the 26.88% (R.T. 15.323 minutes).

3.4.4 Proliferation of PBMC

Cells proliferation was affected by treatment ($P<0.001$). As reported in Figure 3.4.2 a drastic reduction of PBMC proliferation was registered for all treatments at both concentrations as compared with proliferation of cells in presence of ConA and LPS.

3.4.5 Cytokines production by PBMC

As concern the production of pro-inflammatory cytokines IL-1β and IL-6 (Figure 3.4.3 and 3.4.4), their levels were affected by treatment ($P<0.01$, and $P<0.001$, respectively). IL-1β did not show any significant differences (data not show) except for a reduction of its level in supernatants of PBMCs treated with White wine lees extracted in ethanol and water at concentration of 0.8 mg/mL (Figure 3.4.3). An increment of IL-6 was observed when harvesting cells in presence of Nero di Troia red wine lees in water and catalyser at 0.8 mg/mL (ReW/k 0.8) compared to both not stimulated cells and stimulated cells (Figure 3.4.4). Furthermore, IL-6 levels were higher in supernatants of PBMCs cultivated in presence of ReW/k at 0.4 mg/mL than in not stimulated cells. Lastely, supernatants of PBMC stimulated with Rosè wine lees extracted in water and catalyser at 0.4 mg/mL exerted higher concentration of IL-6 than both stimulated and not stimulated cells; IL-6 levels returned to stimulated cells level return to CNS when cells were harvested with the same extract at 0.8 mg/mL. Therefore, a dose dependent effect for Rosè wine lees extracted with water and catalyser can be hypothesized.

Differently, for both IL-10 and IFN-γ (Figure 3.4.5 and 3.4.6) a marked augmentation of their levels was registered when cells were cultivated with Nero di Troia red wine lees extracted in water and tested at 0.8 mg/mL (ReW) ($P< 0.001$) in comparison to both stimulated and not stimulated cells. In addition, the level of IL-10 was higher in supernatants of ovine PBMC treated with ReW at 0.4 mg/mL than in not stimulated cells. When in presence of ReW at 0.8 mg/mL sheep PBMC produced higher levels of IFN-γ than in presence of each of the other wine lees. A dose-dependent effect for IFN-γ production by wine lees was found when comparing ReW extract at 0.8 mg/mL with 0.4 mg/mL, the highest concentration of ReW exhibiting the highest IFN-γ production.

3.4.6 Apoptosis assay

The levels of pro-apoptotic Bax protein were higher in supernatants of cells treated at 0.8 ng/mL of each extract data not show). Figure 3.4.7a reports the level of Bax in supernatants, as it shows the level of this protein was higher in all extracts compared to CS and NCS, but any significant

difference was found between the two different concentrations of the same extracts except for ReW. As concern the level of Bcl2 (figure 3.4.7b), an augmentation of this protein was found in supernatants of cells treated with WhE/k, RoEt/k and RoW/k all at 0.8 mg/mL. Comparison between Bax and Bcl2 did not show any significant differences except for ReW. Figure 3.4.7c shows the trend of the pro-apoptotic Bax protein versus anti-apoptotic Bcl2 ($P<0.01$ for both) in cells treated with Red wine lees extracted in water. Supernatants of cells cultivated with ReW at 0.8 mg/mL reported a marked increment of pro-apoptotic Bax protein compared to the same extract at 0.4 ng/mL, and to stimulated (SC) and not-stimulated cells (NSC), thus, suggesting a dose-dependent effect of this extract (Figure 3.4.7). Moreover, Bax and Bcl2 levels significantly differed in supernatants from cells stimulated with 0.4 ng/mL of this extract; this difference was nullified at 0.8 ng/mL, as a result of Bax augmentation.

3.5 DISCUSSION

3.5.1 Proliferation of PBMC and cytokines production

Polyphenols are ubiquitous molecules found in plants, seeds, fruit skin and steam; plants were traditionally used as supplements for their higher content in these antioxidant substances. It is widely demonstrated that polyphenols have an antiviral, antimicrobial and antioxidant activity (Cushnie and Lamb 2005, Steinmann et al., 2012). Wine is one of the richest product in term of polyphenols and several studies demonstrate that wine by-products still contain bioactive compounds among which phenols, flavonoids and antocyanins (García-Marino et al., 2006, Barros et al., 2015). In this study six extracts from wine lees were tested for their ability of modulating in vitro sheep immune response.

Polyphenols such as quercetin decreased the NFkB activation (Blanco-Colio et al., 2000) and its gene expression in LPS-stimulated macrophages (Park et al., 2000). As currently known, NFkB regulates PBMC proliferation, cytokines production and receptor expression (Lyu and Park, 2005).

The suppression of PBMC proliferation with all the tested extracts can be linked to the presence in wine lees of bioactive compounds that are able to modulate an inflammatory response against insult such as LPS stimulation, which is the major constituent of the outer membrane of Gram-negative bacteria. A study conducted by Hsiang et al., (2015) on *Toona sinensis* which contains gallic acid, a phenol also contained in winery by-products, demonstrated the ability of this extract to inhibit in vivo and in vitro LPS-induced NFkB. Furthermore, flavonoids are able to inhibit macrophage proliferation, but not cells viability (Magrone and Jirillo, 2010), as happened in our study where the PBMCs had an average of 97-100% of viability as determined by trypan blue exclusion assay. Caroprese et al., (2012) demonstrated that bioactive compounds such as phytosterols from *Dunaliella tertiolecta* were able to interfere with ovine PBMC proliferation. The anti-proliferative response obtained in this current study led us to speculate a potential use of wine lees in animal supplementation to boost their immunity against inflammatory syndromes.

Moreover, it is demonstrated that polyphenols such as apigenin and luteolin can induce the depletion of macrophages/monocytes in PBMC population, phenomenon confirmed by the reduction of pro-inflammatory cytokines (Hougee et al., 2005). This evidence was in accordance with our results in which the levels of pro-inflammatory IL-6 and IL-1β did not undergo any significant increase.

Cho et al., (2002) demonstrated that the bioactive compound quercetin suppresses proinflammatory cytokines production through MAP kinases and NF-κB pathway in LPS-stimulated macrophages. As reported by Zhang et al., (2014) IL-6 production could be down-regulated by the decrement of mRNA stability via inhibiting ERK1/2 activation when cells are triggered by LPS stimulation. The aforementioned mechanisms can be ascribed to different polyphenol features mostly dependent of the position of sugar moiety as confirmed by studies conducted on human PBMC stimulated with two similar flavonoids rutin and quercetin (Cherng et al., 2008). It is well known that both anthocyanins and flavonoids can act as antioxidants thanks to their capacity to give hydrogen ions and to their radical scavenging activity; moreover, they can repair damaged cells and stimulate the

activity of immunocompetent cells (Wu et al., 2017). Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. IL-6 is a powerful cytokine with pleiotropic effects; recently, it has been described as both pro-inflammatory and anti-inflammatory cytokine depending on the context (Hunter and Jones, 2015). Different roles are attribute to this cytokine, such as B cells differentiation, T cell activation, and proliferation (Kishimoto, 2006, Dienz and Rincon, 2009, Tanaka et al., 2014). The slight increment of IL-6 recorded in PBMC stimulated with Red and Rosè wine lees in water and catalyzer can due to the high concentration of antocians in term of malvin extract reported. These results are in accordance with Barak et al. (2001) on in vitro human monocytes treated with different concentration of Sambucol formulations. This is a natural remedy based on a standardized black elderberry extract contains *Sambucus nigra* which is rich in anthocyanins and bioflavonoids.

The high anti-inflammatory capacity of grape and of its derivates is attributed mainly to polyphenols, which are able to reduced inflammatory cytokines such as IL-1β, TNF-α, MCP-1 in macrophages LPS stimulated by inhibiting the factor NFκB through the activation of IKK and the degradation of IκBα (Comalada et al., 2006; Wheeler et al., 2010). Moreover, a direct interference with signalling pathways and gene expression events could be involved (Mauray et al., 2010). In this experiment, the reduction of IL-1β due to wine lees is observed just for one of the six extracts tested, probably because of the short incubation period that was chosen for this trial.

The level of anti-inflammatory cytokines in this study had a marked increment in ovine cells when in presence of Red wine lees extracted in water. IFN-ɣ has a pivotal role in differentiation of B cells, activation of MHC I and II, and antiviral activity (Schroder et al., 2004). The augmentation of this cytokine when PBMC were stimulated with Red wine lees is in accordance with Chiang et al. (2003) that found that the level of IFN-ɣ dramatically increased when human PBMC were cultured in presence of phenolic compounds extracted from Plantago spp. Moreover, in a study conducted by Nair et al. (2002) the flavonoid quercetin was able to upregulates the IFN- ɣ gene expression and production. The authors attributed this capacity to the modulation of Th1/Th2 balance. In our study,

the high level of IFN-ɣ can be attributed to the marked anti-oxidant capacity of Red wine less extracted in water (ReW), as demonstrated by its great capacity to scavenge 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). In support of that, many studies reported the radical scavenging activity of phenolic compounds (Ju et al., 2016; Xu et al., 2016). The anti-inflammatory IL-10 is produced by many cells such as macrophages, dendritic cells, B cells, and various subsets of $CD4^+$ and $CD8^+$ T cells (Iyer and Cheng 2012). Different roles are attributed to this cytokine from the inhibition of MHC II (Couper et al., 2008) to the limitation of pro-inflammatory cytokines such as IL-1α and β, IL-6, TNF-α, and chemokines (Mosser et al., 2008). As aforementioned, the levels of IL-10 in our study were higher in supernatants of cells stimulated with Nero di Troia wine lees extract in a dose dependent manner, probably thanks to the high antioxidant capacity of this extract. In accordance with our results, in a study conducted on human monocytes, the level of IL-6 decreased meanwhile it was observed an upregulation of IL-10 in cells LPS stimulated treated with propolis, dihydrocinnamic acid, p-coumaric and caffeic acid alone or in combination (Cardoso et al., 2017). Propolis contains high level of phenolic compounds, also contained in grape, that exert their antioxidant capacity in term of both ABTS scavenging activity (Miguel et al., 2014) and ferric reducing power (Yang et al., 2011). In addition, treatment with strawberry and mulberry ethanol fruit juice extracts were able to decrease the pro/anti-inflammatory cytokines ratio with an increment of IL-10 in mouse primary splenocytes in presence of LPS.

3.5.2 Production of 5-hydroxymethilfurfural and cell apoptosis

The production of 5-hydroxymethilfurfural originates from the triple dehydration of hexoses; firstly, it was reported as an intermediate of the formation of levulinic acid (Lewkowski 2001), primarily absent in fresh food and beverages. This furfural derived compound is produced in food mainly during the Maillard reaction, a non-enzymatic browning reaction which involves amino acids and reducing sugar. This reaction can be divided into two stages: during the first stage sugars and amino acids condense; then, the Amadori rearrangement occurs with the formation of 1-amino-1deoxy-2

ketose. The Hydroxymethylfurfural (HMF) is produced in the second stage, during the dehydration and fragmentation of sugar molecules (Tamanna and Mahmood, 2015).

There have been a lot of experiments demonstrating the harmful effects of 5-HMF such as genotoxicity, mutagenicity, and carcinogenicity (Fu et al., 2008, Michail et al 2007), even though recently many scientists have reported beneficial effects rather than the harmful ones. In details, it has been suggested that 5-HMF has an antioxidant and antiproliferative action (Zhao et al., 2013), an antiischemic effect (Li et al., 2011), besides its ability to protect human hepatocyte cell line against damages from H_2O_2 (Ding et al., 2010).

In our study the effect of Nero di Troia red wine lees extracted in water (ReW) in term of cytokine production could be attributed to the $ABTS^+$ scavenging activity, ascribed both to the phenol content and to the precense of 5-HMF, which was absent in others extracts. A study conducted by Khodaei and Alizadeh (2017), demonstrated the ability of this furan to increase the level of IFN-ɣ by modulating the balance between Th1/Th2 cytokines. Similarly, the Th1/Th2 modulation by 5-HMF can be the motivation for the IL-10 results. During many infections, such as LPS triggering, $CD4^+$ T cells produce both IFN-γ and IL-10 by effector Th1 cells, to help limiting the collateral damage caused by exaggerated inflammation (Trinchieri, 2007). In addition, 5-HMF has an important role in regulating the apoptotic pathway by acting on BCl2 family and can up-regulates the level of pro-apoptotic Bax and Bad through the mitochondrial intrinsic apoptosis way (Zhao et al., 2014). Accordingly, in our study the level of pro-apoptotic Bax tended to increase in supernatants of cells treated with 0.8 of Nero di Troia wine lees extracted in water, with a dose dependent effect demonstrating the ability of this extract to act on the apoptosis pathway.

3.6 CONCLUSIONS

In this study we investigated the effects of wine lees extracts in term of immunomodulatory properties on peripheral blood mononuclear cells isolated from whole sheep blood.

Results from the present experiment demonstrated the ability of wine lees to affect the immune

responses of sheep PBMC. In addition, bioactive compounds of wine lees extracts, such as phenols, exerted significant antiproliferative effects, besides a beneficial immunostimulatory effect through the induction of the Th1 cytokine IFN-γ.

Moreover, the increment of IL-10 in supernatants of cells cultured in presence of wine lees water-extracts from red vinification of Nero di Troia suggested the anti-inflammatory role of the extracts on sheep immune system. Furthermore, considering the high ABTS scavenging activity of wine lees extracts, it can be speculated that these by-products could be used as a novel natural antioxidant in ruminants' nutrition.

3.7 TABLES AND FIGURES

Table 3.4.1: Characteristics of extracts in term of total phenols (TP), Antocians, flavonoids and their reducing power (FRAP) and scavenging capacity (ABTS). Positive controls for antioxidant assay: butylhydroxytoluene (BHT) and gallic acid (GA).

Extract	T (°C)	Solvent	TP (mg GAE/g extract)	Antocians (mg malvin /g extract)	Flavonoids (mg quercetin /g extract)	FRAP (µmol trolox/g extract)	ABTS (µmol trolox/g extract)
WhEt/k	200	$EtOH+Na_2CO_3$ x 10 H_2O	21.44	626.16	73.36	467.4	870.6
WhEtW	200	H_2O:EtOH	54.8	0.07	7.04	300.24	418.56
RoEt/k	200	$EtOH+Na_2CO_3$ x 10 H_2O	4.62	65.42	10.32	179.2	474.4
RoW/k	200	$H_20+Na_2CO_3$ x 10 H_2O	61.16	922.24	24.24	294.56	92.68
ReW	200	H_20	6.08	88.46	42.88	12.36	1238.4
ReW/k	200	$H_2O+Na_2CO_3$ x 10 H_2O	30.08	469.84	12.16	82.8	215.76
CTR: BHT (µmol/L)	-	-	-	-	-	79.1	-
CTR: GA (µmol/L)	-	-	-	-	-	-	220.1

Table 3.4.2: pH, total SO2, free SO2, combined SO2 and Tartaric, Malic and Lactic acid contents of the extracts. Combined SO2 was calculated as: Total SO2 - Free SO2.

Extract	pH	Total SO_2 (mg/L)	Free SO_2 (mg/L)	Combined SO_2 (mg/L)	Tartaric acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)
WhEt/k	8	58	2	58	1.48	0.08	4.3
WhEtW	9	38	2	36	0	0.6	2.6
RoEt/k	8	25	3	25	1.21	<0.05	0.16
RoW/k	9	37	3	34	0	0.14	0.24
ReW	3	19	1	18	0	0.66	0.22
ReW/k	9	19	1	18	0	0.24	0.19

Table 3.4.3: Molecular SO_2 per 100 parts of Free SO_2 in function of pH (Usseglio-Tomasset, 1995)

pH	Molecular SO_2
3	6.06
3.2	3.91
3.4	2.51
3.6	1.6
3.8	1.01
4	0.64
4.2	0.41
4.4	0.26

Figure 3.4.1 GC-MS/MS chromatogram of 5-Hydroxymethylfurfural in extract ReW (Ritention time: 15.323 minutes).

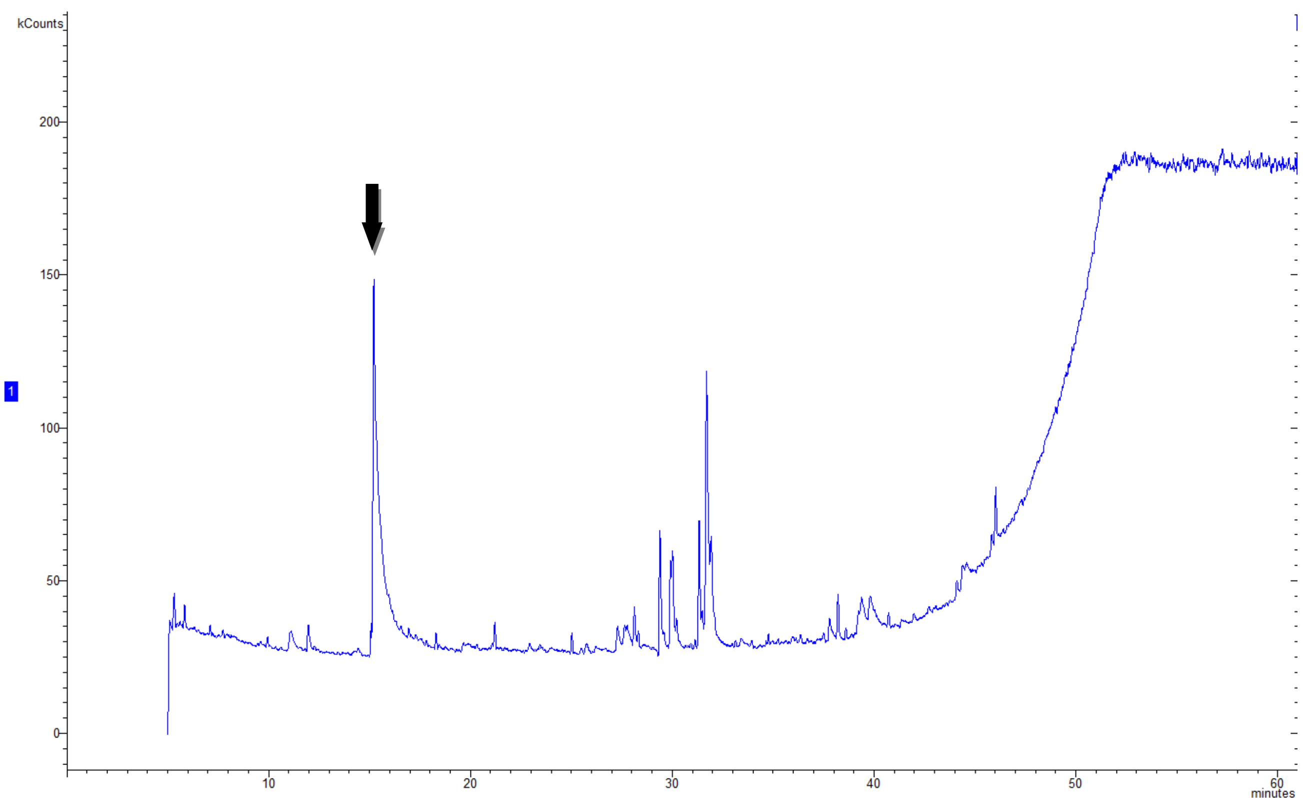


Figure 3.4.2 Proliferation of sheep PBMC (Least Squares means ± SEM) following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

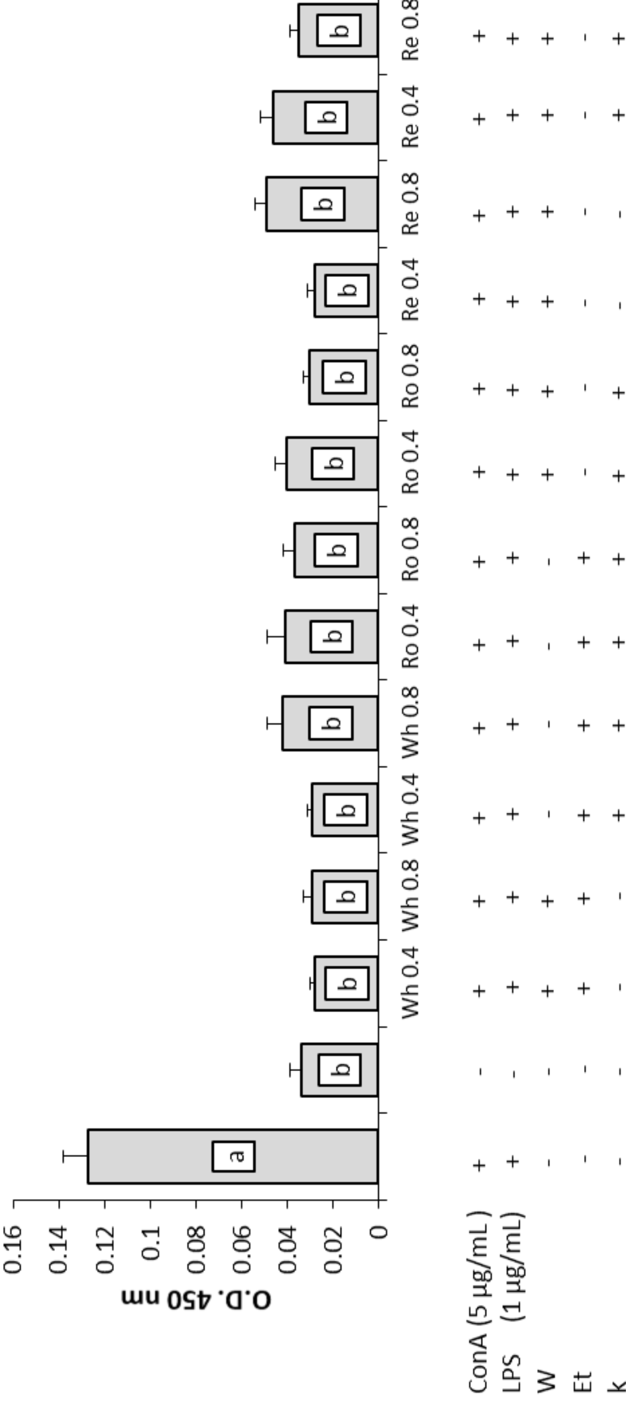


Figure 3.4.3 Least Squares means ± SEM of IL-1β secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

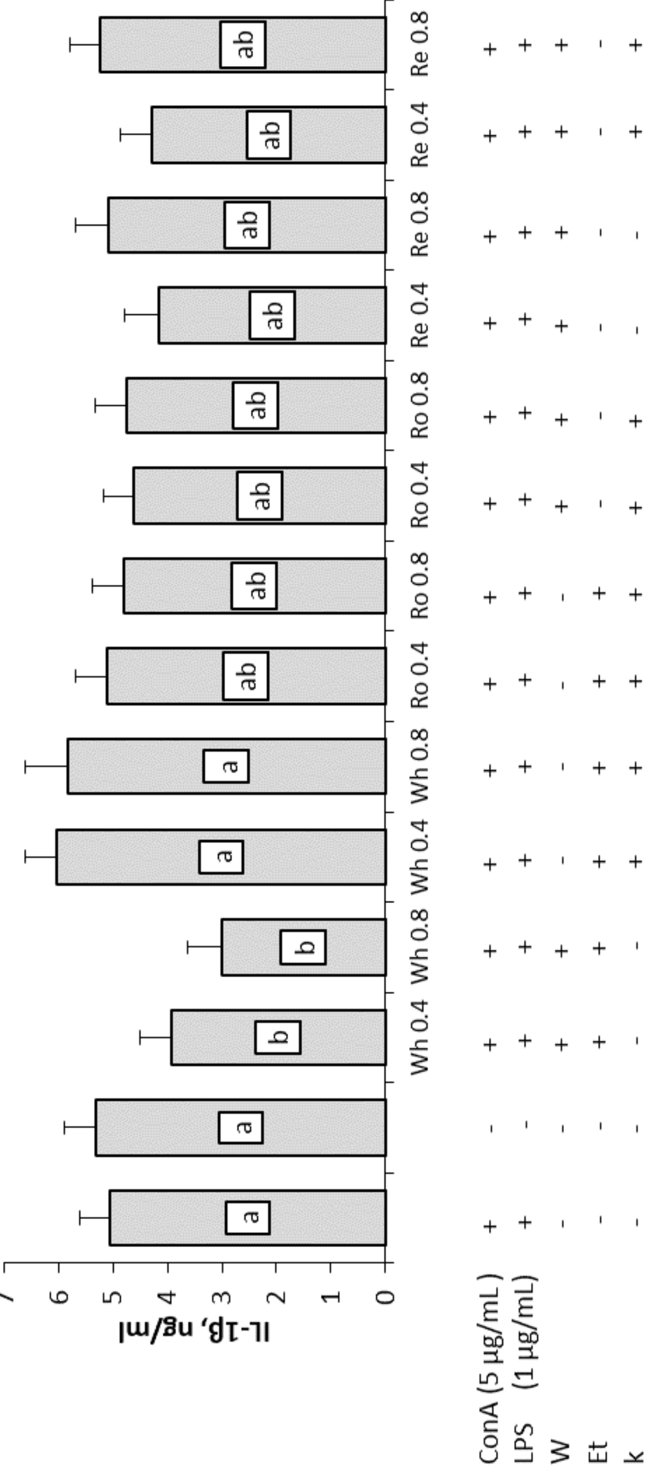


Figure 3.4.4 Least Squares means ± SEM of IL-6 secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

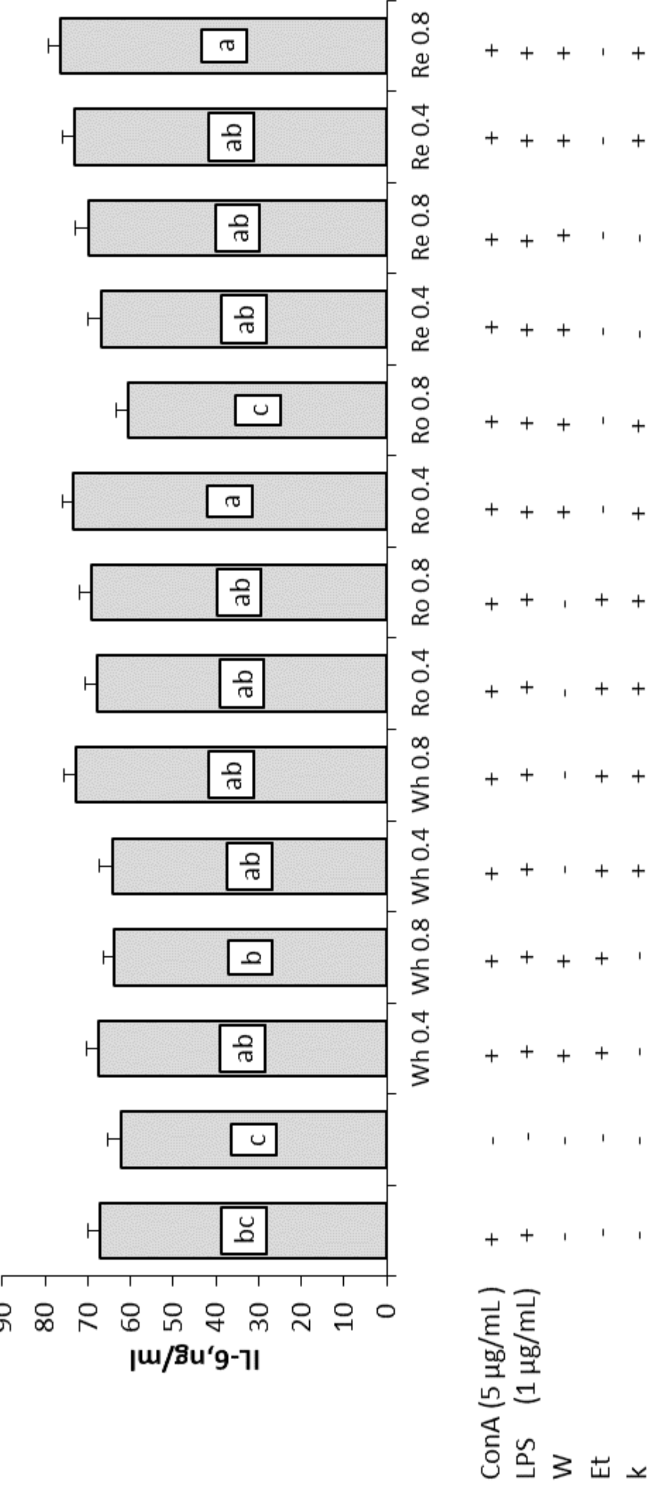


Figure 3.4.5 Least Squares means ± SEM of IL-10 secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

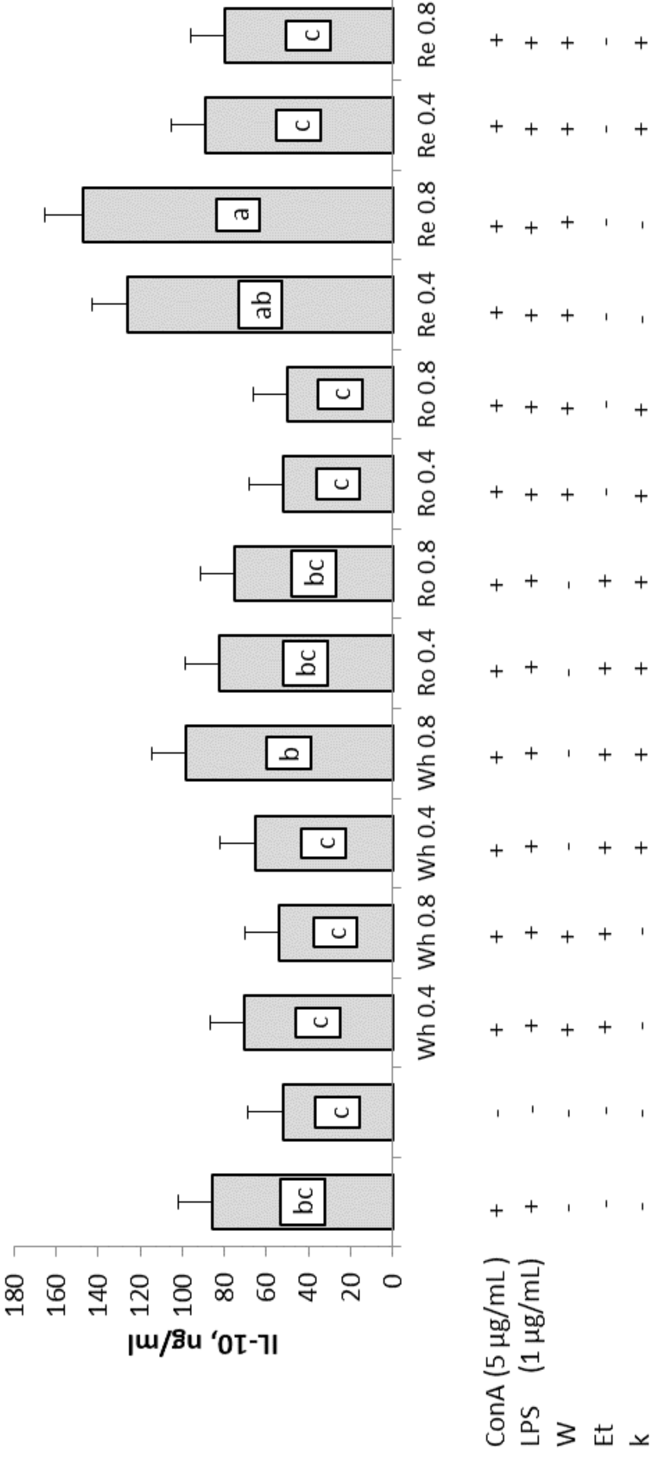


Figure 3.4.6 Least Squares means ± SEM of IFN-γ secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

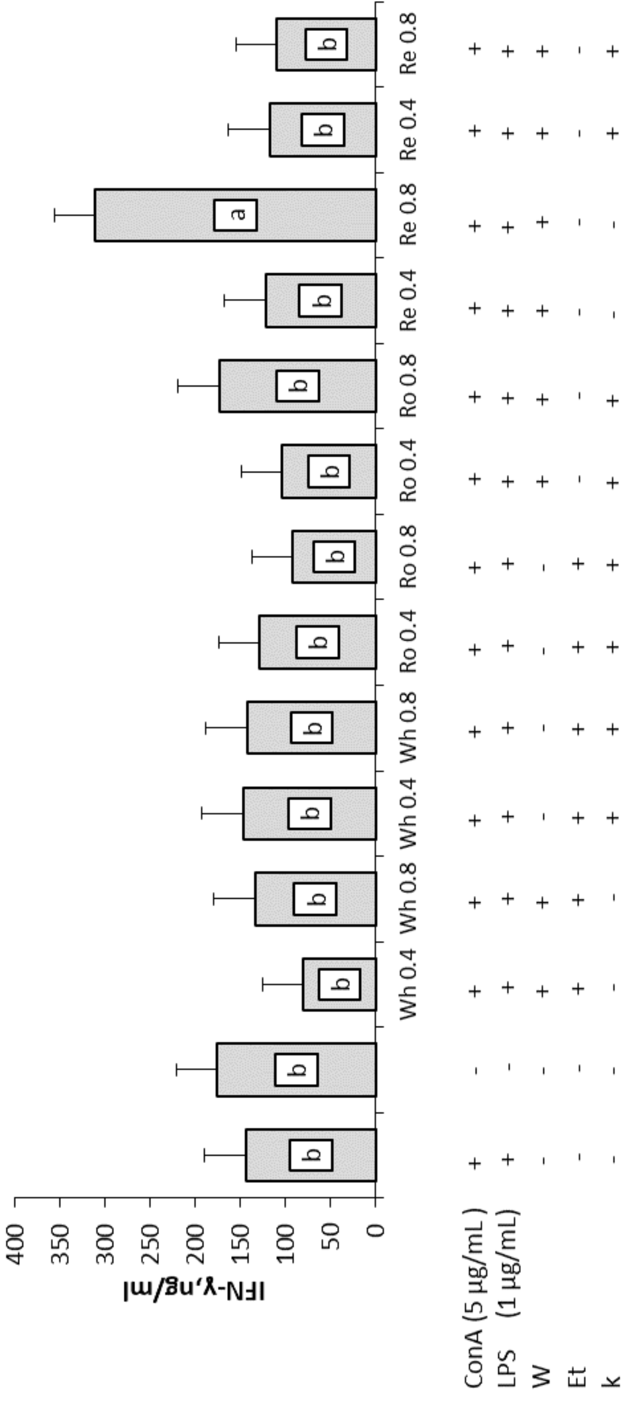


Figure 3.4.7a Least Squares means ± SEM of Bax protein in supernatants of PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

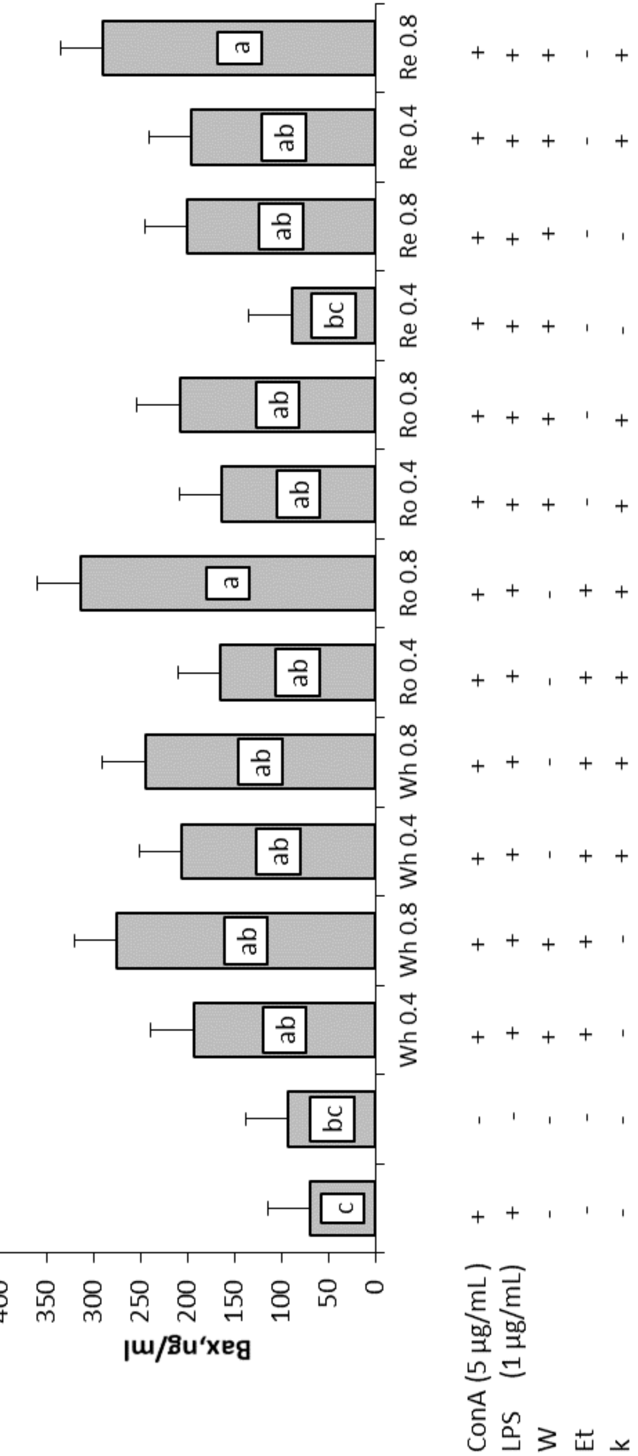


Figure 3.4.7b Least Squares means ± SEM of Bcl2 protein in supernatants of PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).

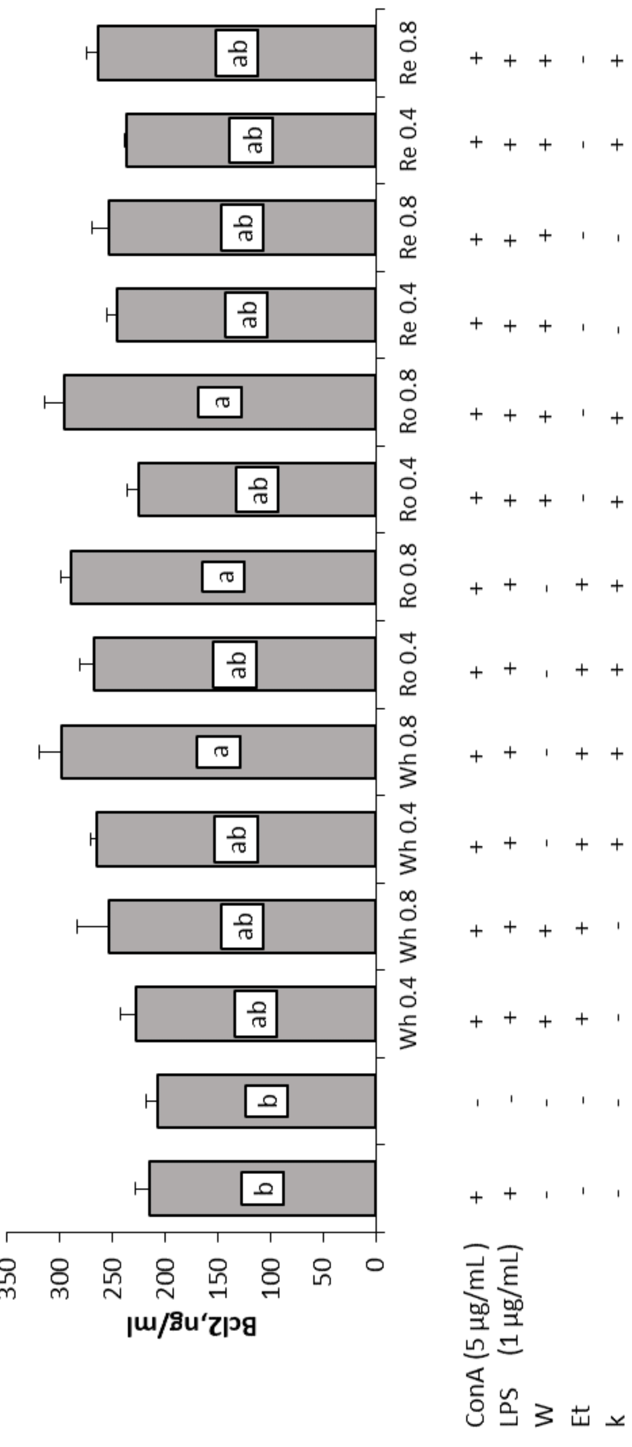
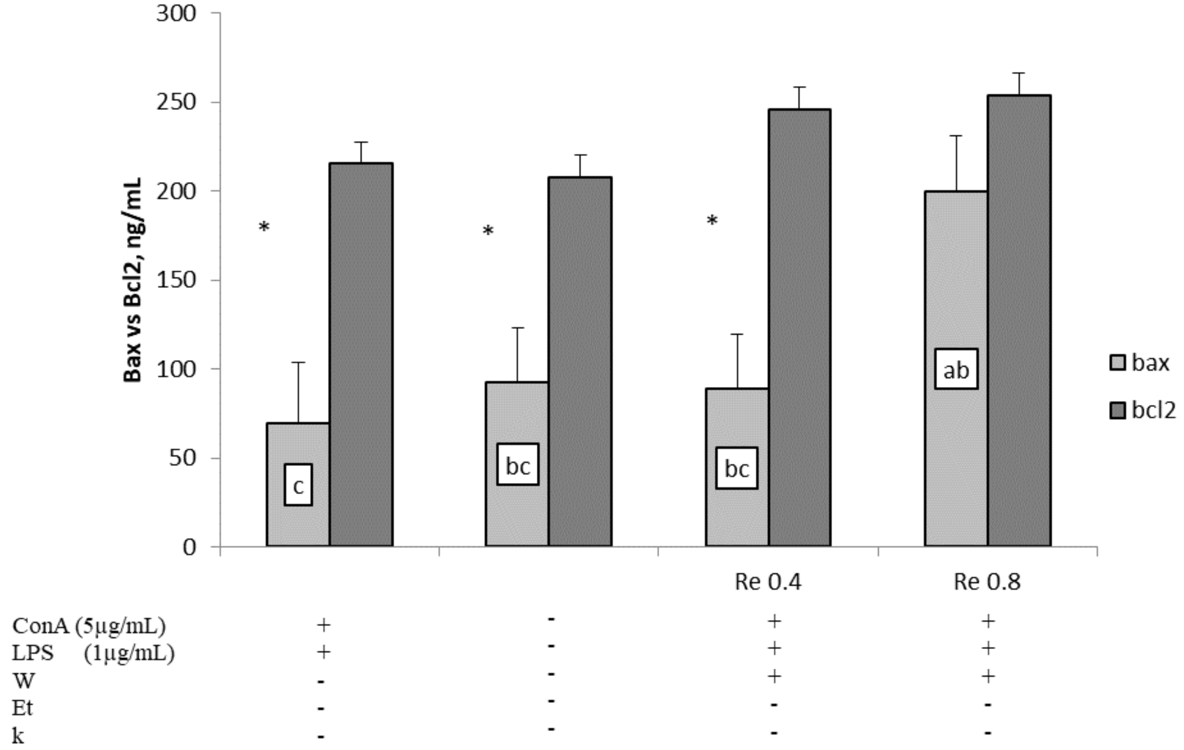


Figure 3.4.7c Least Squares means ± SEM of Bax and Bcl2 protein in supernatants of PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees extracts and in presence of Concanavalin A and LPS. [a, b] Values with different letters differ ($P < 0.05$).



CHAPTER 4

SECOND TRIAL

4.1. INTRODUCTION

Stress can trigger the immune system activating inflammatory processes aroused by both infective and non-infective stressors. A non-infectious inflammation can intervene when inflammatory processes are not provoked by pathogens; therefore, a complex of physio-pathologic phenomena are exhibited in absence of microorganisms. This inflammatory status is named "sterile inflammation" by Fleshner (2013). Similarly to infectious syndromes, sterile inflammation evolved as a physiological response to tissue damage and represents the first step of a series of events aimed at restoring the homeostasis (McDonald and Kubes, 2016). The cellular damaged-molecules are normally recognised as self by the immune system; however, when released in extracellular space they can evoke an immune response comparable to the infectious one. These molecules named "damage associated molecular patterns – DAMPS".

Recently, there is an increasing interest in obtaining new natural ingredients able to modulate the immune responses by reducing inflammation syndromes trough the modulation of the production of cytokines or immune cell activity.

Wine is one of the most widely consumed beverages in the world estimated at 242 million of hectolitres in 2016 (OIV, April 2017), 2,050,000 litres just in Italy in 2015 (TDA, 2015) which is the greatest producer of wine with 39.3 million of hl in 2017 (OIV, 2017).

Such a large and heavily industrialised winery supply chain produces a large amount of different wastes. Our attention focused on wine lees, a wine by-product obtained after the vinification processes. Data reported by the International Organisation of Vine and Wine (OIV, 2015) indicated that Italy produces 2.250.000 hectolitre of wine lees (equal to 5% of wine produced), which are abundant in bioactive compounds with several protective activities such as antioxidant, antimicrobial and anti-hyperlipidaemia (Landeka et al., 2017; Ye et al., 2014).

Scientists focused their attention on the possible reuse of the winery by-product with the aim of obtaining functional feeds or, ingredients useable in cosmetics and pharmacology. These recovery activities are essential to realize a virtuous system with the aim to improve the sustainability of the whole supply chain and to reduce the environmental impact.

In recent times, a new class of bioactive compounds, derived from plants and animals' proteins and also from yeasts and bacteria metabolisms, is attracting considerable attention. Diketopiperazines (DKP), small cycle peptides which is synthetized starting from aminoacids by mammals and microorganisms like secondary metabolites, belong to this class of bioactive compounds (Delaforge et al., 2001). DKPs class enumerate several molecules with different biological activities such as anti-prion antiviral, antibacterial, antifungal, and anti-tumoral (Bolognesi et al., 2010; Kwak et al., 2013; Musetti et al., 2007; Li et al., 2006; Nicholson et al., 2006).

Cyclic dipeptides or cyclodipeptides (CDPs), also called 2,5-diketopiperazines are found in processed food and beverage, (Borthwick et al., 2017), mainly in fermented beverages like in sherry (Oruna-Concha et al.,2015) and in coffee and cocoa (Ginz et al., 2000; Stark et a., 2005), being responsible also for their typical flavour.

This study aimed to evaluate the potential appliance, as immunomodulators, of a cluster of diketopiperazine from wine lees on sheep PBMCs. Two different experiments were performed: i) sheep PBMCs cultured in thermoneutral condition for 48 hours; ii) sheep PBMCs cultured under heat stress for 24 hours.

4.2. MATERIAL AND METHODS

4.2.1 Extracts selection

A GC-MS/MS (method described in 4.2.3) screening of all 72 extracts obtained from wine lees extractions (method described in 3.2.2) let us to identified the precense of diketopiperazine family. Based on this finding we decided to focus our attention on supernatants from MARS extractions in water at 200°C (data not show).

4.2.2 Flash liquid column separation

MARS supernatants from white, rosè and red wine lees extracted in water at 200°C undergo, as fast as possible, to three extractions in ethylacetate/methanol 8/2 in a separating funnel than the extracted phase was concentrate with Rotavapor (Buchi) and purified by flash liquid chromatography (FLC) according to Francavilla et al., (2012) with some modification. A column for FLC (h 70 cm × Ø 2 cm, Sigma Aldrich, Milan, Italy) was packed with silica gel (30Å, 200-300 mesh, Sigma-Aldrich). It was run in gradient elution with etylacetate and methanol ad follow: 100% of etylacetate, ethylacetate/methanol 9/1, ethylacetate/methanol 8/2, ethylacetate/methanol 7/3, ethylacetate/methanol 1/1, ethylacetate/methanol 1/2 and methanol 100%. The extract/silica ratio was 1/60; the run was set under N_2 flow at 2 bars with a corresponding elution flux of 4 mL min^{-1}.

4.2.3 Gas chromatography-mass spectrometry characterization

Extracts were characterized by gas chromatography-mass (tandem) spectrometry GC-MS/MS analysis (GC-MS/MS Ion Trap 240, Agilent Technologies, Santa Clara, CA, USA). A VF-5 ms (Agilent J&W, Santa Clara, CA, USA) capillary column (30 m × 0.25 mm × 0.25 μm film thickness) was used for the analyses. The GC carrier gas was He (1.0 mL/min). The injector temperature was 250 °C. The ion trap was held at 230 ◦C, the manifold at 80 °C, and the transfer line was 250 °C. The GC-MS was operated in electron ionization (EI) and chemical ionization (CI) mode over a mass range of 50–800 m/z. The chemical ionization mode was used to confirm the molecular weight (M + 1) of compounds. A library search was carried out for all peaks using the NIST 05.

Based on results from GC-MS/MS (data reports in results section) wine lees fractions were merged together as follow:

White wine lees extracted in water fractions 1 to 16, fractions 17 to 36, fractions 37 to 43 fractions 44 to 54 fractions 55 to 61, fractions 62 to 70 and fractions 71 to 106.

Rosè wine lees extracted in water fractions 1 to 12, fractions 13 to 24, fractions 25 to 35, fractions 36 to 40, fractions 41 to 47, fractions 48 to 54 and fractions 55 to108.

Red wine lees extracted in water fractions 1 to 11, fractions 12 to 24, fractions 25 to 30, fractions 31 to 34, fractions 35 to 40, fractions 41 to 50 and fractions 51 to 103.

4.2.4 Isolation of PBMC

Blood samples were provided by Segezia research station of the Council for Research and Experimentation in Agriculture (CRA-ZOE). All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. During the entire trial sheep were examined, by veterinarians, to exclude any sign of disease. Blood samples were collected in Na heparinised vacuum tubes from the jugular vein of sheep for the isolation of PBMCs by density-gradient centrifugation according to Ciliberti et al. (2014) with some modification. Each sample was diluted 1:2 in PBS (pH 7.2 at 4°C) and centrifuged 20 minutes at 670 g at 4°C. The buffy coat recovered was suspended in Hank's buffered saline solution (HBSS) with 2% of FBS, 50 µg/ml of gentamicin and 1000U/ml heparin (Sigma Aldrich, Milan, Italy) then layered onto 10 mls of Lymphoprep (density of 1.077 g/mL) (Stemcell) and centrifuges 30 minutes at 1130g at 15°C.

4.2.5 In vitro thermoneutrality assay for 48h

4.2.5.1 Lymphocyte stimulation assay, in vitro treatment and cytotoxicity-trypan blue dye exclusion

PBMC stimulation assay was performed in a 96 U bottom well plate. In each well 100 µl of cells suspension for each sample was added in quadruplicate. Cells were activated by addition of 50 µl per well of ConA 5 µg/mL + LPS 1 µg/mL (Sigma-Aldrich) (SC, stimulated cells). PBMC were treated with 50 µl of wine lees fraction treatments per well at two different concentration 0.4 mg/mL and 0.8 mg/mL. Tested fractions were: white wine lees extracted in water at 200°C fractions 17-36 (WhF1) and fractions 44-54 (WhF2), rosè wine lees extracted in water at 200°C fractions 12-24 (RoF1) and fractions 41-47 (RoF2) and red wine lees extracted in water at 200°C fractions 12-24 (ReF1) and fractions 41-50 (ReF2). Medium only provided negative control wells. After incubation at 37°C for 48 hours, plates were centrifuge at 200 g at 37°C for 5 minutes and cell-free supernatant

from each well was collected and stored at -80 °C until ELISA to measure cytokines production. All treatments were reinserted as above and to test lymphocyte proliferation 20 µl/well of Bromodeoxyuridine (BrDU) (Exalpha Biologicals, Inc., Shirley, USA) was added. After 18h of incubation, BrDU cells proliferation assay was performed. The plates were spin down at 200 g at 37°C for 1 minutes than 170 µl/well of supernatant was aspirated, the plates were dried out by hairdryer and added 200 µl of Fix solution. Plates were washed with washing solution provided for 6 times. Following step were executed as reported inside BrDU instruction manual. BrDU incorporation during DNA synthesis was measured by determining optical density with spectrophotometer (Power Wave XS, Biotek, UK) at 450 nm.

Cells viability was determined by trypan blue exclusion and cells were counted on a hemacytometer considering that the uptake of this acid dye indicates an irreversible membrane damage preceding cell death.

4.2.5.2 Cytokines' determination

IL-6 and IL-1β were detected by ELISA assay into 96-well plates according to Caroprese et al., (2006) with some modification.

All incubations were at 37 °C. Mouse monoclonal antibodies specific for IL-6 and for IL-1β (Clone 4B6 for IL- 6 and Clone 1D4, Serotec Ltd, Killington, UK) were dissolved in carbonate buffer (pH 9.6) at final concentration of 2 µg/ml and used to coat plate overnight at 4 °C.

Then, plates were washed up 4 times with PBST and 200 µl of blocking solution (3% BSA in PBST) was added in order to block nonspecific binding, then plates were incubated for 1h. Subsequently, 50 µl per well of supernatants were added and incubated for 1 h. 3% of BSA in PBST provided negative control wells. After washing step, 100 µl of rabbit polyclonal anti IL-6 and IL 1β were added and plates were incubated for 60 minutes. To detect the binding of IL-6 and IL-1β a sheep anti-rabbit IgG conjugated with HPR (horseradish peroxidase) was added and optical density was measured at 450 nm. Culture supernatants were read against a standard curve obtained using

scalar dilution of recombinant ovine IL-6 (Cusabio Biotech Co., Wuhan, P.R.China) and recombinant bovine IL-1β (Kingfisher Biotech Inc., St Paul, USA).

Data were expressed as ng/mL of IL-6 or IL-1β.

The ELISA detection of IL-10 and IFN-ɣ were done according to Kwong et al. (2002) and Caroprese et al., (2014) respectively.

Plates were coating with specific monoclonal anti IL-10 (Clone CC318, Serotec; 2µg/mL) and anti-bovine IFN-ɣ (Clone MCA2112 Biorad) at final concentration of 2 µg/ml then plates were allowed to stay overnight at 4°C. After washing plates with PBST (0.01%Tween 20 in PBS) blocking solution (BSA 3%in PBST) were added to each well. All steps were performed at room temperature. 50 µL of supernatants were disposed in each well while blocking solution provides negative control wells. After washing step 100µL of Mouse anti IL-10 with biotin (Clone CC320 for IL-10, Serotec, and mouse anti-bovine IFN-ɣ conjugates with biotin (MCA 1783B) were added respectively at 2 µg/mL and 0.5 µg/mL.. After 60 minutes at room temperature plates were washed up and added of 100 µl of streptavidin conjugated to horseradish peroxidase (1/500 in PBS, Serotec). BSA 1% in PBST provided negative control for steps above. After 45 minutes 100 µl tetramethyl benzidine (TMB) was added, with 2µl of H_2O_2 and dimethylsulfoxide (DMSO) in phosphate citrate buffer pH 5.0. Plates were incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µl/well of H_2SO_4 (2M) and the optical density was measured at a wavelength of 450 nm.

4.2.6 In vitro thermal stress assay

4.2.6.1 Lymphocyte stimulation assay, in vitro treatment and cytotoxicity-trypan blue dye exclusion

For the in vitro thermal stress experiment two different temperatures, 39°C and 43°C, were chosen. The first temperature, 39°C, was adopted to simulate normothermia, while 43°C to mimic a severe hyperthermia, according to Lacetera et al., (2006) with some modification. These authors tested an exposure at 39°C continually and 3 cycles of 13h at 40, 41, 42 and 43°C interspersed with 2 cycles

at 39°C. Cells were exposed to 39°C continuously (T39). In each well 100 µl of cells suspension for each sample was added in quadruplicate. Cells were activated by addition of 50 µl per well of ConA 5 µg/mL + LPS 1 µg/mL (Sigma-Aldrich) (SC, stimulated cells). PBMC were treated with 50 µl of wine lees fraction treatments per well at two different concentration 0.4 mg/mL and 0.8 mg/mL. Tested fractions were: white wine lees extracted in water at 200°C fractions 17-36 (WhF1) and fractions 44-54 (WhF2), rosè wine lees extracted in water at 200°C fractions 12-24 (RoF1) and fractions 41-47 (RoF2) and red wine lees extracted in water at 200°C fractions 12-24 (ReF1) and fractions 41-50 (ReF2). Medium only provided negative control wells. After incubation, as described above, plates were centrifuge at 200 g at 37°C for 5 minutes and cell-free supernatant from each well was collected and stored at -80 °C to measure cytokines production with ELISA assay. All treatments were reinserted as above then 20 µl/well of Bromodeoxyuridine (BrDU) (Exalpha Biologicals, Inc., Shirley, USA) was added to test lymphocyte proliferation. BrDU cells proliferation assay was performed as in the previous experiment. BrDU incorporation during DNA synthesis was measured by determining optical density with spectrophotometer (Power Wave XS, Biotek, UK) at 450 nm.

Cells viability was determined by trypan blue exclusion and cells were counted on a hemacytometer considering that the uptake of this acid dye indicates an irreversible membrane damage preceding cell death.

4.2.6.2 Cytokines' determination

IL-6 and IL-1β were detected by ELISA assay according to Caroprese et al., (2006) with some modification, into 96-well plates

All incubations were at 37 °C. Mouse monoclonal antibodies specific for IL-6 and for IL-1β (Clone 4B6 for IL- 6 and Clone 1D4, Serotec Ltd, Killington, UK) were dissolved in carbonate buffer (pH 9.6) at final concentration of 2 µg/ml. Coated plates were incubated overnight at 4 °C.

The following day, plates were washed up 4 times with PBST and 200 µl of blocking solution (3% BSA in PBST) was added, then plates were incubated for 1h. Subsequently, 50 µl per well of

supernatants were added and incubated for 1 h. 3% of BSA in PBST provided negative control wells. After washing step, 100 µl of rabbit polyclonal anti IL-6 and IL 1β were added and plates were incubated for 60 minutes. To detect the binding of IL-6 and IL-1β a sheep anti-rabbit IgG conjugated with HPR (horseradish peroxidase) was added and optical density was measured at 450 nm. Culture supernatants were read against a standard curve obtained using scalar dilution of recombinant ovine IL-6 (Cusabio Biotech Co., Wuhan, P.R. China) and recombinant bovine IL-1β (Kingfisher Biotech Inc., St Paul, USA).

Data were expressed as ng/mL of IL-6 or IL-1β.

The ELISA detection of IL-10 and IFN-ɣ were done according to Kwong et al. (2002) and Caroprese et al., (2014) respectively.

Plates were coating with specific monoclonal anti IL-10 (Clone CC318, Serotec; 2µg/mL) and anti-bovine IFN-ɣ (Clone MCA2112 Biorad) at final concentration of 2 µg/ml then plates were allowed to stay overnight at 4°C. After washing plates with PBST (0.01%Tween 20 in PBS) blocking solution (BSA 3%in PBST) were added to each well. All steps were performed at room temperature. 50 µL of supernatants were disposed in each well while blocking solution provides negative control wells. After washing step 100µL of Mouse anti IL-10 with biotin (Clone CC320 for IL-10, Serotec, and mouse anti-bovine IFN-ɣ conjugates with biotin (MCA 1783B) were added respectively at 2 µg/mL and 0.5 µg/mL. After 60 minutes at room temperature plates were washed up and added of 100 µl of streptavidin conjugated to horseradish peroxidase (1/500 in PBS, Serotec). BSA 1% in PBST provided negative control for steps above. After 45 minutes 100 µl tetramethyl benzidine (TMB) was added, with 2µl of H2O2 and dimethylsulfoxide (DMSO) in phosphate citrate buffer pH 5.0. Plates were incubated in the dark at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µl/well of H2SO4 (2M) and the optical density was measured at a wavelength of 450 nm.

4.2.6.3 Mitocondrial health

Healthiness of mitochondrial membrane is crucial for Ca^{2+} uptake and storage, reactive oxygen species generation, and detoxification, the synthesis of ATP by oxidative phosphorylation. Therefore, mitochondrial membrane depolarization is a good indicator of mitochondrial dysfunction, which is a distinctive feature of the early stages of apoptosis. For this reason cells from both experiment were tested for mitochondrial integrity by a EVOS FL Cell Imaging System (Thermo Fisher) using the HCS Mitochondrial Health Kit (Invitrogen).

4.3 STATISTICAL ANALYSIS

All variables were tested for normality using the Shapiro-Wilk test. In the first experiment, proliferation data were analysed using ANOVA for mixed models using the MIXED procedure of SAS. Concentration, type of extract and their interaction were used as fixed effect. Where significant differences were found ($P<0.05$) Fisher's Least Significant Difference test was used to identify significant differences between means. Cytokines production were analysed using a ONE-WAY ANOVA of SAS. In the second experiment proliferation and cytokines production were analysed using ANOVA for mixed models using the MIXED procedure of SAS. Concentration, type of extract, temperatures and their interaction were used as fixed effect. Where significant differences were found ($P<0.05$) Fisher's Least Significant Difference test was used to identify significant differences between means.

4.4 RESULTS

4.4.1 GC-MS/MS

Two sequential GC-MS/MS analysis demonstrated that diketopiperazine were stable after the solvent extraction carried out in separating funnel. Figure 4.4.1a and b report GC-MS/MS results concerning the two different fractions of White wine lees extracted in water at 200°C WhF1 and WhF2 respectively, while results of the two different fractions of rosè wine lees extracted in water

at 200°C RoF1, RoF2 are reported in figure 4.4.2 a and b. Lastely, figure 4.4.3a and 4.4.3b report results relative to the two different fractions of red wine lees extracted in water at 200°C (ReF1, ReF2).

4.4.2 Proliferation and cytokines production of PBMC cultured for 48h at 37°C

PBMC proliferation decreased for all wine lees fractions ($P<0.001$) compared to stimulated cells with a proliferation even lower than not-stimulated cells at 0.4 mg/mL of White and Red lees F1 and with Rosè wine lees F1 at both concentrations (Figure 4.4.4).

The pro-inflammatory cytokines IL-1β and IL-6 (Figure 4.4.5 and 4.4.6) did not show any significant differences, even though a reduction of IL-6 concentration was observed when cells were treated with White wine lees F1 at both concentrations, RoF1 and ReF1 at 0.8 mg/mL, and RoF2 and ReF2 at both concentrations when compared to not-stimulated cells. Differently, an augmentation of IL-10 ($P<0.001$) was recorded when PBMCs were cultivated in presence of White wine lees Fraction 2 at both 0.4 and 0.8 mg/mL compared to ConA and LPS stimulated cells and not stimulated cells. A decrement of this cytokine was found in supernatants of cell harvested in presence of almost all extracts except for WhF1 0.8 mg/mL, ReF1 0.4 mg/mL and Red wine lees F2 at both concentrations (Figure 4.4.7).

The production of IFN-ɣ was not affected by the treatment (Figure 4.4.8).

4.4.3 Proliferation and cytokine production of PBMC under heath stress

Cells proliferation was affected by treatment ($P<0.001$), temperature ($P<0.001$) and their interaction ($P<0.001$). As reported in figure 4.4.9 a reduction of PBMC proliferation was registered for all treatments at both concentrations as compared with proliferation of cells cultivated in presence of ConA and LPS at both 39°C and 43°C. Moreover, this reduction was more marked when cells were stimulated with F1 of all three wine lees, while the reduction of cells proliferation was attenuated when cells were treated with the fraction F2 of all extracts except for RoF2 0.8 mg/mL. Lymphomonocytes proliferation differed significantly between 39 and 43°C when cells were

harvested with White wine lees F2 at both concentration, Rosè lees F2 at 0.8 mg/mL and Red wine lees F2 at both concentration.

As concern the production of interleukins, IL-1β was affected only by temperature ($P < 0.001$) with a significant difference between the two temperatures with all treatments at both concentrations (Figure 4.4.10). The level of IL-6 in supernatants cultivated with wine lees fractions was affected by temperature and the interaction between temperature and treatment ($P < 0.05$). In condition of thermoneutrality IL-6 levels decreased in supernatants of cells cultivated in presence of Rosè wine lees F1 0.8 mg/mL compared to Rosè F2 0.4 mg/mL and to Red wine lees F1 at 0.4 mg/mL, while at 43°C the fraction 2 of each extract at both concentration led to a decrement of the level of this cytokine compared to stimulated cells and at the same level of non-stimulated PBMC. The same effect was registered for supernatants of cells harvested with Rosè and Red wine lees F1 respectively at 0.4 mg/mL and 0.8 mg/mL. Results are reported in Figure 4.4.9.

The anti-inflammatory cytokine IL-10 concentration was affected by treatment ($P<0.001$) temperature ($P<0.001$) and their interaction ($P<0.001$) (Figure 4.4.12). A marked reduction of this cytokines was registered at 39°C for all wine lees fractions compared to stimulated cells and not-stimulated cells. The levels of this cytokines were different between the thermoneutrality and hyperthermia in CS, NCS and when cells were stimulated with White wine lees F1 and F2 at 0.4 mg/mL, Red wine lees (Re) F1 at 0.4 mg/mL and ReF2 at both concentration.

The level of IFN-ɣ was affected only by temperature ($P<0.05$) (Figure 4.4.13). The highest level was recorded when cells were treated with Red wine lees fraction 2 administered at 0.8 mg/mL compared to White wine lees and Rosè wine lees F1 at 0.8 mg/mL and with RoF2 0.8 mg/mL and Red wine lees F2 at 0.4 mg/mL.

4.4.4 Mitochondrial health

Results of cells viability of heat stress trial are reported in table 4.4.1 Cell cultured in precense of extracts undergone a decrement of mithocondrial membrane potential as indicated by an increment

of fluorescence green staining. This is more evident when cells were treated with the highest concentration of wine lees, except for Re F1 at 43°C

4.5 DISCUSSION

This study was undertaken to assess the immunomodulatory effects of a mixture of isomers of diketopiperazines, separated by FLC on MAE extracts of wine lees, on ovine PBMCs.

Cyclic peptides are formed by two amino acid residues linked to a central diketopiperazine (DPK) ring structure and their biological activity derived from the substitution of amino side-chain groups with any of the twenty-endogenous l-α amino acids (Milne and Kilian, 2010).

Microwave assisted extraction (MAE) can be considered a valid alternative to traditional extraction methods; it is a cost-effective technique that combines microwave and solvent extraction with numerous advantages such as reducing time of extraction, increasing kinetic of extraction, and above all a low environmental impact. Many authors reported the usage of this extraction method on various matrices (e.g. plants or seed) to obtain natural bioactive compounds (Lucchesi et al., 2004; Terigar et al., 2010). Nowadays, microwave assisted extraction can be used to obtain diketopiperazines, also using water as solvent, thanks to the high pressure and temperature generated during the reaction, with the results of having a great yield of these bioactive compounds (Pérez-Picaso et al., 2009; Tullberg, 2006).

To the best of our knowledge, no studies have investigated the opportunity to apply the MAE technique to oenological by-products in order to obtain cyclic peptides.

The marked reduction in lymphomonocyte proliferation observed can be explained with the high potential immunomodulatory activity of wine lees fractions thanks to their content in diketopiperazine mainly in pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl). The different impact of fractions F1 and F2 on monocytes proliferation could be linked to the different isomers distribution in these two fractions. Pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl) also known as Cyclo(leucil-prolyl) or Cyclo(Leucil-Propyl) is a

peptide derivatives of cyclic dipeptides and diketopiperazine with empirical formula $C_{11}H_{18}N_2O_2$. In agreement with our study Lalitha et al. (2016) demonstrated that Cyclo(Leu-Pro) reduced the proliferation of HeLa and A059 cancer cells in dose-dependent manner. Furthermore, another study reported the antiproliferative activity of two different 2,5-diketopiperazines on activated T cells and the authors attributed this effect to a cytotoxic mechanism (Hartung et a., 2011). Our current experiment, where the cells viability decreased in presence of wine lees fractions, was on the same wavelength of the above-quoted study. The cytotoxic effect could be associated to the arrest of cells cycle as demonstrated by Wang et al.(2015) who found that the indole diketopiperazines, isolated from *Chaetomium cochliodes* were able to determine the cell cycle arrest at the G2/M phase and to activate the caspase-3. Furthermore, diketopiperazine can modulate Bax, Bcl-2 and Bcl-xL to induce the mitochondrial-mediated apoptosis (Choi et., 2011).

Stress can influence cellular gene expressions and metabolism, inhibit the cell cycle progression and the cells permeability leading to the "heat shock" or cellular stress response (Collier et al., 2006, Belhadj Slimen et al., 2015). PBMC proliferation is an important indicator of lymphocyte function and several studies demonstrated that heat stress can have an impact on immune system (Do Amaral et al., 2011, Collier et., 2008). Lacetera et. al (2005) demonstrated that animals exposed to high temperatures, such as during summer season, had a depression of immune system; moreover, high temperature can affect blastogenesis of PBMCs and the DNA synthesis in mitogen cultured cells (Kelley et al., 1982; Lacetera et al., 2006). Accordingly, our study demonstrated a proliferation impairment when cells were cultured in condition of hyperthermia, even though a reduction of PBMC proliferation also occurred in normothermia conditions.

The release of inflammatory cytokines, among which IL-6, IL-1β and TNF-α, is the mechanism achieved by immune system to restore homeostasis after an episode of stress.

It is likely that heat stress affects negatively human health and animal welfare (Pearce et al., 2014) with negative consequences on agricultural economy (Key et al., 2014). Guijarro-Munoz et al. (2014) demonstrated that in pigs exposed to heat stress in presence of LPS for 2, 4, or 6 hours a

modulation of NF-kB signalling occurred. NFkB regulates inflammatory immune responses through the transcription of gene responsible for inflammatory mediators such as ILs. In normal condition NFkB is bounded to its inhibitor IkB, which is phosphorylated following inflammatory stimulus such as LPS (Hayden and Ghosh, 2008). From the above consideration NFkB is a critical factor implicated in immune inflammation response and it could be a good target for bioactive anti-inflammatory compounds. The role of DKP on pro-inflammatory cytokines has been largely investigated. The decrease of IL-6 production was achieved in cells supernatants in presence of wine lees fraction rich in Cyclo(Leucil-prolil) in condition of hyperthermia. These results go along with results obtained by Nalli et al., (2017), where IL-6 levels were affected by the presence of a cyclic-peptide containing a residue of proline.

IL-1β resulted higher in supernatants of heat stressed PBMC then in condition of normothermia and was not drive by the presence of wine lees fractions. On the contrary in a study conducted by Kim et al. (2013), IL-1β levels were attenuated when macrophages were stimulated with LPS in presence of different concentration of Neoechinulin A, a diketopiperazine obtained from marine fungus Eurotium sp. SF-5989. Furthermore, these authors demonstrated that DKP are able to reduce inflammatory mediators by inhibiting the NFkB binding and p38 MAPK pathways.

It is widely demonstrated that during thermal stress occurs a shift from Th1 immune-driven response to Th2 with a subsequent increment of Th2 cytokines leading to an augmentation of IL-10 at the expense of IFN-ɣ. Cyclic dipeptides are reported to up-regulate the level of both IL-10 and IFN-ɣ in cells cultured for 44 hours. Results reported herein showed a lower level of IL-10 in thermal stressed cells and in cells cultured at 39°C let us to speculate that the immune response was still in the early phase. Accordingly, our in vitro simulation of heat stress resulted in a higher production of IFN-ɣ from PBMCs compared to the levels in supernatants from cells in condition of normothermia.

4.6 CONCLUSIONS

As the best of our knowledge this is the first study where oenological by-products are investigated as a good matrix to obtained natural bioactive compounds like diketopiperazines interesting for their biological activity.

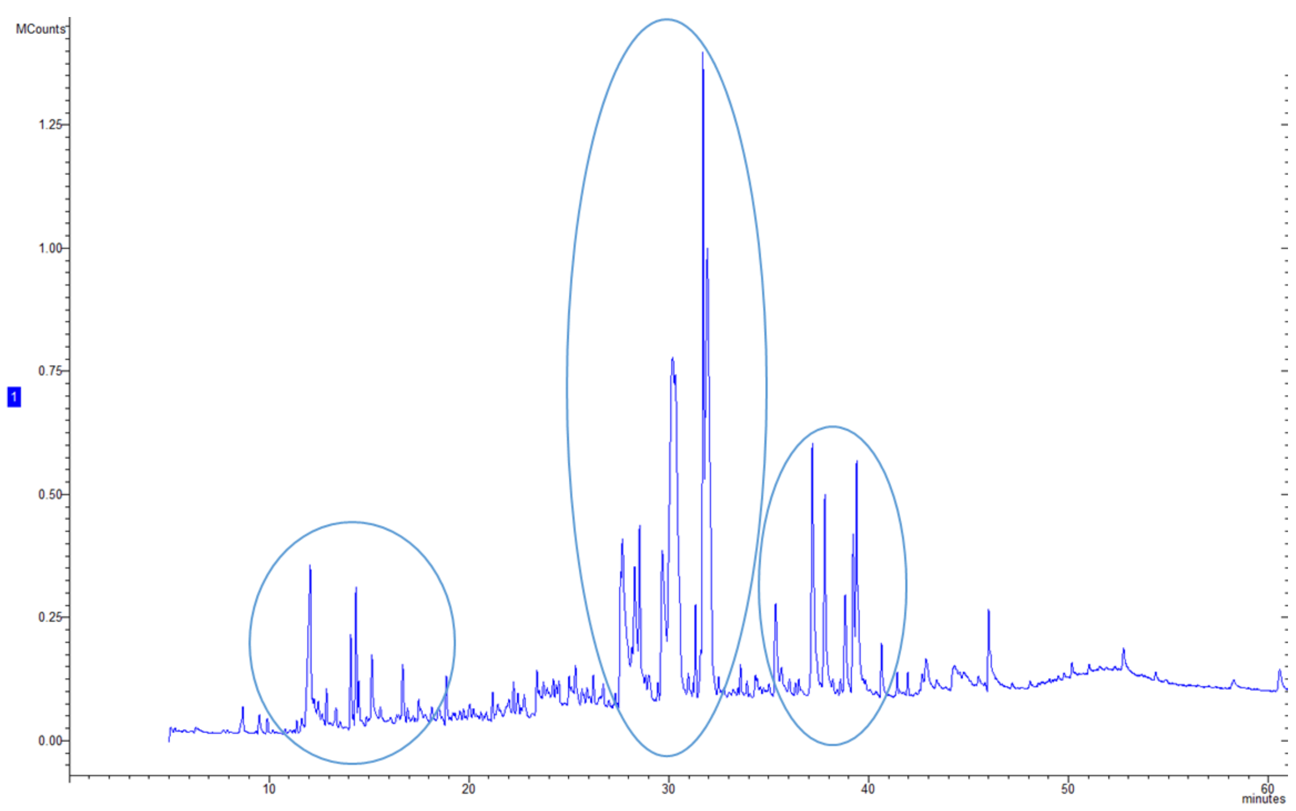
Results from the normothermia assay report a reduction of lymphomonocytes proliferation in addition to a reduction of anti-inflammatory cytokines. These outcomes, added to the increment of the anti-inflammatory IL-10 show off the capacity of certain extracts to drive the immune system and let us to hypothesize a possibile reuse of wine lees waste as a new source of anti-inflammatory compounds useful in animal nutrition.

The reduced proliferation, obtained in the heat stressed cells trial, in addition to the reduced viability obtained in this study can lead us to speculate the possible role of Cyclo(leucyl- propyl) a cyclic diketopiperazine as a chemotherapeutic agent.

This role can be supported thanks to the ability of DKPs to modulate the immune response, even in stressful situation such as during heat stress by up-regulating anti-inflammatory cytokines and down-regulating inflammatory mediators such as IL-6.

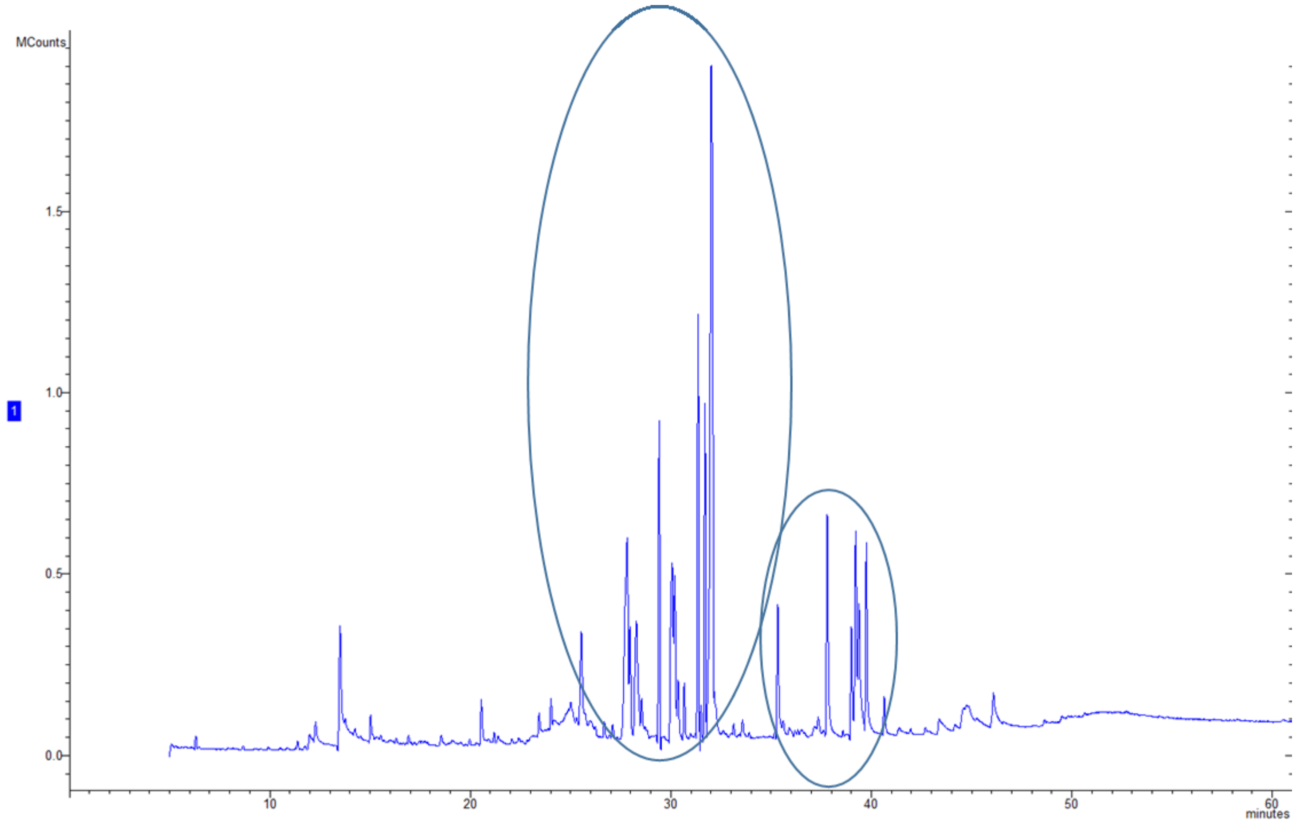
4.7 TABLES AND FIGURES

Figure 4.4.1a: Chromatogram of White wine lees fraction F1. Fraction compositions is reported in table below.



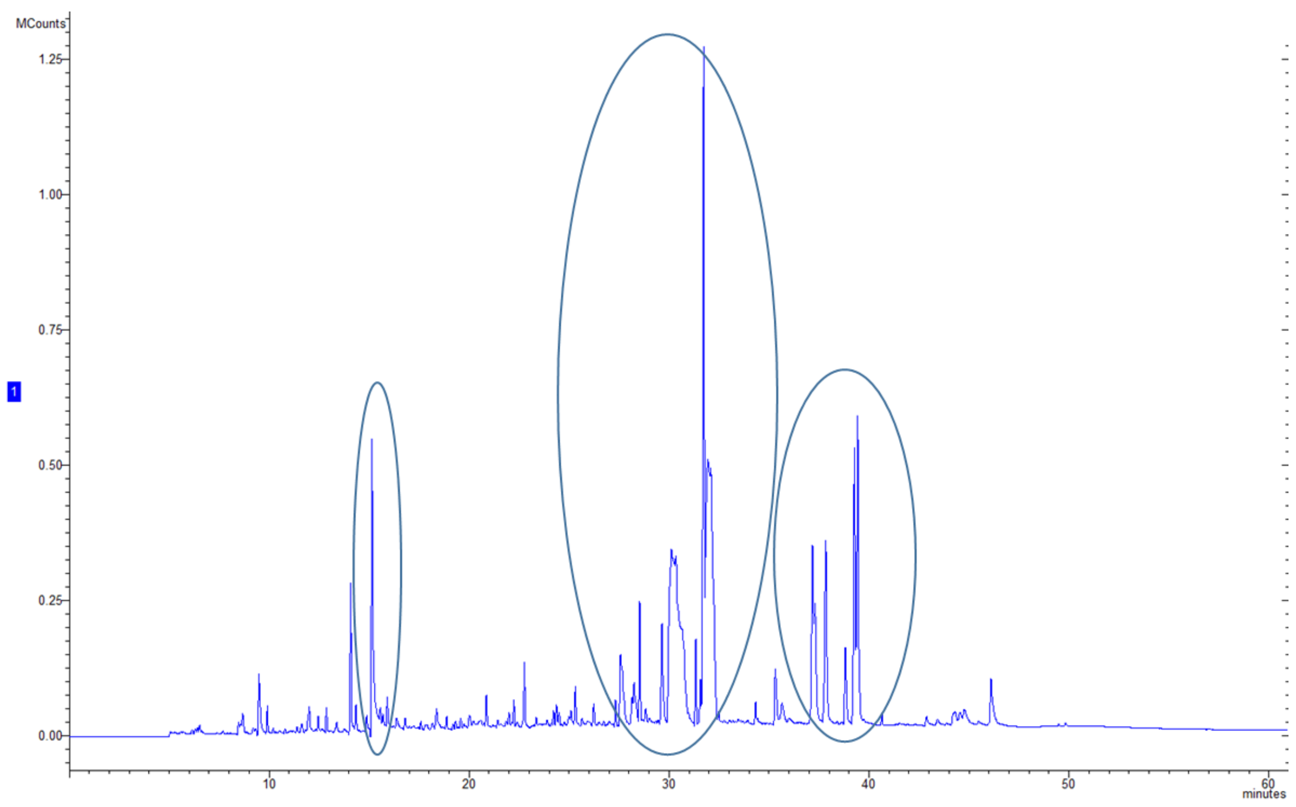
RT	Peak Name	% Amount
8.69	Pyrazine, 2-ethyl-5-methyl-	0.197435336
23.4	2-Acetylpyrido[3,4-d]imidazole	0.400577397
27.573	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)	1.263897112
27.678	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)	3.268059134
28.162	3,6-Diisopropylpiperazin-2,5-dione	0.261844573
29.68	3,6-Diisopropylpiperazin-2,5-dione	5.092725484
30.204	3,6-Diisopropylpiperazin-2,5-dione	21.84099748
31.339	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	0.972495173
31.722	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	8.510570661
31.924	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)	15.30045867
35.32	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)	0.755170478
37.18	2,5-Piperazinedione, 3-benzyl-6-isopropyl	4.148206878
37.805	2,5-Piperazinedione, 3-benzyl-6-isopropyl	3.653046403
38.812	Cyclo-(l-leucyl-l-phenylalanyl)	1.672404504
39.22	Cyclo-(l-leucyl-l-phenylalanyl)	2.46094756
39.391	Cyclo-(l-leucyl-l-phenylalanyl)	4.245694072

Figure 4.4.1b: Chromatogram of White wine lees fraction F2. Fraction compositions is reported in table below.



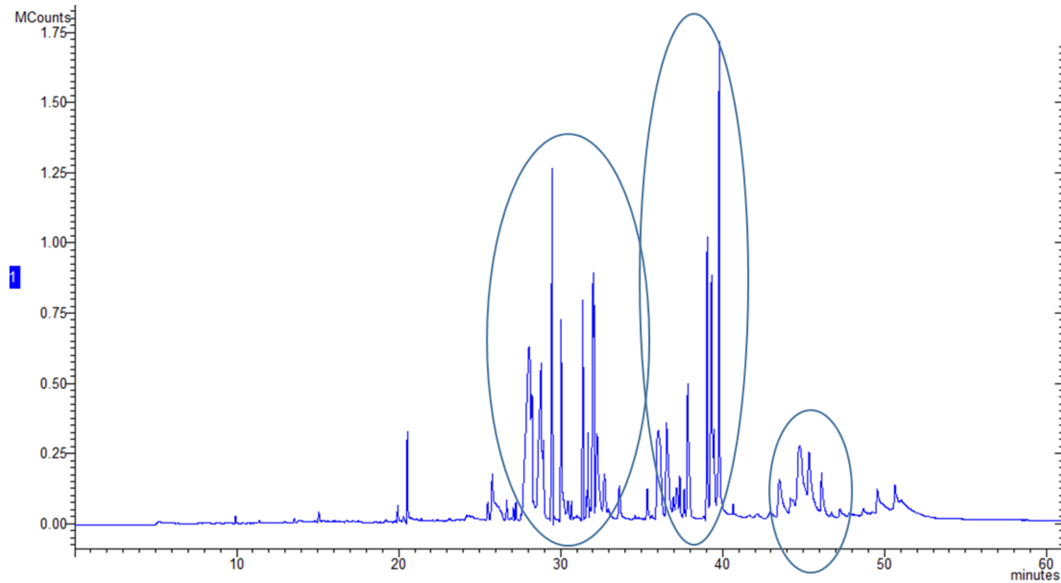
RT	Peak Name	% Amount
24.025	Piperidine, 1-(2-methyl-1-propenyl)-	0.41924073
25.549	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	2.202456579
27.837	3-Butyl-6-methylpiperazine-2,5-dione	10.27210349
28.242	3,6-Diisopropylpiperazin-2,5-dione	4.224319685
28.543	Cyclo-(glycyl-l-leucyl)	0.65102041
29.428	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	5.154996322
30.072	3,6-Diisopropylpiperazin-2,5-dione	8.941781213
30.358	N-(6-Methoxy-2-methyl-3-pyridinyl)acetamide	1.157333183
31.368	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	7.038833505
31.501	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	0.488837827
31.71	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	5.348823122
31.864	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	1.05638792
32.016	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	22.3600083
35.339	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	2.834385049
37.801	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	4.415533805
39.009	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	2.146698185
39.221	Cyclo-(l-leucyl-l-phenylalanyl)	4.461999134
39.381	Cyclo-(l-leucyl-l-phenylalanyl)	3.342848503
39.747	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	3.495520298

Figure 4.4.2a: Chromatogram of Rosè wine lees fraction F1. Fraction compositions is reported in table below.



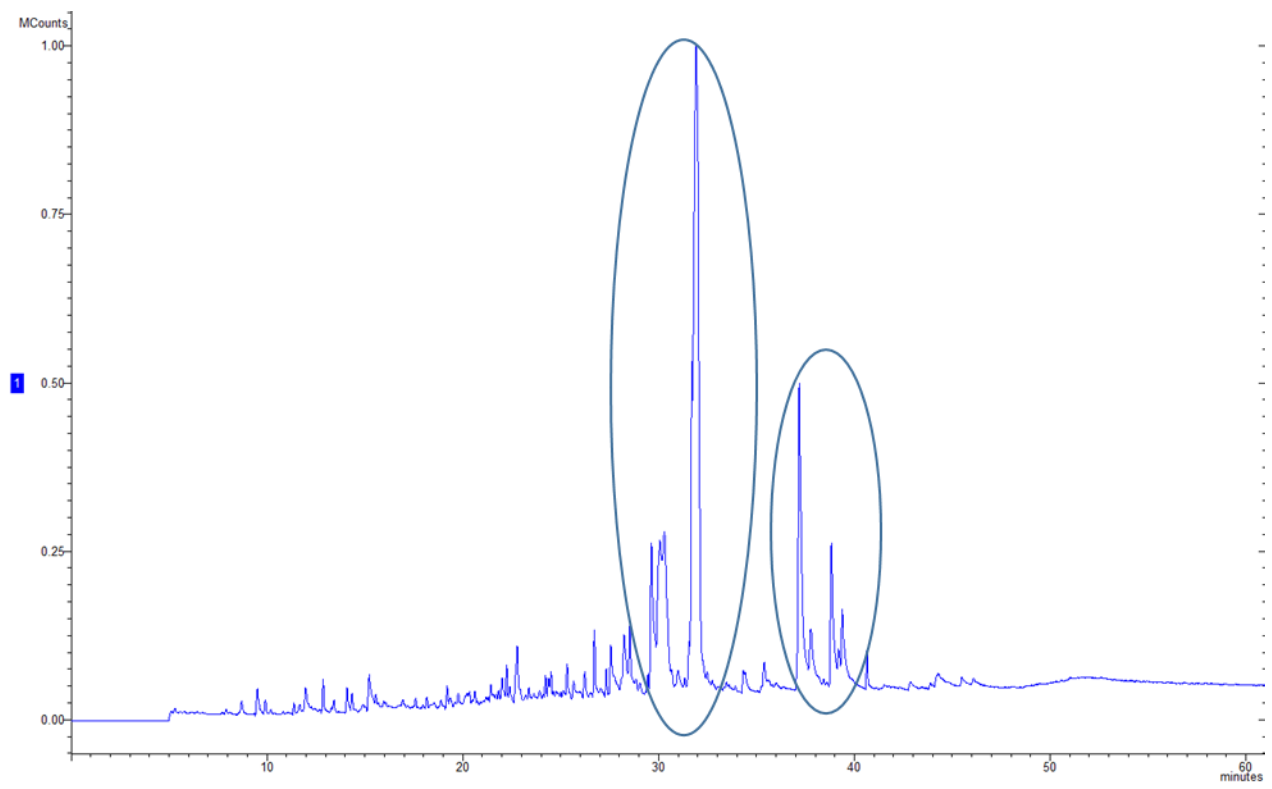
RT	Peak Name	% Amount
17.592	1H-Pyrrole-3-carboxamide, 2,	0.101873061
27.573	3-Butyl-6-methylpiperazine-2,5-dione	1.160547465
27.662	3-Butyl-6-methylpiperazine-2,5-dione	0.856136768
30.131	3,6-Diisopropylpiperazin-2,5-dione	21.01839685
31.336	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	0.915986954
31.731	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	9.731667499
32.094	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	20.59841635
35.308	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	1.137684511
37.299	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	2.625567689
37.835	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	4.070233298
38.806	Cyclo-(l-leucyl-l-phenylalanyl)	1.463420403
39.273	Cyclo-(l-leucyl-l-phenylalanyl)	4.9701324
39.434	Cyclo-(l-leucyl-l-phenylalanyl)	5.589028527
46.097	2,5-Piperazinedione, 3,6-bis(phenylmethyl)-	0.885867917

Figure 4.4.2b: Chromatogram of Rosè wine lees fraction F2. Fraction compositions is reported in table below.



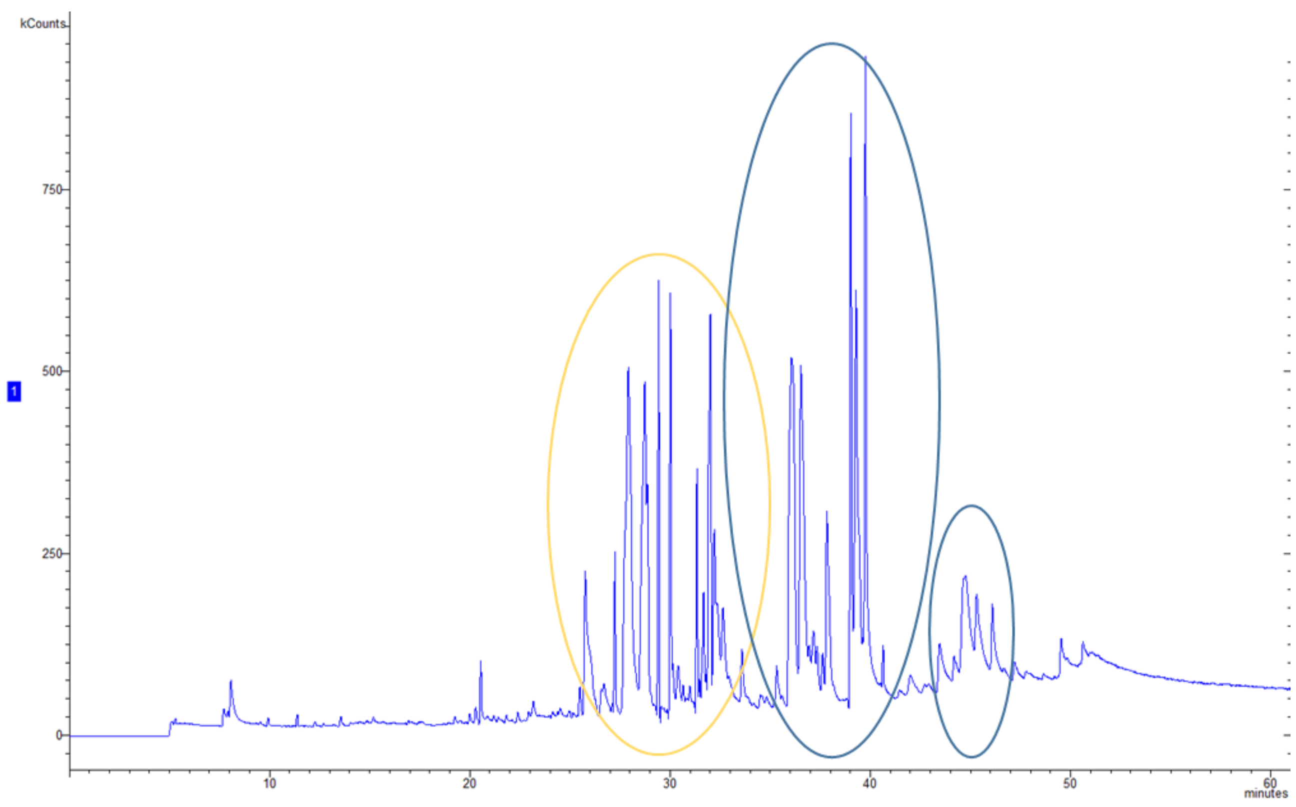
RT	Peak Name	% Amount
15.06	2-Pyridylacetamide	0.173803046
25.789	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	0.88169348
28.071	3-Butyl-6-methylpiperazine-2,5-dione	11.96083552
28.253	3-Butyl-6-methylpiperazine-2,5-dione	2.601127904
28.801	Cyclo-(glycyl-l-leucyl)	6.896362986
28.949	Cyclo-(glycyl-l-leucyl)	1.41644465
29.487	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	6.395939504
30.039	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	3.721683004
31.379	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	3.255910101
31.596	5-Hydroxy-2,2'-bipyridyl	0.340422614
31.699	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	1.15430676
31.995	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	4.215582291
32.062	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	3.352664859
32.248	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	3.65530474
32.701	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	1.364692105
33.63	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	0.833866253
35.357	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	0.689896299
36.055	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	6.300118543
36.537	2,5-Piperazinedione, 3-(phenylmethyl)-	4.353964095
37.354	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	0.809040783
37.63	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	0.556318481
37.854	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	4.07382532
39.056	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	5.060498837
39.326	Cyclo-(l-leucyl-l-phenylalanyl)	6.480318653
39.46	Cyclo-(l-leucyl-l-phenylalanyl)	2.271486695
39.8	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	8.819308667
43.486	Cyclo-(l-leucyl-l-tyrosyl)	0.484262062
44.713	Cyclo-(l-leucyl-l-tyrosyl)	0.259039488
46.112	2,5-Piperazinedione, 3,6-bis(phenylmethyl)-	1.081048783

Figure 4.4.3a: Chromatogram of Red wine lees fraction F1. Fraction compositions is reported in table below.



RT	Peak Name	% Amount
13.414	5-Acetyl-2-methylpyridine-	0.192433285
14.086	5-Hydroxymethyldihydrofuran-2-one	0.422205275
29.636	3,6-Diisopropylpiperazin-2,5-dione	4.048623259
30.057	3,6-Diisopropylpiperazin-2,5-dione	4.016840052
30.297	3,6-Diisopropylpiperazin-2,5-dione	4.010762069
31.686	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	5.982068032
31.922	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	28.39564772
37.178	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	10.18213605
37.79	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	1.677889312
38.835	Cyclo-(l-leucyl-l-phenylalanyl)	4.939623265
39.196	Cyclo-(l-leucyl-l-phenylalanyl)	0.992753217
39.374	Cyclo-(l-leucyl-l-phenylalanyl)	2.221409413
44.303	Cyclo-(l-leucyl-l-tyrosyl)	0.840987898

Figure 4.4.3b: Chromatogram of Red wine lees fraction F2. Fraction compositions is reported in table below.



RT	Peak Name	% Amount
20.543	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	0.55706598
25.769	2,5-Piperazinedione, 3-methyl-6-(1-methylethyl)-	1.298578886
27.909	3-Butyl-6-methylpiperazine-2,5-dione	11.72547033
28.733	Cyclo-(glycyl-l-leucyl)	8.701967733
28.887	Cyclo-(glycyl-l-leucyl)	2.376346262
29.435	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	3.844609833
30.018	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	4.10111371
31.342	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	1.923779077
31.676	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	0.832235674
31.944	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	5.709711437
35.32	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	0.49000201
36.112	2,5-Piperazinedione, 3-methyl-6-(phenylmethyl)-	12.84167643
36.522	2,5-Piperazinedione, 3-(phenylmethyl)-	8.221391503
37.82	2,5-Piperazinedione, 3-benzyl-6-isopropyl-	3.676555569
39.026	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	6.558538784
39.29	Cyclo-(l-leucyl-l-phenylalanyl)	7.36490442
39.76	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	8.227288144
44.728	Cyclo-(l-leucyl-l-tyrosyl)	0.629979418
46.088	2,5-Piperazinedione, 3,6-bis(phenylmethyl)-	0.591380007

Figure 4.4.4: Least Squares means ± SEM of PBMC proliferation following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ ($P < 0.05$).

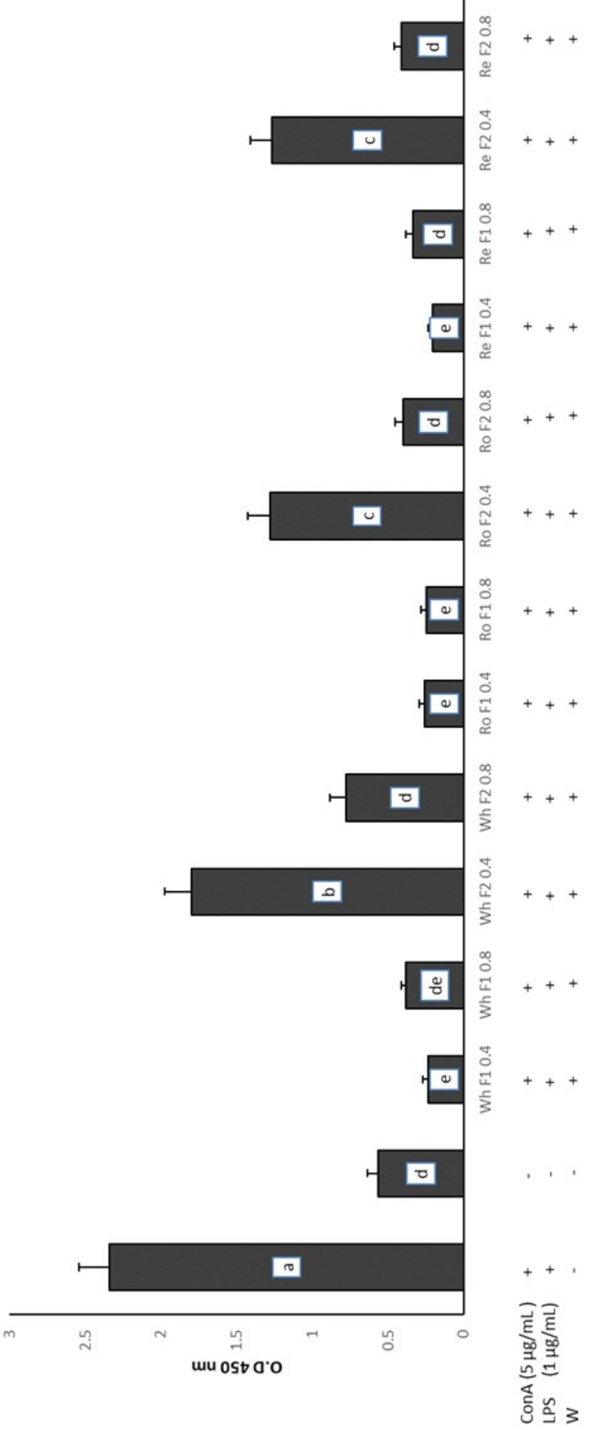


Figure 4.4.5: Least Squares means ± SEM of PBMC IL-1β production following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ ($P < 0.05$).

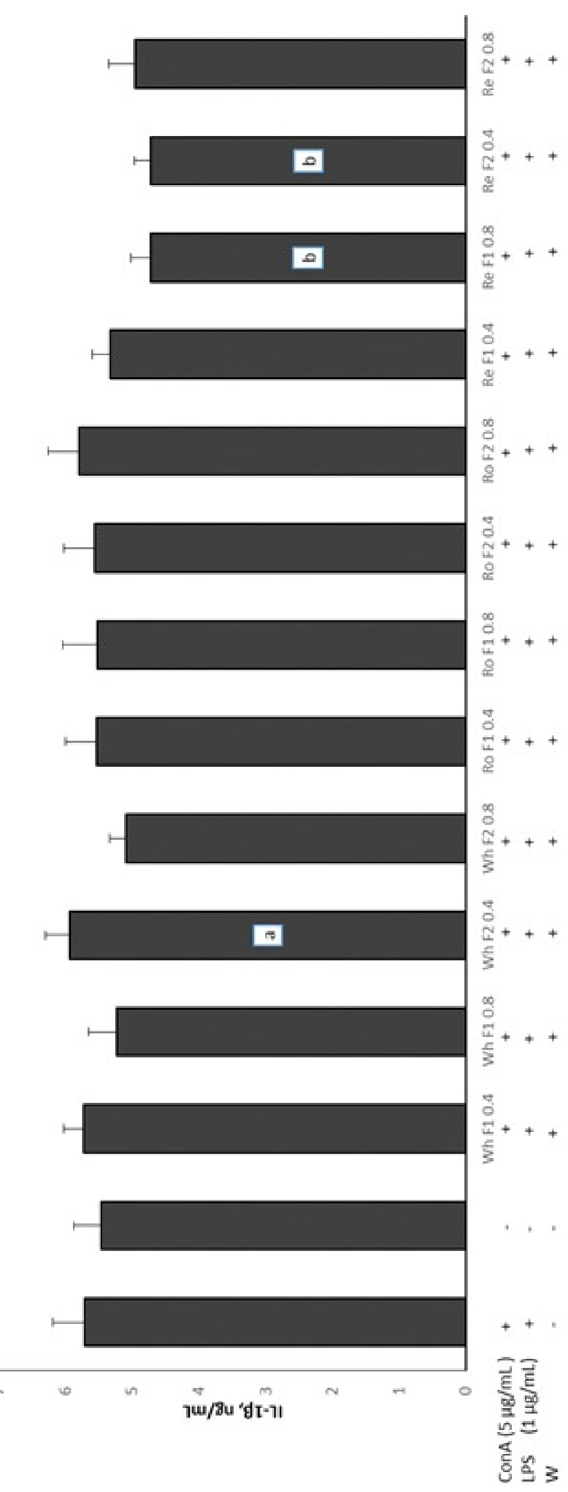


Figure 4.4.6: Least Squares means ± SEM of PBMC IL-6 production following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ (P < 0.05).

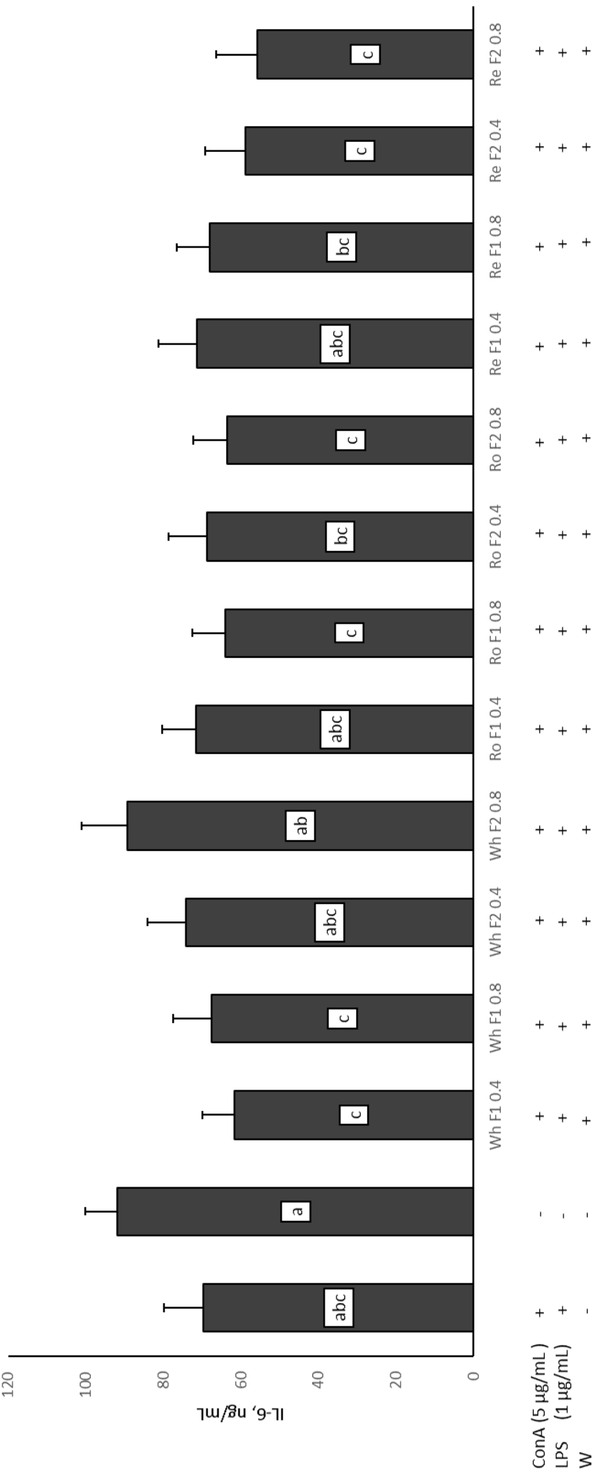


Figure 4.4.7: Least Squares means ± SEM of PBMC IL-10 production following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ ($P < 0.05$).

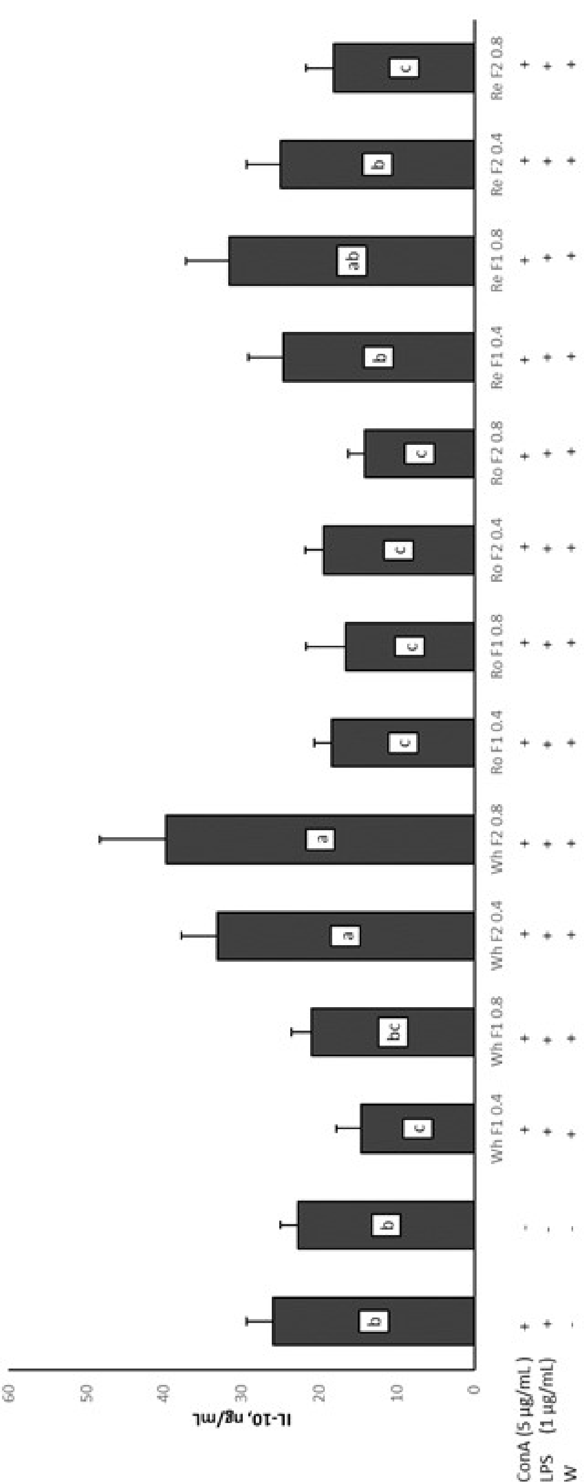


Figure 4.4.8: Least Squares means ± SEM of PBMC IFN-ɣ production following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/Ml) of six different wine lees fractions and in presence of Concanavalin A and LPS. A, b Values with different letters differ (P < 0.05).

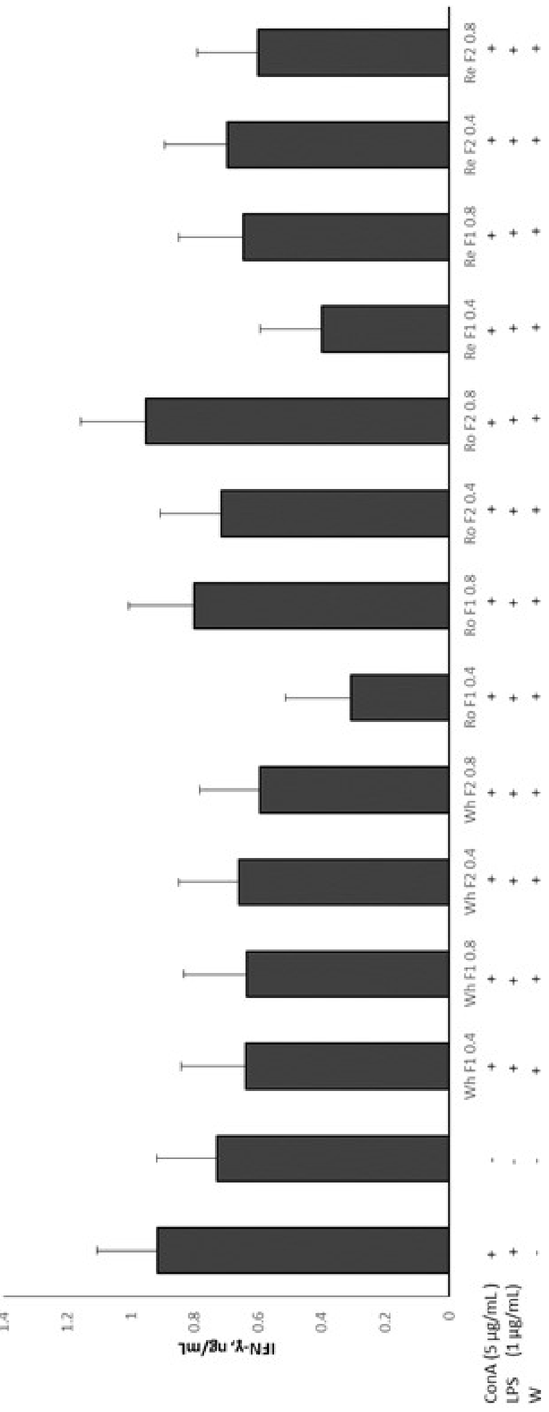


Figure 4.4.9: Least Squares means ± SEM of PBMC proliferation following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ ($P < 0.05$).

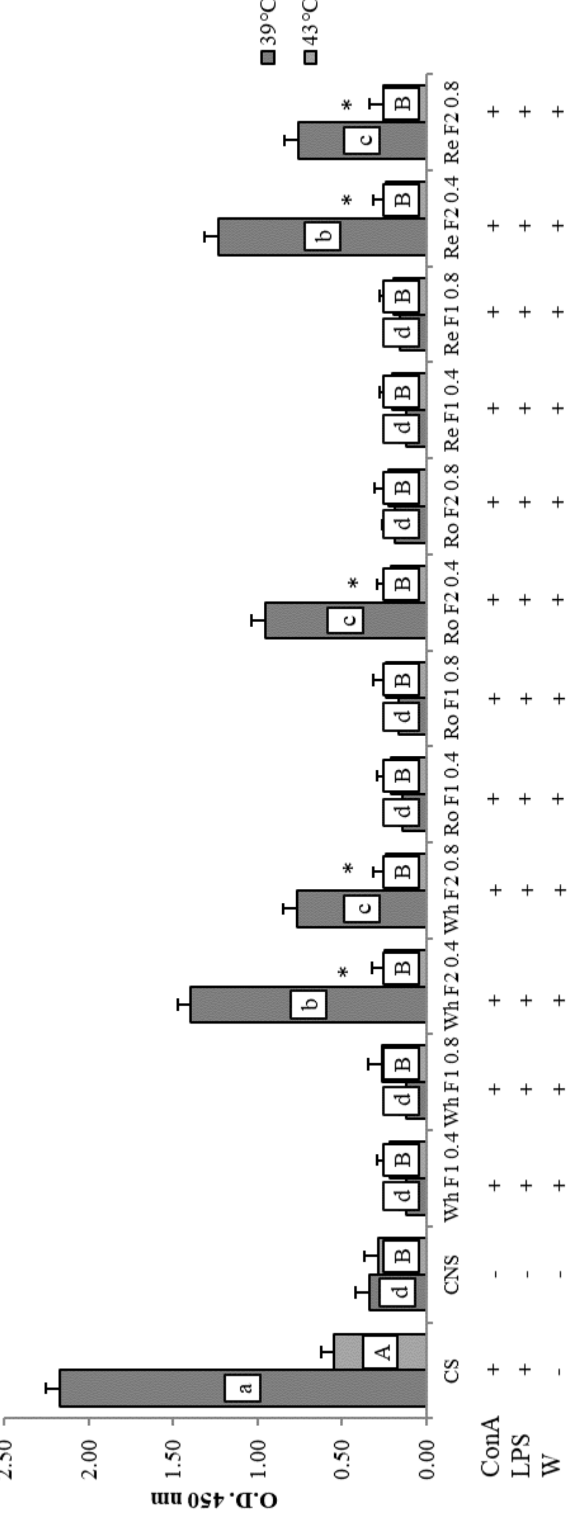


Figure 4.4.10. Least Squares means ± SEM of IL-1β secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ (P < 0.05).

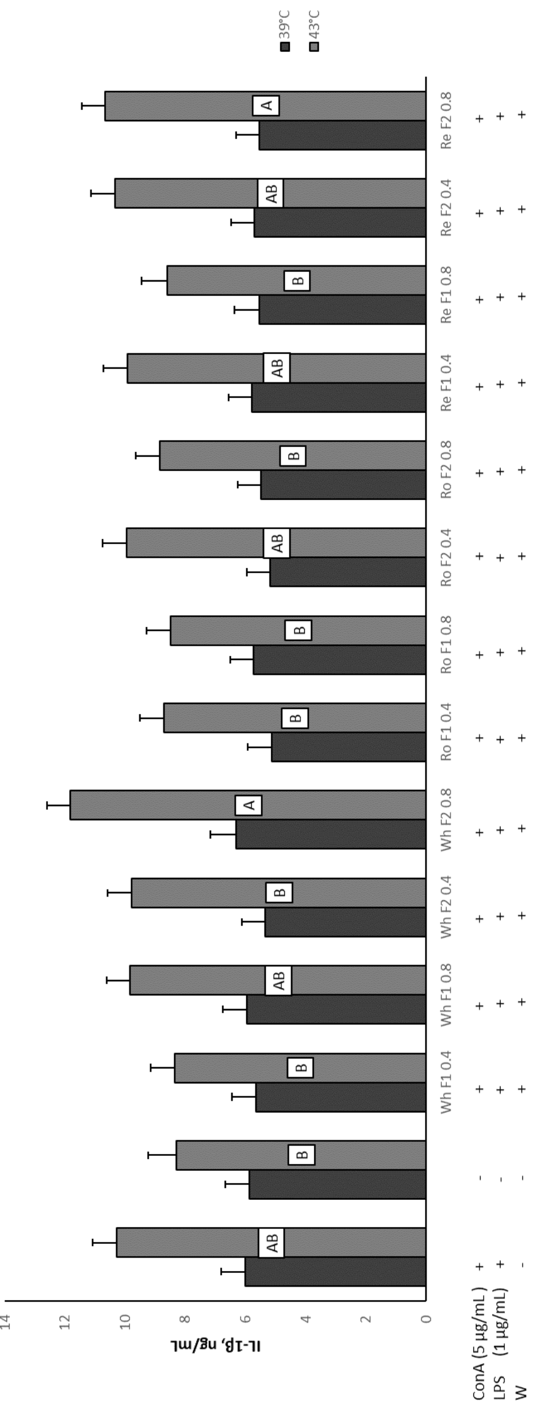


Figure 4.4.11. Least Squares means ± SEM of IL-6 secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ (P < 0.05).

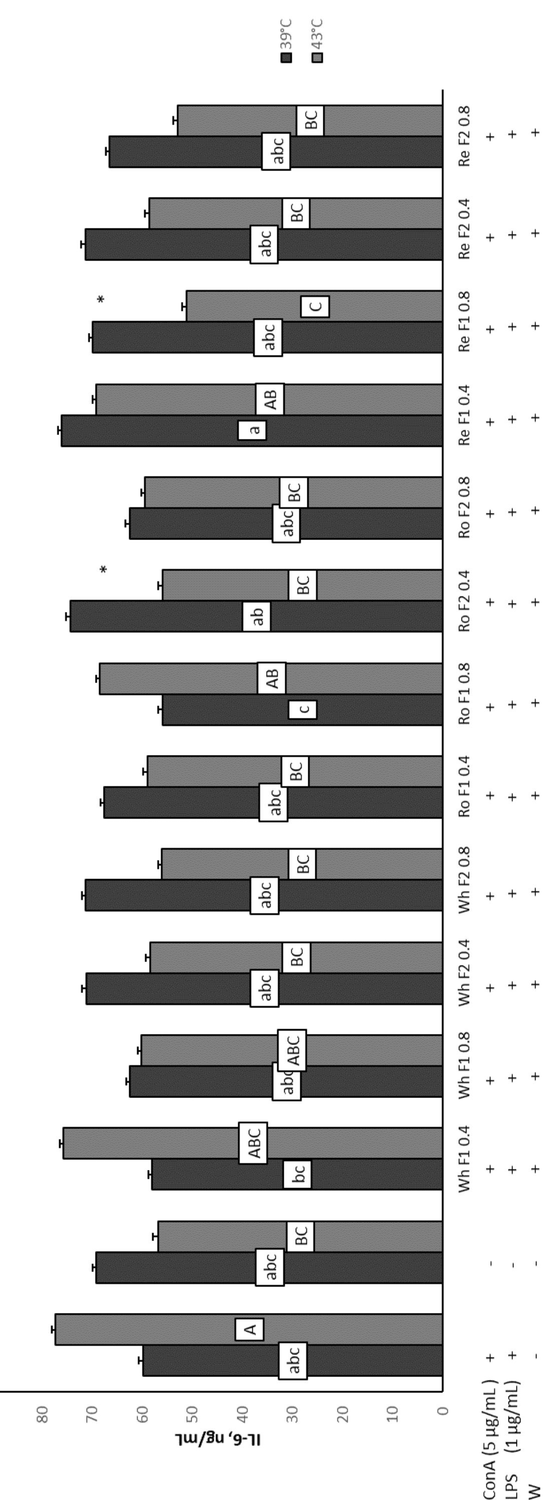


Figure 4.4.12. Least Squares means ± SEM of IL-10 secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ (P < 0.05).

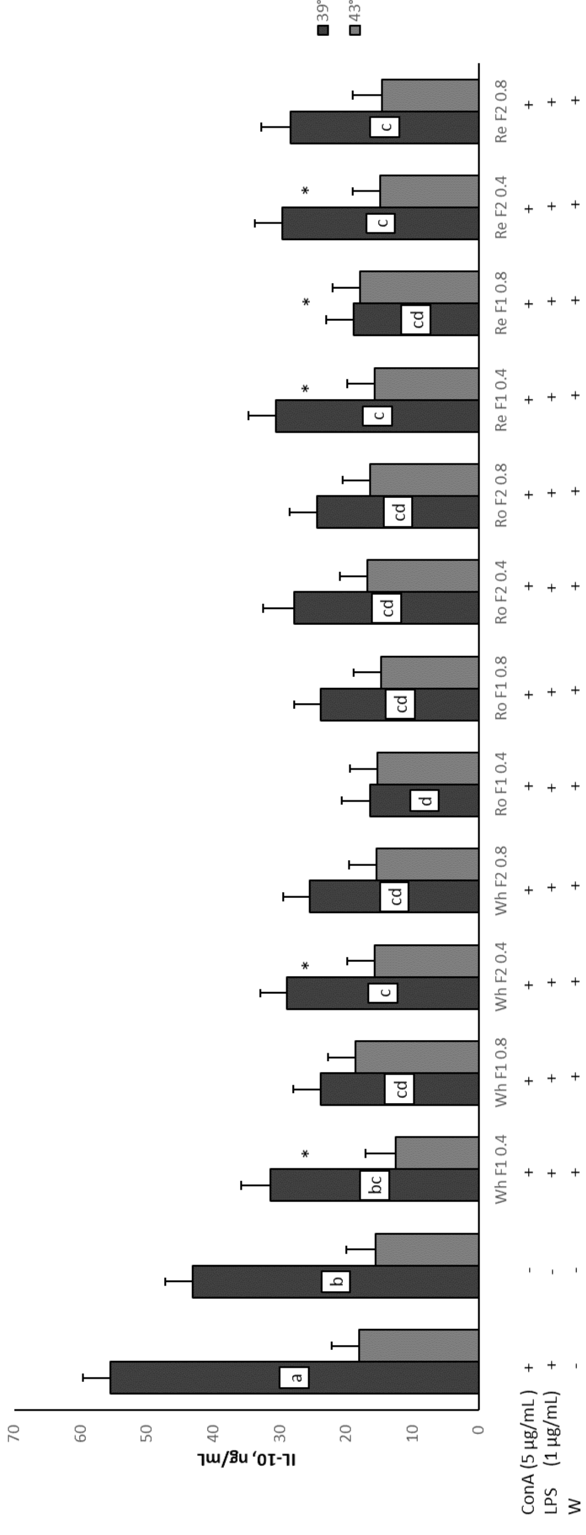


Figure 4.4.13. Least Squares means ± SEM of IFN-ɣ secretion by PBMC following in vitro stimulation. PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine lees fractions and in presence of Concanavalin A and LPS. a, b Values with different letters differ (P < 0.05).

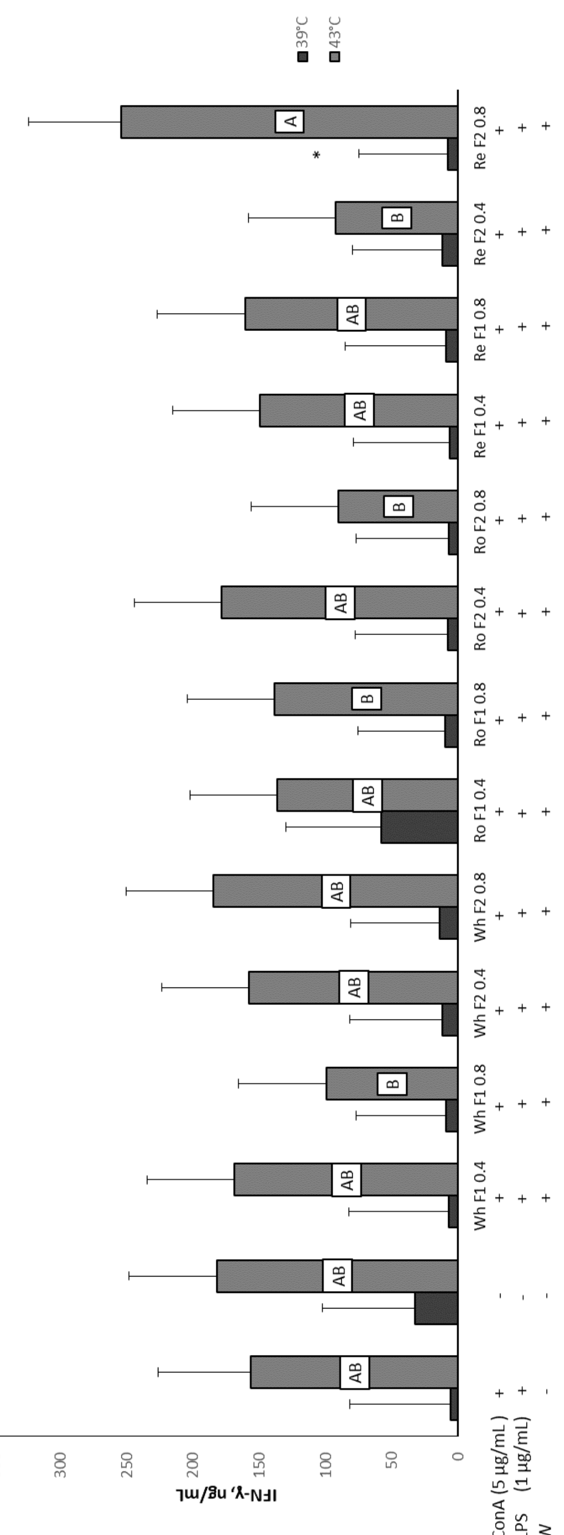
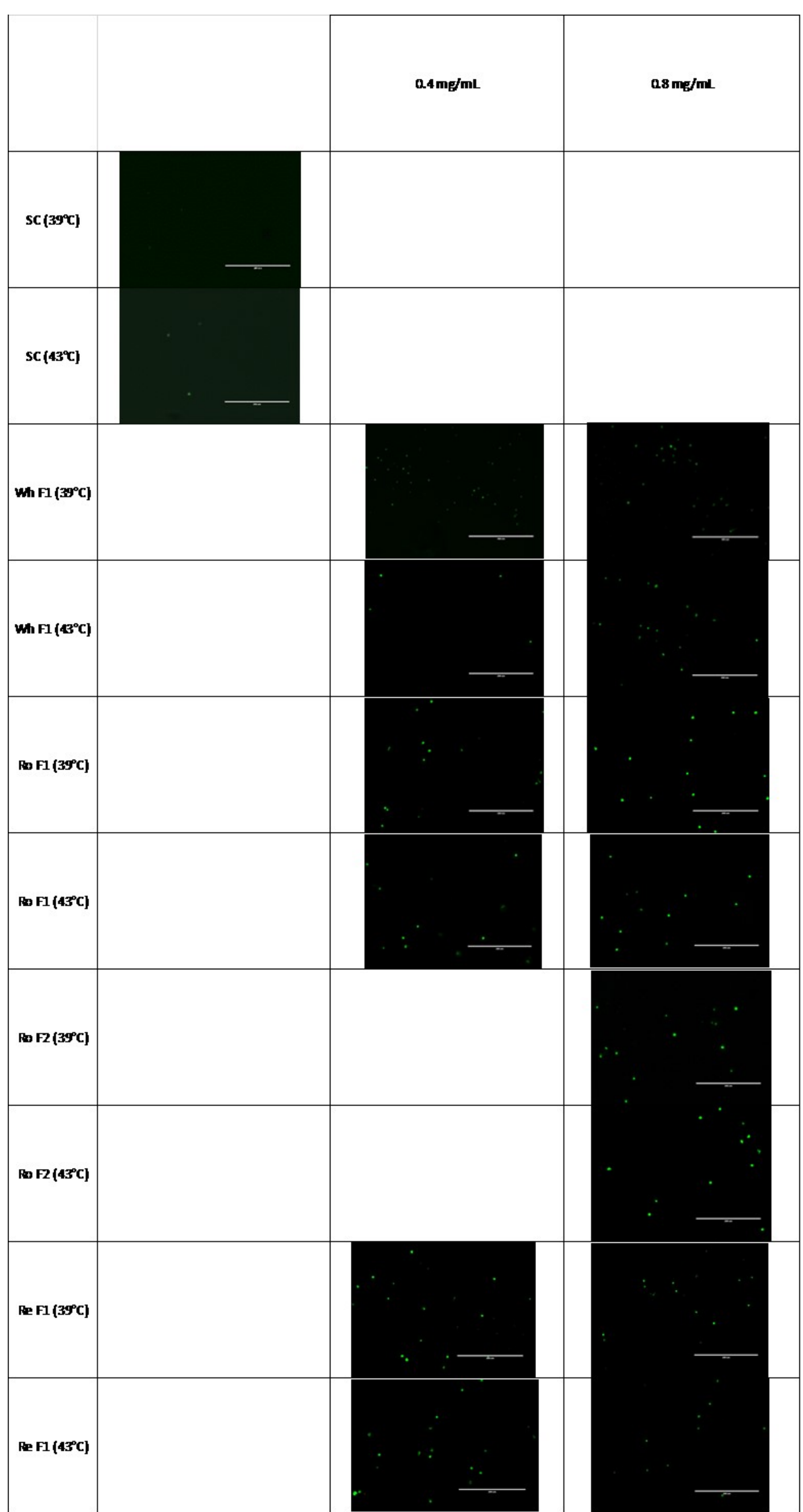


Table 4.4.1: Cells viability of PBMC cultured in condition of thermoneutrality and heat stress compared to LPS ConA stimulated cells.



CHAPTER 5

THIRD TRIAL

5.1 INTRODUCTION

The transition period in dairy animals represents an intensive period with an increased physiological stress. It is consideredas the lapse intercurrent from three weeks before parturition to three weeks later (Caroprese et al., 2006), during which dairy animals undergo metabolic and endocrine changes that could partially explain their immune suppression (Lacetera et al., 2005).This status undermined animals' homeostasis, with the results of increasing susceptibility to pathogens; ruminants became easily prone to illness, therefore, health problems during this period could dramatically affect dairy production and reproduction.

Several studies were undertaken in the last decades in order to understand causes and consequences of molecular and biochemical changes connected to transition period.

Hormonal variations occur during this period, leading to an increase in energy supply along with a decrement in dry matter intake. The augmentation of GH can explain the activation of hepatic gluconeogenesis; meanwhile, a condition of insulin-resistance responsible for NEFA mobilization used for milk synthesis or as a form of energy in post-partum phase. The increased request of energy lead to the partial oxidation of NEFA to obtained ketonic corps such as β-hydroxybutyrrate (Wankhade et al., 2017, Block, 2010). These hormonal and biochemical changes are responsible for an impairment of both innate and adaptive immunity (Aitken et al., 2011).

It has been demonstrated that lymphocytes proliferation and phagocytic activity marked decreased one week after calving (Meglia et al., 2005), while neutrophils-mediated immunity was dramatically altered at calving (Crookenden et al., 2016). In addition, around lambing, in vivo sheep production of pro-inflammatory cytokines, such as IL-6 and IL-1β increased (Caroprese et al., 2006). Jahan et al. (2013) demonstrated that the level of pro-inflammatory cytokines such as IL-6 and IL-1 after parturition were significantly correlated to clinical and subclinical pathology.

The metabolic dysregulation during the peripartum period is strictly connected to the unbalance of antioxidant status by an increment of ROS (Gabai et al., 2004), and it is demonstrated that cattle have high oxidative stress and low antioxidant defences during the post-partum period (Sharma et al., 2011); therefore, a strategy to sustain the immune responses of ruminants during transition period could be the reduction of ROS production.

Nutritional strategies such as feeding antioxidants can improve the state of immunosuppression preventing the progression toward inflammation. Furthermore, during post-partum, dietary changes have an effect on blood parameters and antioxidant status as demonstrated in a study conducted on cows fed with verbascoside (Casamassima et al., 2012).

Polyphenols are known for their antioxidant capacity and recently a study conducted in dairy cattle during transition period demonstrated the anti-inflammatory ability of polyphenols contained in curcuma and tea (Winkler et al., 2015). Polyphenols are largely found in grape and in its by-products (Yi et al., 2009), and the beneficial impact of its waste-derived bioactive compounds has been recently investigated (Hogan et al., 2010).

Grape marc (or pomace) is one of the grape by-products and represents approximately 25% of the grape and it is comprised of skins and seeds (Dwyer et al., 2014). It was reported that feeding grape by-products to ruminants can positively impact on rumen emission (Moate et al. 2014) and have an immunomodulatory activity (Nudda et al. 2015).

This thesis focused on the valorisation of grape pomace, the major component of winery organic waste, by the evaluation of their ability to influence in vitro immunocompetence of sheep PBMCs during the transition period. To reach this aim grape pomace were collected, submitted to microwave assisted extraction, and analyzed to be tested in vitro on PBMCs of periparturient sheep.

5.2 MATERIAL AND METHODS

5.2.1 Chemicals

Red Wine pomace of Nero di Troia vite were provided by Pirro's winery situated in Troia (Foggia) during the vintage 2016.

5.2.2 Moisture test:

Wine pomace were tested for its content of humidity by the thermogravimetric analyzer LECO TGA and then indirectly total solids were determined as follow:

% TS = 100 - % H2Où

5.2.3 Microwave assisted extraction of wine pomace

Microwave-assisted extractions (MAE) were performed in MARS™ 6 (CEM) configured with a 12 positions carousel. 1 g of wine pomace were transferred to the teflon extraction vessels. Extractions were performed using water, water:ethanol 1:1 and ethanol with a total solid:solvent ratio of 1:40. For the catalyzer extraction, 2 mol/kg s.s of Na_2CO_3 were added in each position. All vessels were closed and refer vessel was provided of a fiber optic probe to measure the temperature in the systems and a probe to measure the pressure. The operational parameters employed in the MAE apparatus were the following: magnetron power 100%, ramp temperature time 30 min and maintaining temperature time 10 min. Four temperature programs were tested 50°C, 100°C, 150°C and 200°C. From the combination of MAE extraction 24 extracts were obtained.

5.2.4 Antioxidant capacity assay

5.2.4.1 FRAP Assay

FRAP reagent was prepared according to Francavilla et al, (2013). Briefly, the FRAP reagent was prepared adding 20 mL of TPTZ (10mmol/L) in HCl (40 mmol/L) to 20 mL of $FeCl_3$ (20 mmmol/L) and 200 mL of aceate buffer (0.3mmol/L, pH 3.6) and then it was warmed up to 37°C.

The reaction is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous-blue-coloured form in the presence of antioxidants. 100 μL sample of each extract was mixed with 3 mL of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm after

incubation at 37 °C for 10 min. For the present study, the standard curve was constructed using Trolox solution (3-120 μM) and the results were expressed as μmol Trolox/g dry weight of extract. Butylated hydroxytoluene (BHT) whose concentration was 0.1 mg/mL was used as positive control.

5.2.4.2 ABTS Assay

The scavenging activity of wine lees extract was detected by the ABTS [2-2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)] assay according to Francavilla et al., 2013 and Re et al.,1999. Firstly, the ABTS stock solution was prepared by a reaction of the colorless ABTS 7mM with 2.45 mM of $K_2S_2O_8$. The solution was allowed to stand in the dark for 16h meanwhile the blue-green ABTS+ form was generated. Secondly, the working solution was prepared adding 1 mL of ABTS stock solution to 88 mL of PBS (5mM ph 7.4) and then 2 mL of this solution was added to 200 μl of samples in methanol. In presence of antioxidant the $ABTS^+$ could also be reduced to its colorless form. After 6 min of reaction the absorbance was measured at 645 nm and the results were expressed as mmoL Trolox g^{-1} dry weight of extract. For the present study, the standard curve was constructed using Trolox solution (3-1200 μM). Gallic acid (GA) at the concentration of 0.1 mg mL^{-1} was used as positive control.

5.2.4.3. Phenolics determination

Total phenols (TP) were dermined following the Folin-Ciocalteu method according to Francavilla et al., (2013). Briefly, 200 μL of each extract was mixed with 1.5 mL of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min. A 1.5 mL sodium bicarbonate solution (60 g/L) was added to the mixture. After incubation for 90 min at room temperature, the absorbance was measured at 750 nm. For this study the standard curve was constructed using a gallic acid standard solution (25–150 μg/mL in 50% methanol). The results were calculated as gallic acid equivalent (GAE)/g dry weight of extract.

Antocians and flavonoids were determined according to Cliff et al., 2007. Briefly, 0.5 mL of each extract was diluted 1/10 with ethanol 10%, then 0.25 mL was added of 0.25mL of 0.1% HCl in ethanol 95%. Finally, 0.25 mL of each diluted extract was added to 0.25 mL of 0.1% HCl in 95%

ethanol, and 4.55 mL of 2% HCl. Each sample was vortexed and allowed to stand for 15 min then the absorbance was measured at 520 nm and 360 nm for antocians and flavonoids, respectively. For this study the standard curve of antocians and flavonoids were constructed using malvin (oenin chloride 0-250 µg/mL), (Sigma-Aldrich) and quercetin (0-100 µg/mL), (Sigma-Aldrich), respectively. The results were expressed as mg/mL of malvin and mg/mL of quercetin respectively for antocians and flavonoids.

Among the 24 extracts obtained, based on the results of the above determination we choose to focus our attention on these Nero di troia wine pomace extracts: Water/Ethanol + catalyzer extracted at 50°C (WEt/k), Ethanol+ catalyzer extracted at 100°C (Et/k), Water/Ethanol extracted at 150°C (WEt), Water extracted at 200°C (W), Ethanol extracted at 200°C (Et) and Water/Ethanol + catalyzer extracted at 200°C (WEt/k).

5.2.5 Animals

The experimental site was a commercial farm (Agri Raffa, Lucera, Foggia, Italy). During the entire trial sheep were examined, by veterinarians, to exclude any sign of disease. Sheep were balanced for age, parturition time (± one week), BW, and BCS. Blood samples were taken into duplicate heparinized vacuum tubes (Becton Dickinson, Plymouth, UK) for each animal. Sheep were bleed 15 days before the lambing (t1), 7 days before the lambing (t2), lambing time (t3) and 7 days after the lambing (t4). All procedures were conducted according to the guidelines of the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. The assay was carried out as in the second trial.

A sample of sheep diet was frozen and mixed for chemical analyses. The chemical composition of diets was determined by standard procedures (AOAC 2000).

5.2.6 Lymphocyte stimulation assay, in vitro treatment, cytotoxicity-trypan blue dye exclusion and cytokines' determination

PBMC stimulation assay was performed in a 96 U bottom well plate. To perform this in vitro treatment PBMC were treated with 50 µl per well of two different concentration (0,4 mg/mL and

0,8 mg/mL) of Nero di Troia red wine pomace, extracted with water (W), water/ethanol 1:1 (WEt) and ethanol (Et), with or without Na_2CO_3 x 10 H_2O as catalyzer (W/k, WEt/k, Et/k). The test was performed for 24 h at 37°C and 5% of CO_2. In each well 100 µL of cells suspension for each sample was added in quadruplicate. Cells were activated by addition of 50 µl per well of ConA 5 µg/mL + LPS 1 µg/mL (Sigma-Aldrich) (SC, stimulated cells). Medium only provided negative control wells (NSC, non-stimulated cells). After incubation, as described above, plates were centrifuge at 200 g at 37°C for 5 minutes and cell-free supernatant from each well was collected and stored at -80 °C until ELISA to measure cytokine production. All treatments were reinsert as above and to test lymphocyte proliferation 20 µl/well of Bromodeoxyuridine (BrDU) (Exalpha Biologicals, Inc., Shirley, USA) was added. After 18h of incubation, BrDU cell proliferation assay was performed. The plates were spin down at 200 g at 37°C for 1 minutes than 170 µL/well of supernatant was aspirated, the plates were dried out by hairdryer and added 200 µl of Fix solution. Plates were washed with washing solution provided for 6 times. Following step were executed as reported inside BrDU instruction manual. BrDU incorporation during DNA synthesis was measured by determining optical density with spectrophotometer (Power Wave XS, Biotek, UK) at 450 nm.
Cells viability was determined by XTT Cell Proliferation Assay Kit (ATCC).
The determination of cytokines on PBMC supernatants was done as in the second trial; for this test were determined the level of IL-6, IL-10 and IFN-ɣ. Moreover, IL-6, IL-1β, IL-10 and IFN-ɣ were determined, also, on plasma of sheep obtained centrifuging blood samples at 1400 g for 15 minutes at 20°C according to Caroprese et al., (2012). Plasma cytokines results were expressed as ng/mL for IL-1β. IL-6, IL-10 and in pg/mL for IFN-ɣ. Plasma was collected and store at -80°C until cytokines determinations.

5.2.7. β-hydroxybutyrate and NEFA detection:

Blood samples were collected in EDTA vacuum tubes to determine non-esterified fatty acids (NEFA) from plasma, while blood samples collected in Na-heparin tubes were used to determine

plasma β-hydroxybutyrate. Blood samples were centrifuged at 1400 g for 15 minutes at 20°C according to Caroprese et al. (2012), then plasma was collected and stored at -80°C until assays. NEFA and β-hydroxybutyrate detections were done following the instruction manuals (Assay kit from Bioo scientific).

5.3 STATISTICAL ANALYSIS

All variables were tested for normality using the Shapiro-Wilk test. Proliferation data were analyzed using ANOVA for mixed models using the MIXED procedure of SAS. Concentration, time of sampling, type of extract and their interaction were used as fixed effect. Where significant differences were found ($P<0.05$) Fisher's Least Significant Difference test was used to identify significant differences between means.

5.4 RESULTS

5.4.1 Extracts characteristics

Table 5.4.1 reported the amount of total phenols, antocians, flavonoids and the antioxidant capacity of the extracts in terms of reducing power (FRAP) and scavenging activity ($ABTS^{+}$). The highest concentration of total phenols was found in 50WEt/k while the lowest is contained in 100Et which has a good FRAP power, probably dependent on the antocians precense. The extracts 100Et/k was chosen thanks to its FRAP power and TP content, while the 150WEt demonstrate the highest level of reducing power in term of FRAP. In addition, 200Et shows a good reducing and scavenging activity and concurrently a good antocians content. As table shows, 200WEt/k reports interesting results of all determinations. Lastely, 200W demonstrate a good FRAP and $ABTS^{+}$ activity.

5.4.2 PBMC proliferation

PBMC proliferation was affected by treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P<0.001$). Figure 5.4.1 showed the proliferation of PBMCs following in vitro treatment with extracts of grape pomace. As reported cells proliferation increased starting from 7 days before

lambing to 7 days after lambing, even though extracts obtained at 200°C in water at both concentrations and extracts in ethanol at 0.8 mg/mL lead to a decrement of PBMC proliferation compared to stimulated cells at t2, t3 and t4. At t1 occurred an increment of PBMC proliferation 15 days before the lambing only in presence of wine pomace at 0.8 mg/mL extracted in water and ethanol at 150°C. At 7 days before the lambing (t2) this extract showed a reduction of PBMC proliferation at the same level of not stimulated cells. Seven days before the lambing (t2) the lowest lymphomonocyte proliferation was registered when cells were harvested in presence of extracts obtained at 100°C in ethanol and catalyser and administered at 0.8 mg/mL. Moreover, a marked reduction of proliferation was also observed when cells were cultured with extracts obtained at 200°C in ethanol or in water and administered at both concentrations, or in water/ethanol and catalyser only at 0.8 mg/mL, with a dose dependent effect. In fact, at 0.4 mg/mL the extract 200WEt/k resulted in an augmentation of cell proliferation compared to not stimulated cells. Higher level of cells proliferation was registered after stimulating cells with pomace extracted in water/ethanol and catalyser at 50°C (50WEt/k).

At lambing (t3) the extracts 50WEt/k exerted an augmentation of cell proliferation at both concentration comparing to stimulated cells. In addition, cells treated with 0.8 mg/mL of pomace extracted at 200°C only with ethanol and only with water at 0.8 mg/mL showed a marked reduction of proliferation even lower than stimulated cells. PBMCs proliferation seven days after the lambing (t4) was comparable to lambing and showed an increase in proliferation in cells in presence of 50WEt/k and 100Et/k as compared with stimulated cells. No extracts resulted in cell proliferation lower than non-stimulated cells

5.4.3 Cytokines production in supernatants and plasma

As concern the production of IL-6, its level was affected by treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P<0.001$). This in vitro study demonstrated a marked increment of the level of this cytokines in supernatants of cells cultured with grape pomace extracted in water/ethanol at 150°C and ethanol at 200°C administered at both concentrations as compared to SC and NSC

(figure 5.4.2). In addition, at t1 the level of this cytokine slightly increased in presence of water/ethanol/catalyser extract, added at 0.8 mg/mL, and obtained at 50°C and 200°C. Seven days before lambing (t2) the level of IL-6 remained higher in the supernatants of cells harvested with the previous reported extracts (150WEt, and 200Et); in addition, IL-6 increased in supernatants of cells cultured with pomace extracted in 100Et/k at 0.4 mg/mL and with pomace extracted in 200Wet/k at 0.4 mg/mL. At lambing (t3) the production of IL-6 had a similar scenario to the one observed seven days before, but a slight decrement of this cytokine was registered at 0.4 mg/mL of grape pomace extracted at 200°C with water, ethanol and catalyser. Seven days after lambing (t4) there was a further reduction of IL-6 in presence of this latter extract. A decrement of IL-6 was also observed in presence of 50°CWet/k added in concentration of 0.4 mg/mL and in presence of extract obtained at 200°C with water administered at 0.8 mg/mL.

Figure 5.4.3 reported the production of IL-10 in presence of grape pomace which was affected by treatment ($P<0.001$), time ($P<0.001$) and their interaction ($P<0.001$). The production of this cytokine increased at lambing (t3) as well as 7 days after lambing (t4) compared to t1 and t2. Fifteen days before lambing IL-10 level decreased at value lower than stimulated and not stimulated cells with 50WEt/k. The lowest level was found in supernatants of cells harvested with 50WEt/k at 0.8 mg/mL as compared to stimulated and not stimulated cells.

At t2 the lowest level of IL-10 was found when cells were harvested with 0.8 mg/mL of grape pomace 200Et.

As concern the time of lambing, all extracts, except for the one extracted at 50°CWEt/k at 0.4mg/mL, were able to reduce the level of IL-10 compared to stimulated PBMCs. In addition, the pomace extracted in 100Et/k, 150WEtin ethanol/water at 150°C, WEt/k and W both at 200°C, were able to reduce IL-10 production at the same level of not stimulated cells. The level of IL-10 seven days after lambing increased in all extracts when compared to not stimulated cells and reached the same level of stimulated cells, except when PBMC were treated with 0.8 mg/mL of grape pomace

extracted in only ethanol and only water both at 200°C and in WEt/k at 150°C and 200°C. These last two extracts lead to a reduction even lower than not stimulated cells.

Lastly, as concern the production of IFN-ɣ results are reported in figure 5.6.4 and showed the highest increment of this cytokine seven days after lambing(t4). Results showed an increment of IFN-ɣ in supernatants 15 days before lambing when in presence of grape pomace extracted in 200Et at 0.8 mg/mL compared to both stimulated and not stimulated cells. The lowest production was observed in presence of 200W at both concentrations. Seven days before lambing (t2) the IFN-ɣ production decreased when PBMCs were in presence of all extracts obtained at 150°C and 200°C at both concentration compared to stimulated cells, but not in supernatant of cells cultured with 0.8 mg/mL of 200WEt/k. At lambing time (t3) a slightly decrement of this cytokine was observed in 200Et at 0.8, 200Wet/k at 0.4, 200W at both concentrations.

Seven days after lambing (t4) the level of IFN-ɣ rose when cells were treated with 0.4 and 0.8 mg/mL of 100Et/k and 0.4 mg/mL 200Et.

Results from this in vitro study demonstrate that any significant differences were found in plasma in term of cytokine production (Figure 5.4.5).

5.4.4 β-Hydroxybutirrate and NEFA

No significant differences were found in term of b-hydroxybutyrrate and NEFA in sheep plasma (data not show).

5.5 DISCUSSION

It is well established that during transition period ruminants have to cope with a metabolic disarray and an immunosuppressed immune system thus resulting in a “transition disorders” following by failure in adaptation to fetal growth, calving and high-energy demands for lactation (Sordillo et al., 2014).

The experiment herein focused on the effect of extracts from grape pomace in in vitro sheep PBMC in order to sustain immune response during the transition period and therefore opening a possible use of this by-products in ewe's diet.

Nutritional strategies involving grape by-products are widely investigated but, to the best of our knowledges, there are not many studies that explore the immune impact of grape waste in sheep around lambing.

Lymphocytes proliferative response give information on "healthy" status of innate immune response since monocytes and their derived cells have an essential role in this type of immunity which is the first line of defence against disease outbreak (Aitken et al., 2011, Pinedo et al., 2013). Our current study reported increased PBMC proliferation at day of parturition as well as seven days after lambing in presence of LPS and ConA and with almost all extracts, even though 200°C extracts in Water at both concentrations and in Ethanol at 0.8 mg/mL reduced PBMC response. Around parturition monocyte and macrophage populations increased in blood and tissues of cows (O'Boyle et al., 2012); in addition, Nonnecke et al., (2003) underlined that during transition period, in dairy cows, a change in functions and subset of peripheral blood mononuclear leukocyte populations occurs, probably caused by the physiological demand for lactation. Despite the number of circulating leucocytes increased, a breakdown of immune defence arise (Meglia et al., 2001). During winemaking, the polyphenols of the grape are transferred to the wine, but a high proportion still remained in the solid winemaking by-products. Reduced proliferation was reported by Gómez-Alonso et al. (2012) where the anti-proliferative activity was mediated by the direct cytotoxic actions of flavonols and consequent blockage in G2/M of cell cycle preceded by reduction of cyclooxygenase-2 (COX-2) and cyclin D1 expression. Furthermore, it has been demonstrated the ability of fermented grape pomace to induce T regulatory cells in terms of Foxp-3 molecule expression, a transcription factor that play an immunosuppressive role in immune system by acting on NFAT and NFkB (Marzulli, et al., 2012, Kim C. H. 2009). Reduced PBMC proliferative

response in presence of aforementioned extracts let us to speculate an immunosuppressive role of these extracts on lymphomonocyte mitogen proliferation.

Inflammation is necessary to eradicate invading pathogens and in repairing processes. It has been established that during peripartum cell-mediated and humoral immunity are activated by the secretion of inflammatory cytokines, among which IL-6 (Caroprese et al., 2006).

In a previous study by Jahan et al. (2015), ex vivo stimulation of whole blood with two different concentrations of LPS, high and low, demonstrated the pivotal role of IL-6 in periparturient cows by regulating the immune response as showed by its increment with the low dose of LPS. Studies conducted by Ishikawa et al. (2004), demonstrated that the level of IL-6 was higher in peripheral blood before calving than after calving, while the plasma level of IL-6 in sheep was reported to be the highest at parturition (Caroprese et al., 2006).

Data from this experiment showed that grape pomace extract in water/ethanol or in ethanol at 150-200°C respectively, induced higher concentrations of IL-6 by PBMCs as a result of their high level of reducing power. Therefore, the administration of these grape pomace can be suggested to attenuate the immunosuppressive trend of transition period.

Secretion of inflammatory cytokines are accompanied by the production of anti-inflammatory ones (Islam et al., 2013). As reported by Marzulli et al. (2014) fermented grapes pomace added to human peripheral blood mononuclear cells were able to induce T regulatory cells in terms of Foxp-3 expression and release of IL-10. Results of the present study demonstrated a sharp increment of IL-10 at day of lambing and seven days after. These results are consistent with Crookenden et al. (2016) who demonstrated the up-regulation of IL-10 in neutrophils separated from whole blood of dairy cow, at day of calving. The concomitant increment of IL-6 and IL-10 in some extracts suggest the activation of innate immune response and the diminishing of the immune depression state around parturition.

Interferon-gamma is a T-cell derived cytokine often produced in response to antigen or mitogen stimulation. This cytokine is a potent immunomodulatory factor to many aspects of the immune

system and it could elicit functional changes in mammary phagocytic cells leading to the control of bovine mastitis (Sordillo et al., 1997). During the peripartum, $CD4^+$ cells produced less IFN-ɣ and IL-2, but more IL-4 and IL-10 compared to $CD4^+$ cells obtained during later stages of lactation (Sordillo et al., 2005). In our study the level of IFN-ɣ increased 7d after lambing in cells treated with ethanol and catalyser, and ethanol probably in relation to the high content of total phenols in ethanol/catalyser and to the high FRAP ability in Et extract.

Studies conducted by Yamanaka et al., (2012) reported the ability of high lignin phenylpropanoids to increase IFN-ɣ production thanks to their ability to bind to CD4 molecules and induce IFN-ɣ production.

5.6 CONCLUSIONS

In this study, we showed that cellular immune functions of sheep are modulated during the transition period as shown by a different behaviour of proliferative response which was reduced in presence of 200°C extracts in Water at both concentrations and in Ethanol at 0.8 mg/mL.

The concomitant increment of IL-6 and IL-10 with some extracts let us to speculate the activation of innate immune responses to overcome the immunodepression resulting from the transition period, whereas the general increment of IL-10 suggested the anti-inflammatory activity of grape pomace extracts useful to modulate immune system during peripartum.

Lastly, the increased level of IFN-ɣ registered 7 days after lambing let us to hypothesized the potential use of these extracts for functional foods as immunostimulants.

5.7 TABLES AND FIGURES

Tabel 5.4.1: Total phenol (TP), reducing power (FRAP), radical scavenging activity (ABTS), total antocians and total phenols of Nero di Troia extracts. CTR: positive control butylhydroxytoluen (BHT) e gallic acid (GA).

Extract	T (°C)	Solvent	TP (mg GAE/g extract)	Antocians (mg malvin /g extrtact)	Flavonoids (mg quercet. /g extrtact)	FRAP (µmol trolox/g extract)	ABTS (µmol trolox/g extract)
WEt/k	50	H_20:EtOH+Na_2CO_3 x 10 H_2O	390.89	32.60	1.51	103.34	160.85
Et/k	100	EtOH+Na_2CO_3 x 10 H_2O	204.34	6.30	9.20	338.73	68.65
WEt	150	EtOH+ H_2O	185.52	7.08	32.58	2665.23	183.44
Et	200	EtOH	10.02	50.52	20.17	674.18	194.73
WEt/k	200	H_20:EtOH+Na_2CO_3 x 10 H_2O	294.55	60.59	62.67	648.82	177.05
W	200	H_2O	91.02	32.11	21.68	754.36	180.57
CTR: BHT (µmol/L)	-	-	-	-	-	79.1	-
CTR: GA (µmol/L)	-	-	-	-	-	-	220.1

Table 5.4.2: Ingredients and chemical composition of the sheep diet (DM basis).

Diet chemical composition		
	Hay	Oat + Fava bean
Moisture %	59.59	6.54
DM%	40.41	93.46
Ash %	9.33	3.42
Ether extract, % of DM	7.49	5.40
CP, % of DM	13.49	20.70
CF %	27.68	10.57
ADF, % of DM	33.88	13.95
NDF, % of DM	44.32	26.53
ADL, % of DM	11.28	1.46

Figure 5.4.1: Least Squares means ± SEM of PBMC proliferation following in vitro stimulation 15 days (t1), 7 days (t2) before the lambing, at lambing (t3) and 7 days after lambing (t4). PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine pomace extracts and in presence of Concanavalin A (5 µg/mL) and LPS (1µg/mL). a, b refers to significance intra ime between treatment. Values with different letters differ ($P < 0.05$).

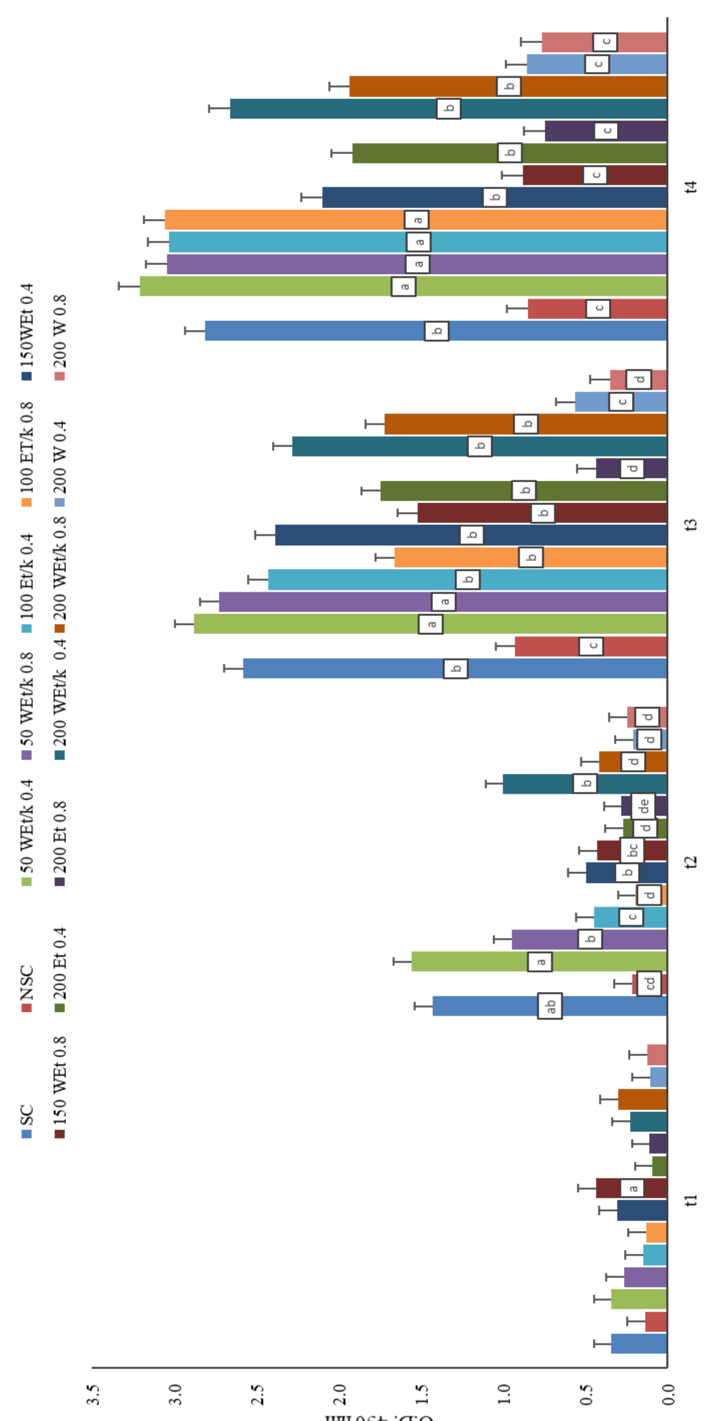


Figure 5.4.2: Least Squares means ± SEM of IL-6 production by PBMC following in vitro stimulation 15 days (t1), 7 days (t2) before the lambing, at lambing (t3) and 7 days after lambing (t4). PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine pomace extracts and in presence of Concanavalin A (5 µg/mL) and LPS (1µg/mL). a, b refers to significance intra-time between treatment. Values with different letters differ ($P < 0.05$).

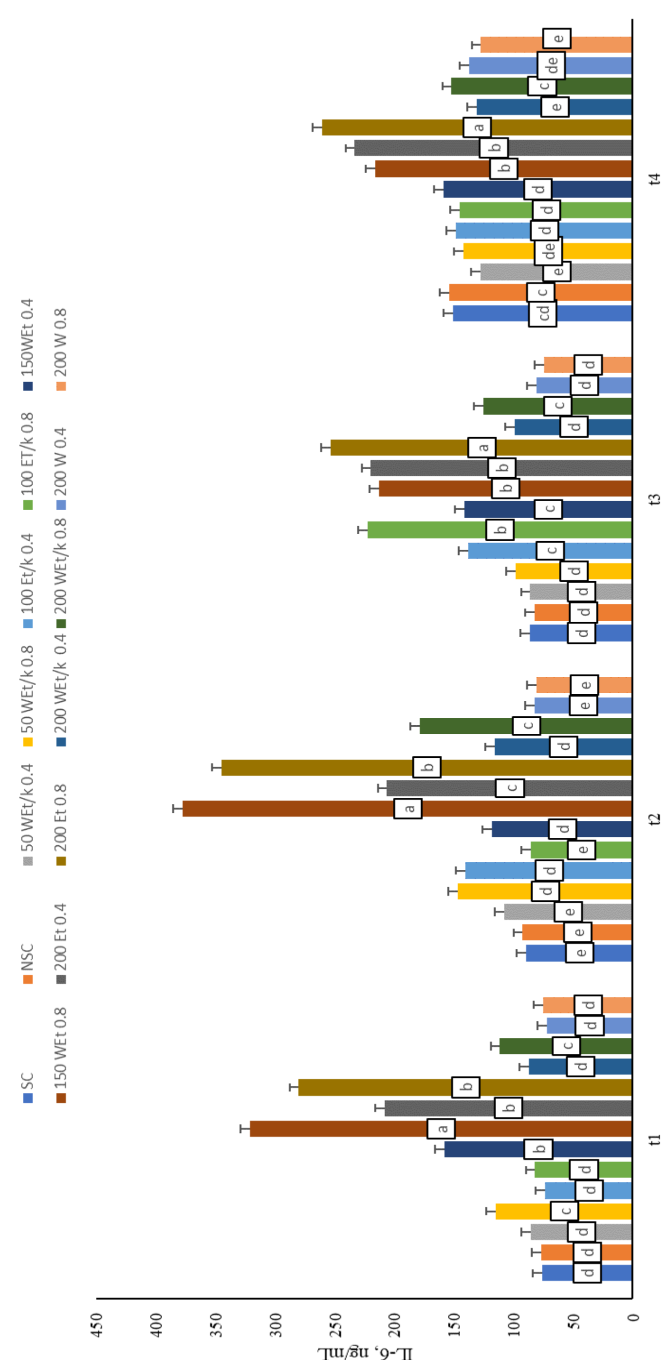


Figure 5.6.3: Least Squares means ± SEM of IL-10 production by PBMC following in vitro stimulation 15 days (t1), 7 days (t2) before the lambing, at lambing (t3) and 7 days after lambing (t4). PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine pomace extracts and in presence of Concanavalin A (5 µg/mL) and LPS (1µg/mL). a, b refers to significance intra-time between treatment. Values with different letters differ ($P < 0.05$).

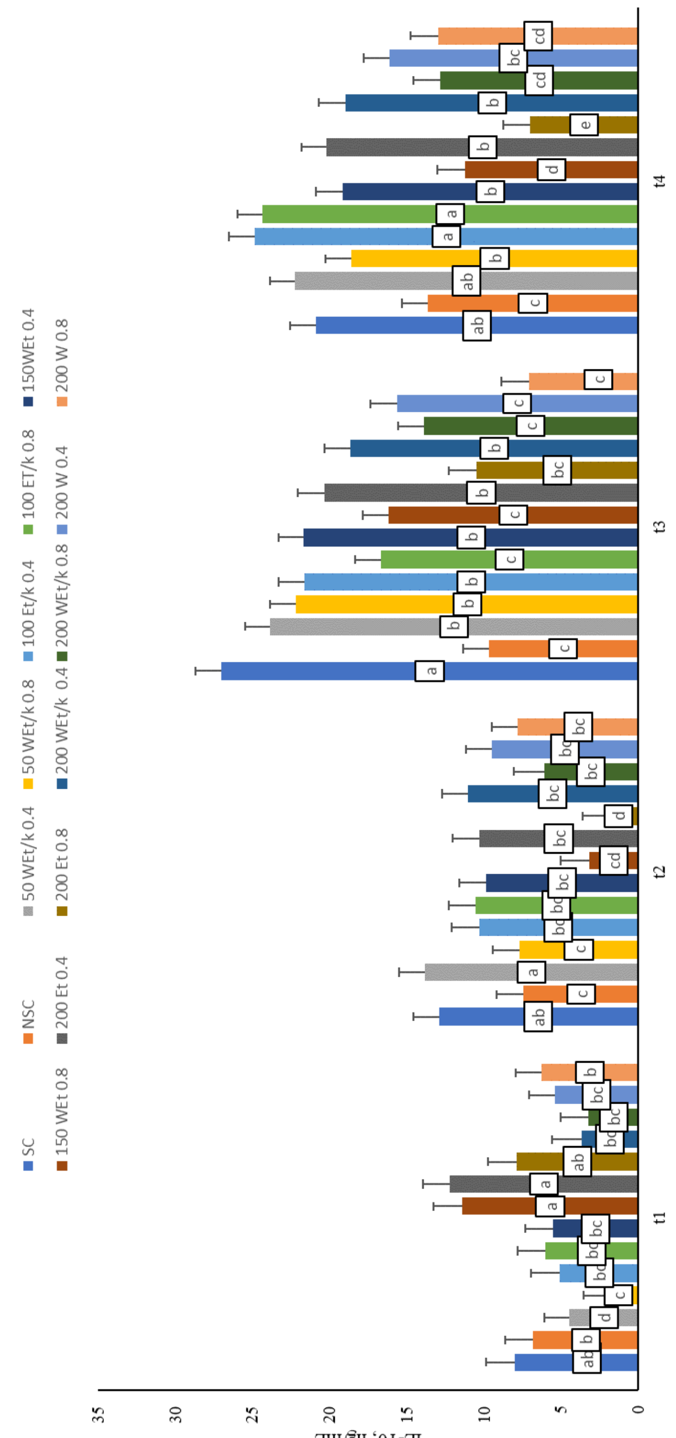


Figure 5.4.4: Least Squares means ± SEM of IFN-ɣ production by PBMC following in vitro stimulation 15 days (t1), 7 days (t2) before the lambing, at lambing (t3) and 7 days after lambing (t4). PBMC were treated with two different concentrations (0.4 and 0.8 mg/mL) of six different wine pomace extracts and in presence of Concanavalin A (5 µg/mL) and LPS (1µg/mL). a, b refers to significance intra-time between treatment. Values with different letters differ ($P < 0.05$).

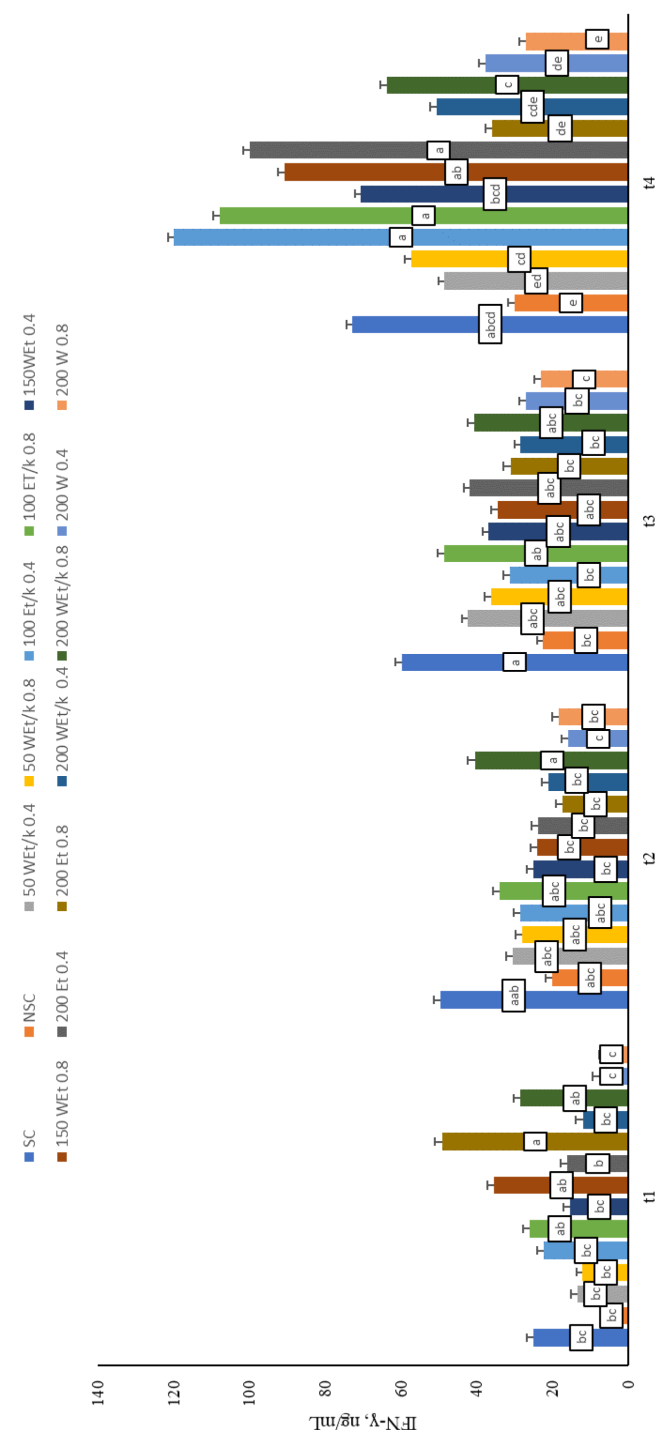
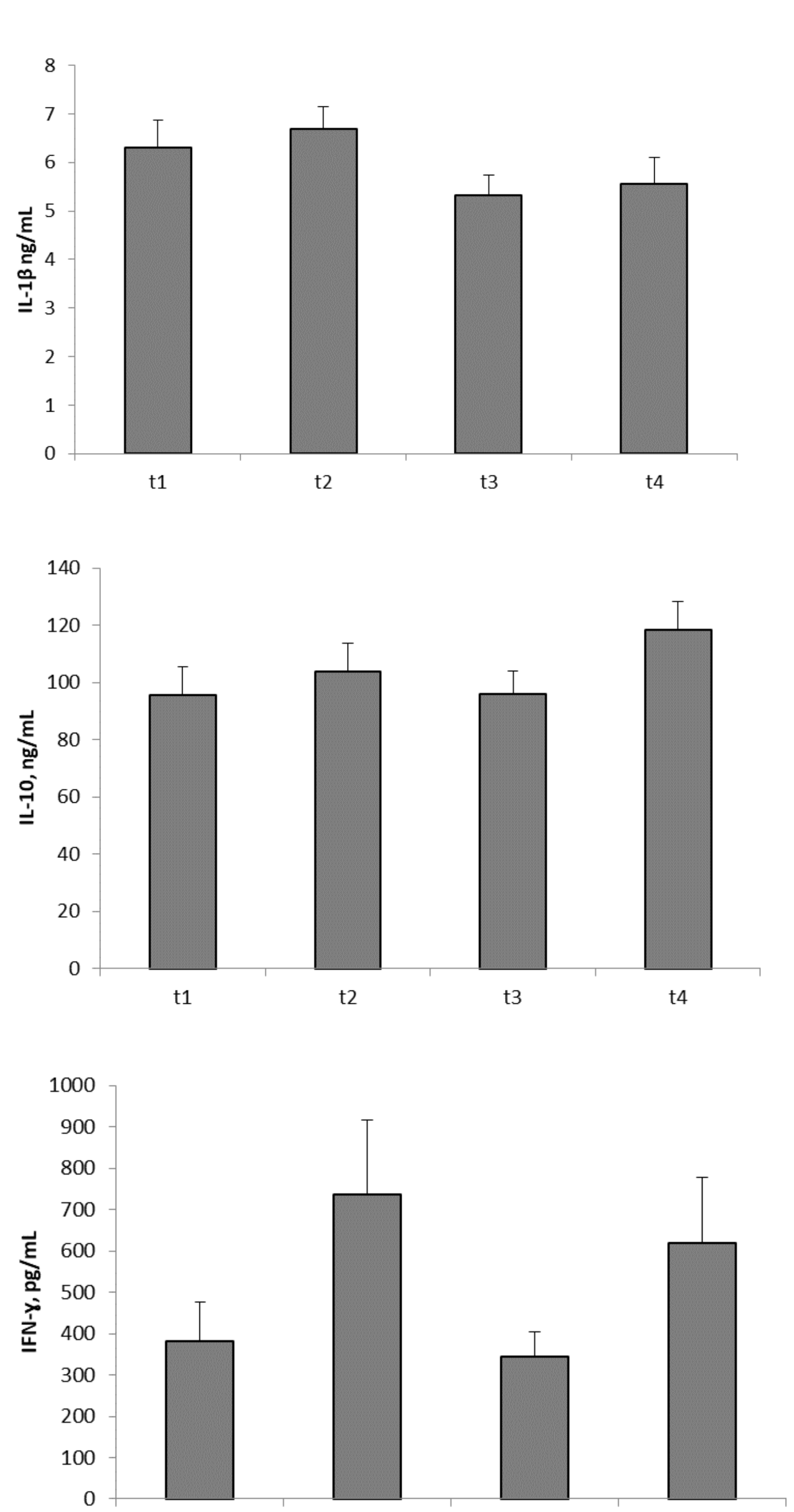


Figure 5.4.5: Least Squares means ± SEM of IL-1β, IL-10 and IFN-ɣ in sheep plasma 15 days (t1), 7 days (t2) before the lambing, at lambing time (t3), and 7 days after the lambing (t4). a, b Values with different letters differ ($P < 0.05$).



CHAPTER 6

CONCLUDING REMARKS

This dissertation described the potential application of winery by-products as immunomodulants in sheep.

Wordwild is generally accepted that industries generate a large amount of waste leading to a huge environmental impact. Recently, scientists focus their attention to what is called "zero waste economy" which is based on the circular economy concept. In this point of view waste can be used as new material to generate products.

This thesis aimed to characterize two different oenological by-products, wine lees and grape pomace, with the porpuse of extract bioactive substances which can be used as immunimodulants in sheep.

In order to support our economy, winery by-products were collected at local wineries and in order to valorizate the Apulia Region production, local cultivar of Vitis Vinifera were chosen.

First of all, winery by-products were collected and submitted to extraction procedures in order to isolate bioactive compounds. These procedures were conducted in an "enviromentaly friendly" approch using a microwave assisted extraction (MAE) with low environmental impact solvents such as water, ethanol and their mixture.

Three different trials were conducted, in two of them wine lees were assessed while in the tird trial grape pomace were studied.

In the first trial, three different wine lees from Bombino/Trebbiano d'Abruzzo in white vinifiaction and Nero di Troia in rosè and red vinification were submitted to MAE extraction with water, ethanol and water/ethanol 1:1 and catalyser/no catalyser and then total phenols, antocians, flavonoids content and antioxidant capacity were assessed. MAE extracts were tested on in vitro PBMC proliferation and cytokines' production. Results from this in vitro trial confirm the hypothesis that wine lees are able to affect sheep immune system, in particular PBMCs proliferation were marked reduced in precense of all wine lees extracts. Even though no significative variation of

pro-inflammatory cytokines were found, anti-inflammatory IFN-γ and IL-10 result augmented when Nero di Troia red wine lees in water (ReW) were added to PBMC, demonstrating an immunostimulatory effect of this wine lees extract which can be associated to the high scavenging activity of this extract. Furthermore, a gas chromatography-mass spetrometry analysis of wine lees extracts let us to found in Nero di Troia red wine lees in water the precense of 5-hydroxymethylfurfural (5-HMF). This “sugar derivate” has a large antioxidant and antiproliferative action and in this study its precense is connected to the high $ABTS^+$ capacity. Moreover, 5-HMF affect the apoptotic pathway through the BCl2 protein family and accordingly to this statement this trial demonstrates that PBMC stimulated with ReW had an increased level of pro-apoptotic Bax proteins.

In the second trial white, rosè and red wine lees MAE extracted in water were further purified by flash liquid chromatography (FLC), then a GC-MS/MS analysis of FLC fractions were conducted. Results from gas chromatography report the presence of a family of diketopiperazines in these wine lees fractions with a different isomers distribution in different fractions. PBMCs proliferation assay were conducted in vitro with two different wine lees fractions in condition of thermal stress (43°C) for 24 h and in normothermia (37°C) for 48 hours.

Diketopiperazine are recently investigated as a novel antioxidant, antiviral and antimicrobial molecules, besides to their application as immunostimulants. Results from this second trial report a marked decrement of lymphomonocytes proliferation in condition of normotheria. In addition, this experiment demonstrates that when wine lees fraction, rich in diketopiperazine, were added to PBMC an augmentation of IL-10 was obtained.

In the thermal stress assay, a conspicuous reduction of PBMC proliferation and viability was observed, besides to an increment of anti-inflammatory cytokines and a reduction of the pro-inflammatories ones.

Lastely, in the third trial red Nero di Troia grape pomace were MAE extracted with water, ethanol and water/ethanol 1:1 and catalyser/no catalyser and then total phenols, antocians and flavonoids

content and antioxidant capacity were assessed. Grape pomace extracts were tested on in vitro PBMC of sheep during transition period.

Results demonstrate a different impact on PBMC proliferation depending on the types of extracts; differently from the overall increment of PBMC, proliferative response in presence of 200°C extracts in Water at both concentrations and in Ethanol at 0.8 mg/mL undergone to a decrement. This result could be associated with an immunosuppressive role of these extracts. In addition, the level of IL-6 resulted higher when cells were harvested in precense of grape pomace extracted in water/ethanol or in ethanol at 150-10 200°C respectively. At day of lambing and seven days after it was registerd an increment of the level of IL-10. This cytokine and the IL-6 resulted both higher in some extracts and this effect can be linked to the activation of the innate immune response. Lastly, in this trial the level of the anti-inflammatory IFN-ɣ was higher in cells harvested with ethanol and catalyser extract and ethanol extracts. seven days after lambing.

To sum up, this thesis demonstrates the opportunity to reuse winery by-products as novel natural innunomodulants thanks to their content in bioactive compounds. In particular, wine lees extracts have an antiproliferative, immunostimulatory and antioxidant effect while wine lees frations thanks to the precese of diketopiperazine can be used as anti-inflammatory and chemotherapeutic agent. At last grape pomace can be used as anti-inflammatory and immunostimulants in sheep during transition period.

From the above discussion, grape by-products could be useful to enhance animal performances also in an environmentally friendly approach. In this direction, further studies are required in order to implement new low impact extraction procedures from other oenological and agricultural by-products with the aim of obtaining bioactive substances useful to produce novel feed formulations wich are able to ameliorate ruminants performaces and meanwhile to reduce the environmental impact resulting from the “industrialized world”.

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