UNIVERSITÀ
DI FOGGIA

**DIPARTIMENTO DI SCIENZE AGRARIE, DEGLI ALIMENTI E DELL'AMBIENTE**

Ph.D. thesis in

**Management of Innovation in the Agricultural and**

**Food Systems of the Mediterranean Region**

XXX cycle

Academic Year 2016-2017

# *FERMENTATION – BASED FOOD: INFLUENCE OF PROCESSING ON PRODUCT QUALITY*

Supervisor

**Dr. Sandra Pati**

Candidate

**Maria Luisa Savastano**

Coordinator

**Prof. Giancarlo Colelli**

*To my family*

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Doctorate course in "**Management of Innovation in the Agricultural and Food Systems of the Mediterranean Region**" XXX cycle.

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# CONTENTS

## ABSTRACT

Fermented foods and beverages represent an important part of human nutrition in every food culture around the world. Some of the most popular fermented products derive from grain, fruit and vegetables and are alcoholic based, for instance the notable variety of traditional beers and wines. Originally, fermentation can be regarded as a biological method of food preservation, but it is increasingly understood that some fermented foods also promote human health through the contribution of microorganism leading to further properties unlike to the starting food materials, beyond basic nutrition. Indeed, there is evidence that some fermented foods provide beneficial effects through direct microbial/ probiotic action and indirectly via the production of metabolites and breakdown of complex proteins. Kefir, for example, can help to relieve gastrointestinal disorders and reduce the symptoms of lactose intolerance besides having antioxidant, antimicrobial, anti-tumor and immunomodulation effects. Moderate wine consumption has undeniable health benefits as a decrease in the probability of cardiovascular disease, to delay the onset of noninsulin-dependent diabetes mellitus, combat hypertension, and reduce the frequency of certain cancers and several other diseases.

In the last years, there is a growing interest in winemaking without added of sulfites, owing to the perception that sulfites may cause negative health effects. The absence of sulfites in wine can be regarded as a quality factor, because consumers are attracted to foods without added additives, which are considered as healthy and genuine. Several methods for sulfites removal or reduction during winemaking have been studied, but the complete substitution with a product performing the same roles without the disadvantages of sulfites has been to date unsuccessful. Conversely, the wine production without addion of sulfites is becoming increasingly feasible due to technological improvement in winemaking. Furthermore, in the last years, there is a increased interest in the world wine market of red wines with fruity characteristics and old techniques developed some decades ago such as thermovinification, are becoming more widespread. Thermovinification is a winemaking process consisting of heating grapes to improve the extraction of grape anthocyanins and polysaccharides, responsible for color and roundness, whereas it reduces the tannins extraction, thus exogenous tannins are often added.

Within fermented foods, milk kefir is becoming more popular in European countries due to various health benefits linked for example to bioactive peptides. In particular, phosphopeptides are a subgroup of mulitphosphorylated bioactive peptides, which for instance can enhance the absorption of minerals in the gastrointestinal tract because binding and

solubilizing bivalent metal ions such as $Ca^{2+}$. Formation of phosphopeptides during the manufacture of kefir is affected of production technology. Finally, a great attention is paid to offer an alternative to conventional dairy-based drink, such as soy drink kefir, which could be a substitute food for vegans and people suffering from lactose intolerance or allergies.

The purpose of the present Ph.D. work was the study of influence of several technological parameters on the composition of two fermented foods: wine and kefir. In the first part, the work was focused on the study of several factors affecting the quality of white and red wines, whereas, in the second part, Ph.D. research regarded the influence of some technological parameters on the production of phosphopeptides in kefir. Finally, the characterization of microbiological and peptide profiles of kefir prepared with kefir grains from soy-drink was achieved.

As concerning wine, a study of the evolution in bottle of white wine produced by reductive winemaking, with and without sulfites, under several experimental conditions, was carried out over 15 months. Dark storage at 12 °C and 30 °C were compared to investigate the temperature effect, meanwhile uncontrolled temperature and light conditions were selected to simulate inadequate storage conditions. Results showed that volatile acidity and the absorbance at 420 nm increased at 15 months of storage particularly in both samples without sulfur dioxide addition and stored at 30°C and uncontrolled temperature and light conditions. Total $SO_2$ and free $SO_2$ decreased with time in wine samples with sulfites, with the exception of wine stored at 12°C, due to $SO_2$ action as antioxidant. Seventeen phenolic compounds were determined by HPLC-DAD-MS/MS analysis including hydroxycinnamate derivatives and minor compounds resulting from oxidation processes. At 15 months, the sample without sulfites addition and stored at the constant temperature of 30°C was the most browned, followed by samples stored at inadequate temperature and light conditions and at constant temperature of 12°C both without sulfites added. Besides the oxidation compounds, the absence of sulfites seems to have favored the presence of cis forms of hydroxycinnamate derivatives and caffeic acid derivatives. Furthermore, the combination of inadequate conditions of light and temperature favored hydrolysis reactions more than the effect due to the only high temperature. Finally, results concerning volatile profile showed that the presence of sulfites helps the typical aroma of young wines; contrarily the storage without sulfites accelerated the hydrolysis of acetate esters and contributed to increase furfural, 5-methyl-2-furancarboxaldehyde, and benzaldehyde contents. The appropriate storage in the dark, at low temperature seems to be the most important condition as improper storage conditions favor aroma degradation regardless sulfite addition.

In the second work, the influence of thermovinification on ochratoxin A (OTA) content in red wines was studied. Two heating treatments were investigated (60-65°C for 2 h and 80-85°C for 30 min), both treatments applied with and without added tannins. To achieve this goal, a rapid and automatable method for the determination of OTA in wine using a microextraction by packed C18 sorbent followed by high performance liquid chromatography with fluorescence detection was developed and validated for a successful application in the context of wine production. Important experimental parameters, such as sample and eluent volumes, extraction mode, draw and dispense speeds, number of eluent passes up and down through the stationary phase, were optimized. The validation included the comparison of the sensitivities related to solvent-matched, matrix-matched and standard addition calibrations and the participation to a proficiency test in an inter-laboratory circuit. Matrix effects were also investigated. Accuracies relevant to real samples were estimated to range between 76 and 100%, at 0.2 μg/L, and between 84 and 108%, at 1.0 μg/L, in compliance with the EU Regulation 401/2006; the limits of detection and quantification were of 0.08 and 0.24 μg/L, respectively, i.e. much lower than the maximum level currently permitted for OTA in the European Union (2.0 μg/kg, corresponding to ca 2.0 μg/L). As concern thermovinification process, no significate differences on OTA concentration among the wines studied were found.

As regards the second part of the thesis, first, the profiling of multi-phosphopeptides in kefir was carried on, and phosphopeptides were selectively enriched on hydroxyapatite and further analyzed with mass spectrometry analysis. Thus, 22 phosphopeptide sequences were identified in kefir, mainly derived by β-casein, and 9 of them showed the polar acidic motif pSpSpSEE (pS= phosphoserine), which is the most active in binding minerals.

Then, the influence of temperature (18 or 25°C), pH (4.0 or 4.7) and the repeated use of grains with 2-step fermentation (back-slopping approach) on phosphopeptide profile in milk kefir prepared using kefir grains was studied. Microbiological characterization was carried out in each sample, considering both grains and kefir, before and after fermentation. The total lactic acid bacteria, acetic acid bacteria and yeasts are increased after fermentation steps and, in particular, microbial growth, at the investigated conditions, was affected only quantitatively by the fermentation temperature whereas both quantitatively and qualitatively by the final pH. The LC-ESI-QTOF-MS/MS study revealed a phosphopeptide profile of kefir that include 93 phosphopeptides with lengths ranging from 10 to 43 amino acids, and of which, 82 showed the typical acid motif “SSSEE”, crucial for exhibiting the bioactive properties of mineral binding. Furthermore, kefir processing, in particular pH, largely influenced the proteolysis of kefir grains microorganisms and the profile of microbial population. A reduced proteolysis rate was

observed in kefir prepared with back-slopping approach, indicating a low reproducibility of proteolytic activity of kefir grains. Moreover, a sensory evaluation was carried out on the kefir samples and no statistically significant differences ($p < 0.05$) were observed in odor, taste and acidity between kefir products.

Finally, the microbiological evaluation and a comprehensive analysis of peptide profile in kefir, prepared with kefir grains from soy-drink were performed. As concerning microbial population, only lactic acid bacteria and yeasts were found in soy drink and grains kefir; acetic acid bacteria were not detected. One hundred seventy-nine peptides were identified mainly derived from glycinin and lipoxygenase and ranged from 9 to 37 amino acids. The released peptides from the parent proteins are not distributed equally among the protein sequences, indeed, there were distinct parts in the proteins where more peptides are released than in other parts. However, the specificity of lactic acid bacteria proteinases in soy-based fermented foods has not been equally investigated as the proteolytic behavior on milk proteins. Results showed 62 bioactive sequences, encrypted within the investigated peptides, which are known for ace-inhibitory, antihypertensive, antioxidative, hypocholesterolemic and antimicrobial activities.

All results described in the present Ph.D. thesis contributed to increase the knowledge on wine and kefir, on relationships between quality and processing and potential health benefits, providing important tools to improve quality of wine and kefir productions.

# CHAPTER 1

## INTRODUCTION

### 1.1 Fermented foods: an overview

The production of fermented foods is among the oldest food processing technologies known to man. Based on archeological findings, the history of fermented foods can be traced back to thousands of years. For instance, evidence from jars and vessels show that winemaking was popular in Neolithic Egypt and Middle east (Marshall and Mejia, 2011; Prajapati and Nair, 2008) and fermented rice, honey and fruit beverages in China date back to 7000 BC (McGovern et al., 2004), whereas recent proteomic analysis show kefir milk to have been fermented some 3500 years ago in Asia (Yang et al., 2014). Fermented foods and beverages represent an important part of human nutrition in every food culture around the world (Tamang and Samuel, 2010). The variety of fermented products originates from the heterogeneity of traditions and cultural preferences found in the world, and the staple and/or by-products used for fermentation. In many cases, it is highly likely that the production methods were unknown and came about by chance, and passed down by cultural and traditional values to subsequent generations. Some of the most popular fermented products derive from grain, fruit and vegetables and are alcoholic based, for instance the notable variety of traditional beers and wines. Fermented/pickled fruit and vegetable are very common in Europe, Asia, America, Africa and Middle East, while fermented fruit juice, tea leaves and product of brina are widely consumed in Asia (Josephsen and Jespersen, 2004). Fermented cereals, tubes and roots belong to dietary staples in Asia, Africa, Europe and Latin America, while fermented seeds and fish are present in different countries in the world (Josephsen and Jespersen, 2004). Fermented foods are made through a controlled microbial growth, with enzymic activities, due to amylases, proteases, lipases, hydrolyzing polysaccharides, proteins and lipids to nontoxic products with flavors and textures pleasant to the consumer (Figure 1) (Steinkraus, 1997). From a biochemical point of view, fermentation is a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidizing agent.

Fermentation plays different roles in food processing:

1) enrichment of the human dietary through development of the new and desirable tastes and textures in foods;

2) preservation of food through lactic acid, alcoholic, acetic acid, alkaline fermentations and high salt fermentations;
3) enhance nutritional value of food with vitamins, protein, essential amino acids and essential fatty acids;
4) improving food safety during food fermentation through inhibition of pathogens (Adams and Nicolaides, 2008) or removal of toxic compounds (Ray and Panda, 2007);
5) decrease in cooking times and fuel requirements.

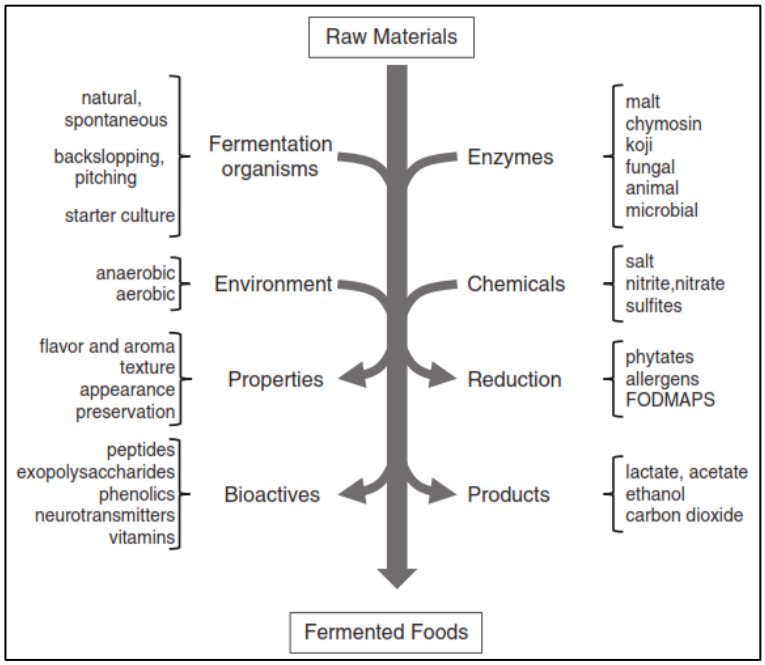


**Figure 1.** Overview of fermented foods processing. Raw material are fermented in specific condition to create desirable foods (Marco et al., 2017).

### *1.1.1 Classification of fermented foods*

Fermented foods can be classified by primary metabolites and microorganisms involved (Yokotsuka, 1982), by fermentation type (Steinkraus, 1997) or by food substrate. They are the products of acidic, alkaline or alcoholic fermentations, which are mediated by either bacteria, yeasts, moulds, or mixed (bacteria and yeasts) cultures (Ray and Joshi, 2014). Cereals, milk, meat, fish, grape (fruit), vegetables and soybean are the substrates mainly used in their commercial production. Fermented foods from around the world are clustered in the following categories (Ray and Joshi, 2014):

a) Cereal-based fermentation. Products play an important role in human nutrition in all countries where cereals grow. The major cereal based foods include wheat, maize, barley, oats, rice, sorghum and millet. In terms of texture, fermented cereal foods are liquid or solid.

Bread is one of the oldest artificial foods, and it is prepared from a dough of flour and water, usually by baking. Among cereal based beverages there is boza, made with the fermentation of a variety of cereals including barley, oats, rye, wheat or rice semolina and millet flour for best quality and taste (Arici and Daglioglu, 2002). Another beverage are kvass, a soft drink common in Russia and produced from rye, barley malt, rye fluor and stale rye bread (Dlusskaya et al., 2008), and amazake that is a sweet non-alcoholic fermented rice drink, precursor to sake and produced in Japan (Yamamoto et al., 2011). Beer is another product known in the world and there are different types of starchy plants that are used for brewing, including maize (in South America), soy (in India and Iran), millet and sorghum (in Africa), and rice (in the Far East), but beer production from barley malt is currently the most common worldwide (FAO, 2009).

b) Dairy-based fermentation. Products can be made from milk or skimmed milk (pasteurised or unpasteurised) from cow, camel, sheep, goat, mare and yak. They can be obtanined through defined starter cultures, back-slopping or by lactic acid bacteria (LAB), such as *Lactobacillus, Lactococcus*, and *Leuconostoc* and allowed types of yeasts, such as *Saccharomyces* and *Candida* that are the species most commonly detected. Lactic acid fermentation involves production of lactic acid and consequently a lowering of pH that makes fermented foods safe with desiderable texture and particular flavour that are unlike those present in the starting materials (Wouters et al., 2002). Yoghurt is the most popular fermented milk in the world and it is obtained from fermentation of cow milk by *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Kefir and koumiss are fermented drinks made with kefir grains composed by a symbiotic combination of bacteria, yeasts and protein milk. Other fermented milk products close to the yoghurt are acidophilus milk (North America), laban and ayran (Middle East), leben (Arab World) anddahi (India). Other fermented milk products are cheeses made from milk and natural lactic flora or appropriate starter culture.

c) Meat-based fermentation. Products can be produced with the addition of microorganisms and different ingredients, such as fat, salt, curing agents (nitrate / nitrite), sugar and spices, that are mixed together with meat (Leroy et al., 2013) obtaining products with a shelf-life and expediency for consumption longer than meat and with satisfactory sensory characteristics, particularly distinct flavours. LAB control the fermentation process and prevent harmful bacterial growth due to production of acids and alcohols, which leads to a pH decrease; meantime, proteins are fragmented into peptides and amino acids (Leroy et

al., 2013). Several fermented meat products are available such as fermented sausage, bacon, and ham (Liu et al., 2011; Leroy et al., 2013).

d) Fish-based fermentation. Products are worldwide and depend on the culture, climate, and availability of both salt and fish. There are two types of fermented fish products available in the world: fish sauce and fish paste. The first type has been called “the mother of all condiments” and the most known are garos and garum, prepared by fermenting fish blood and intestines in a salt brine. Other products are patis, budu, bakasang, which are very popular in southeast and east of Asia. The most common Fish pastes (Panda et al., 2011) are hentak and ngari in India, terasi from Indonesia, bagoong from the Philippines, ngapi from Myanmar and belacan from Malaysia.

e) Fruit-based fermentation. Wine is the most commonly available and best known in the world, but there are some fruits like plum, peach, pear, apple, citrus, strawberry, etc., which are fermented to produce low alcoholic beverages and are consumed all over the world (Joshi, 2009; Jackson, 2011). Winemaking involves grape must fermentation by *Sacchromyces cerevisiae* yeasts (FAO, 2011), with the transformation of fermentable sugars to ethanol. Production of ethanol preserves and makes the fermented products stable and safe. There are made several types of wines like table wines, sweet and dry wines, fortified wines,sparkling wines (Joshi et al., 2011). A non-alcoholic fermented beverage is hardaliye made from red grape juice, crushed black mustard seeds, cherry leaf and benzoic acid and it is widely consumed in Turkey (Amoutzopoulos et al., 2013).

f) Vegetables-based fermentation. In the world there are fermented mixed vegetables such as cabbage, radishes, cucumbers, turnips and beets (Ray and Panda, 2007). In particularly, a large number of fermented vegetable juices and blends are producedin Europe, through fermentations of olives, cucumbers (pickles), and cabbage (sauerkraut). The LAB grow in the vegetables without decomposing cellulose or proteins (Li, 2001), and contribute to the characteristics of the final product in addition to preservation.The addition of salt is required and facilitates aroma production, controls against undesirable microorganisms, extracts water and nutrients, and constitutes soft tissue (Panda et al., 2007). Finally, anaerobic environment, salt addition and acid production result in unique features of the products and a high degree of hygienic safety.

g) Soybean-based fermentation. Soy is an important source of protein and essential amino acids in Asian countries, and China is the place of origin. Soybean has been utilized in various forms such as tofu, miso, natto, soy sauce, cheese. During fermentation process, complex organic compounds are broken down into smaller molecules through metabolic

processes of microorganisms, providing additional health benefits. The characteristic aroma and flavor of soybean-based fermented foods are partially generated by LAB (Liu et al. 2011).

### *1.1.2 Health benefits of fermented foods*

Originally, fermentation can be regarded as a biological method of food preservation, but it is increasingly understood that some fermented foods also promote human health through the contribution of microorganism leading to further properties unlike to the starting food materials, beyond basic nutrition. However, most of the traditional fermented beverages and foods are poorly studied (particularly for non-dairy products) and lack of scientific evidence that links them to positive effects on human health. Nevertheless, there is still a perception that many of these fermented foods are "healthy", particularly in societies where them are steeped in local tradition, which in turn contributes to their market potential and justifiers investing in related research (Marsh et al., 2014). A strong association between the microbial content and the enhancement of gastrointestinal health exists for many of the fermented foods, particularly for dairy products. There is evidence that some fermented beverages provide beneficial effects through direct microbial/ probiotic action and indirectly via the production of metabolites and breakdown of complex proteins. Indeed, several bioactive peptides are released by proteolytic system of some LAB in milk products (Hafeez et al., 2014) and they display several activities, such as hypotensive, immunomodulatory, antioxidant, antimicrobic, antithrombotic or opioid activities, which appear to be capable to show beneficial health effects by acting on the nervous, digestive, cardiovascular and immune system (Haque et al., 2009; Nagpal et al., 2001). Naturally fermented milks, for instance, have been shown to have antihypertensive effects, to enhance systemic immunity, to lower cholesterol and to help lower blood pressure. Kefir, in particular, can help to relieve gastrointestinal disorders and reduce the symptoms of lactose intolerance besides having antioxidant, antimicrobial, anti-tumor and immunomodulation effects (Otles and Cagindi, 2003). Moreover, fermented dairy products usually possess β-galactosidase activity and reduced lactose content compared to milk. Most cheeses and yogurt, indeed, are generally well-tolerated by lactose-intolerant individuals (Savaiano, 2014). Fermented cereal food is another category which has received attention regards its health effects, because it is considered source of macronutrients and micronutrients such as vitamins, minerals, fibres, flavonoids and phenolic compounds which can affect oxidative stress, hyperglycemia, inflammation and carcinogenesis (Wang et al., 2014). Moreover, cereal based food have a lower fat percentage than dairy-based one. As concerns fermented vegetable, the

growth of LAB enhances the conversion of phenolic compounds (flavonoids) to biologically active metabolites that induce the expression of antioxidants and detoxifying enzymes protecting against oxidative and chemical damage (Senger et al., 2016). As an example, sauerkraut that were shown to provide antimicrobial, antioxidant and anti-tumor effects (Tamang et al., 2016). Furthermore, consumption of fermented soy products led to improve total cholesterol, non-HDL and LDL concentrations (Cavallini et al., 2016). Also moderate wine consumption has undeniable health benefits. Polyphenols in red wine probably act synergically with tocopherol (Vitamin E) and ascorbic acid (Vitamin C), thus helping the inhibition of lipid peroxidation (Feher et al., 2007). Moderate consumption of wine has been reported to decrease the probability of cardiovascular disease, to delay the onset of noninsulin-dependent diabetes mellitus, combat hypertension, and reduce the frequency of certain cancers and several other diseases (Jackson, 2000). Despite this, it is known that the excessive alcohol consumption, both acute and chronic, can have devastating effects on the physical and mental well-being of individuals. Ethanol consumption can cause liver cirrhosis, increase the likelihood of hypertension and stroke, favor the development of breast and digestive tract cancers, and augment the potential for fetal alcohol syndrome (Jackson, 2000). The presence of biogenic amines, at high concentrations, constitutes another important health risk in fermented foods. Biogenic amines have been found in fermented dairy and cereals products, fish products, wine and beer, sauerkraut, dry sausages, etc. (Spano et al., 2010; Zhai et al., 2012). They are produced by LAB (*Enterococcus*, *Lactobacillus*, *Leuconostoc* and other genera) by microbial decarboxylation of amino acids or by transamination of aldehydes and ketones (Zhai et al., 2012). At high concentrations, biogenic amines are risk factors for food intoxication, while moderate levels may lead to food intolerance (Ladero et al., 2010).

### *1.1.3 Commercialization of fermented foods*

Over the last decade, more and more people are recovering interest in traditional and natural foods and the developments in this way are leading to an increased interest in fermentation technology by food industries (Hugenholtz, 2013). Dairy products represent about the 43% of the functional foods market and, within dairy products, the main contribution is due to fermented products (Özer and Kirmaci, 2010). The majority of fermented milks and yogurt like drinks fall within the category of probiotic beverages due to the presence of specific live microorganism, such as *Lactobacillus* spp., *Bifidobacterium* spp., and other species. For instance, popular functional beverages are kefir in Western Europe and North America, ymer in Denmark and yoghurt in North America, Europe and Asia.

However, in recent years non-dairy probiotic products has been attracting more attention due to the success of bio-functional foods and the desire to expand and offer an alternative probiotic choice to conventional dairy-based beverages. Indeed, this market is projected to have an annual growth rate of 15% between 2013 and 2018 (Marketsandmarkets, 2013). Non-dairy products are particularly attractive due to their lack of dairy allergens, low cholesterol content and vegan-friendly status (Prado et al., 2008). Additionally, different substrates, such as cereal-based, can provide different combinations of antioxidants, dietary fibre, minerals and vitamins. Soy-based foods contain low cholesterol and low saturated fats, are lactose-free, are rich in isoflavones and antioxidants, and have been shown to exert several beneficial effects. Therefore, industry has developed probiotic soy derivatives with several varieties already available commercially (Haelan951®and Jiva™).

### 1.2 Fruit-based fermentation: wine and winemaking

Wine is produced by the alcoholic fermentation of grape must and is a very complex hydroalcoholic beverage, which is constituted of a high number of substances. Some of these exist in the must, while others originate from the fermentation process and chemical reactions during all winemaking process. Furthermore, the quality characteristics of a wine are affected by the quality and variety of grape, and the applied technology.

Winemaking formally begins when the grapes or juice reach the winery and ends with bottling of the finished wine. A schematic flow chart of the basic winemaking operations is shown in Figure 2 (Jackson, 2000). After harvest, crushing and releasing of juice, maceration and fermentation processes begin. Maceration consists of extraction and dissolution of different substances from the seeds and skins, and is induced by the action of hydrolytic enzymes released from cells ruptured during crushing (Jackson, 2000). During maceration, aromatic compounds are released from pomace and must and other compounds are synthetized. Moreover, enzymes also may hydrolyze macromolecules into compounds readily usable by yeasts and bacterial cells (Jackson, 2000). Fermentation begins spontaneously by yeasts naturally present on the grape surface, but this can give unpredictable results depending on the types of yeast, or through inoculation with yeast strains of known characteristic into must. During the first part of fermentation, yeasts convert sugars into alcohol, produce carbon dioxide, and in the second part the remaining sugars are slowly converted into alcohol and the wine becomes clear. The main differences in maceration and fermentation processes between white and red winemaking are:

- White wine. White wine is exclusively produced by the fermentation of grape juice. Indeed, juice extraction and clarification always precede alcoholic fermentation. Furthermore, white wines can be produced from white or red grapes having white juice, if the grapes are pressed in conditions that prevent grape skin anthocyanins from coloring the must. This is the case of *blancs de noirs* from Champagne, made from Pinot grapes (Ribéreau-Gayon et al., 2006). However, all winemaking process includes a selective extraction of grape components and white winemaking does not escape from this general principle. Thus pre-fermentation operations, such as harvest, crushing, pressing and clarification affect greatly the taste and flavor of white wines as control the passage of compounds responsible for the qualities and flaws of grapes into must (Ribéreau-Gayon, 2006). The major physical factors influencing the extraction and types of compounds during maceration are temperature and duration of the process; for instance, cool maceration temperatures and short duration minimize flavonoid uptake, and thereby limit wine bitterness and astringency (Jackson, 2000). After maceration operation, clarification is an important technological operation because wine contains many suspended solids, which affect wine with green aroma and bitter taste. White wines must be protected from oxygen during all the winemaking process to preserve fruity aroma of young wine and to avoid browning (Ribéreau-Gayon, 2006). Indeed, after fermentation, wine is protected from oxidation with several techniques, among which the most important is the addition of sulfur dioxide, while protecting must from oxidation is not universally considered necessary. Some winemakers both limit air contact and add sulfur dioxide to juice to prevent the enzymatic oxidation of phenolic compounds, whereas other winemakers perform various complementary techniques, such as adding of ascorbic acid, cooling grape or heating grape for few minutes (Ribéreau-Gayon, 2006). Finally, fermentation is generally carried out in stainless still tanks allowing to control the temperature during fermentation, which is typically between 15 and 18 °C.
- Red wine. Red wine is produced by the extraction of solids from grape clusters (specifically from skins, seeds and possibly stems) accompanied by the alcoholic fermentation of the juice. Indeed, red wine is a macerated wine. The length and intensity of maceration are adjusted according to grape variety and the type of wine desired (Ribéreau-Gayon et al., 2006). A short maceration (< 24 hours) produces, commonly, rosé wine, while wines for long aging have often been macerated on the seeds and skins for as long as 3 weeks (Jackson, 2000). Extraction efficiency rises as ethanol increases and it should be taken into account that an excessive extraction, obtained by increasing the process temperature and time, could give to wine both an herbaceous character and excessive astringent character.

During the alcoholic fermentation at 22-25 °C, the suspended solids are carried upwards by the $CO_2$ produced, forming a large mass called cap. This floating mass needs to be broken-up and re-submerged several times: this operation is known as "cap punching" or "pumping-over". This operation is very important since allows both a good extraction from grape solid parts and a total fermentation of sugars; besides, if the cap is exposed to air for too long time, the surface can dry out and allow the colonization of airborne bacteria.

After the alcoholic fermentation, malolactic fermentation can take place, during which specific strains of bacteria convert malic acid into the milder lactic acid. This fermentation is often initiated by inoculation with desired bacteria. At the end of the fermentation, wine is racked, some wines are then allowed to age in wood barrels before bottling, which add extra aroma to the wine, while others are directly bottled.

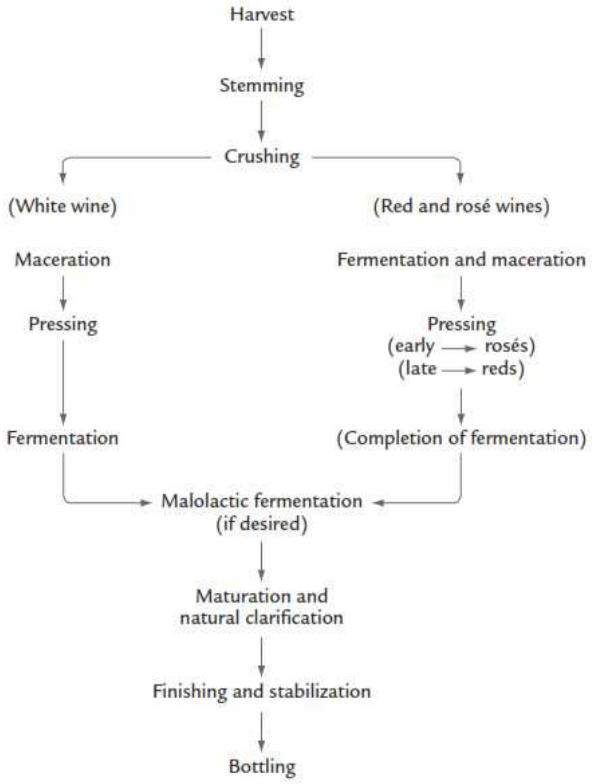


**Figure 2.** Flow diagram of winemaking (Jackson, 2000).

### *1.2.1 The role of sulfur dioxide in winemaking*

Since the end of the 18th century, sulfur dioxide, often abbreviated to sulphite or $SO_2$, was used to protect musts and wines during winemaking, especially the white wines. Sulfur dioxide is added to wine as form employed of gas or liquid solution, and as salts such as potassium bisulfite ($KHSO_3$) or potassium metabisulfite ($K_2S_2O_5$) (Ribéreau-Gayon et al., 2006). Small

quantities of $SO_2$ are also produced naturally by yeasts during fermentation, in concentrations rarely more than 10 mg/L, but in some cases, it can exceed 30 mg/L (Ribéreau-Gayon et al., 2006). Concentration of added $SO_2$ depends on wine type and generally ranges from 50 to 200 mg/L.

In wine, there is an equilibrium between molecular and ionic forms of sulfur dioxide. At pH of wine, 94 to 99% of sulfur dioxide exists in the ionic form as the bisulfite ion $HSO_3^-$ and thus only a small proportion is present as free $SO_2$. The two fractions of $SO_2$ in wine are "free $SO_2$" referred to ion bisulfite and sulfur dioxide, and the "bound $SO_2$" due to the sulfur dioxide that is bound to unsaturated compounds (Oliveira et al., 2011).

Principal properties of $SO_2$ in wine:

- Antiseptic: $SO_2$ regulates the growth of harmful yeasts and bacteria in wine. It has a greater activity on bacteria than on yeasts. Antimicrobial activities are affected by several factors such as the concentration of $SO_2$, the growth and development of microorganism, the pH and the temperature. Indeed, at low $SO_2$ concentration, the inhibition is temporary, while at high concentration, $SO_2$ destroys a percentage of the microbial population. During storage, $SO_2$ hinders the development of all types of microorganisms (yeasts, lactic bacteria, and, to a lesser extent, acetic bacteria), preventing yeast haze formation, secondary fermentation of sweet white wines, *Brettanomyces* contamination and the subsequent formation of ethyl-phenols, the development of mycodermic yeast, and various types of bacteria spoilage (Ribéreau-Gayon et al., 2006).
- Antioxidant: $SO_2$ protects polyphenols and other wine constituents from chemical oxidation, but it has no effect on quickly enzymatic oxidations. $SO_2$ reacts directly with oxygen (Ribéreau-Gayon et al., 2006, Clarke and Bakker 2004) or with the oxygen reduced form (Oleiveira et al., 2011). Boulton et al. (1996) stated that the main function of $SO_2$ is to scavenge hydrogen peroxide produced by the oxidation of polyphenols. Studies in model wines have shown that the reaction of $SO_2$ with oxygen is extremely slow when compared with the uptake of oxygen by wine itself (Danilewicz, 2007). An example of reaction between $SO_2$ and oxygen reduced form, hydrogen peroxide, is shown in Figure 3. In this way, $SO_2$ can inhibit aldehydes formation by competing for hydrogen peroxide and plays an important role in reducing quinones formed during oxidation process back to their phenol form (Danilewicz et al., 2008). Furthermore, $SO_2$ prevents the production of "grape reaction product" (GRP), which takes place through the reaction between caftaric acid quinone with tripeptide thiol glutathione (Singleton, 1985) maintaining a high level of free

hydroxycinnamates with high browning potential (Danilewicz et al., 2008; Oliveira et al., 2011).

- Antioxidasic: $SO_2$ inhibits the functioning of oxidation enzymes (tyrosinase, laccase) and can assure their destruction over time. Before fermentation, $SO_2$ protects musts from oxidation by this mechanism. It also helps to avoid oxidasic casse in white and red wines made from rotten grapes (Ribéreau-Gayon et al., 2006).
- Binding ethanal and other similar products to form non volatile bisulfite adducts, thus $SO_2$ protects wine aroma preventing unpleasant sensory properties. In particular, the reaction between ethanol and $SO_2$ represent the most significant portion of bound $SO_2$ in wine (Ribéreau-Gayon et al., 2006).

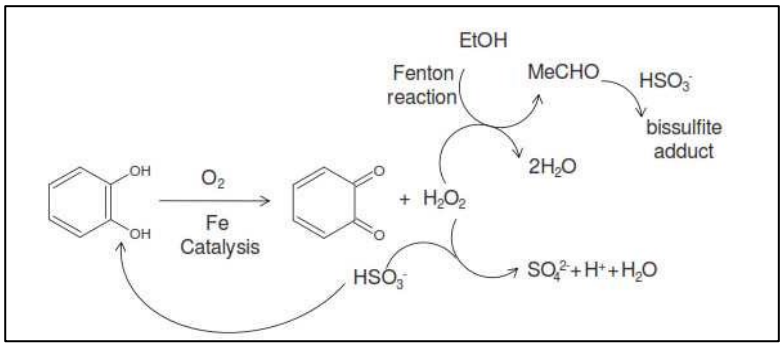


**Figure 3.** The interaction of $SO_2$ with hydrogen peroxide and quinones catechol oxidation, so preventing oxidation of ethanol by Fenton reaction (Oliveira et al., 2011).

*1.2.2 Sulfur dioxide and health*

The addition of sulfur dioxide to wine raises health-related objections due to serious allergic reactions incurred by sulfite-sensitive individuals, and thus the use of this additive is strictly controlled. Furthermore, winemaking techniques have always sought methods of lowering its concentrations (Ribéreau-Gayon et al., 2006). Since the beginning of the 20th century, the potential toxicity of sulfur dioxide has been studied (Ribéreau-Gayon et al., 2006). Some studies have shown that the role of sulfites has been overestimated, as in the case of wine-sensitive asthmatics (Vally and Thompson, 2001), while there is evidence that sulfites may induce relevant adverse reactions in drinkers suffering from sulfite sensitivity. Indeed, several adverse reactions can occur in drinkers such as anaphylactic shock, asthmatic attacks, angioedema, urticarial, nausea, gastric irritation, diarrhea and even death (Dalton-Bunnow, 1985; Vally and Thompson, 2003). Allergenic reactions occur at very low ingested concentrations (around 1 mg) and primarily concern asthmatics. Although $SO_2$ sensitivity has not been clearly demonstrated for non-asthmatics, these allergic reactions led the Food and

Drug Administration (FDA) in the USA to require the mention of the presence of sulfites on wine labels in the United States when the concentration exceeds 10 mg/L (Ribéreau-Gayon et al., 2006). In many cases, the effects of sulfites on health are very mild and depending on the subjects. Beyond respiratory and gastrointestinal symptoms (Lester, 1995), in some cases severe reactions include adverse dermatologic signs and headache (Costanigro et al., 2014). Recently, sulfites have been reported to promote low-density lipoprotein (LDL) oxidation by $Cu^{2+}$ at concentrations found in vivo and to stimulate the LDL-oxidase activity of ceruloplasmin (Laggner et al., 2005). Both to prevent risks for human health of drinkers and guarantee the stability and quality of wines, the European Commission Regulation (Reg. n° 666/2009) indicates the maximum sulfite dioxide content in white and red wine to 200 mg/L and 150 mg/L, respectively (EC, 2009).

### *1.2.3 Evolution of phenolic compounds in white wine without added sulfite*

In the last years, there is a growing interest in winemaking without added sulfites, as a consequence of the perception that sulfites may cause negative health effects. Several methods for sulfites removal or reduction during winemaking have been studied, such as sulfite absorption through the use of anion and cation exchanges or membranes, the use of lysozyme, bacteriocins or ascorbic acid (Comuzzo and Tat, 2003; Ribéreau-Gayon et al., 2006; Sonni et al., 2009). Nevertheless, the substitution with a product performing the same roles without the disadvantages of sulfites was unsuccessful (Li et al., 2008). The absence of sulfites in wine can be regarded as a quality factor, because consumers are attracted to foods without added additives, which are considered as healthy and genuine. Furthermore, this is becoming increasingly feasible due to technological improvement in winemaking without added of sulfites (Lustrato et al., 2006; Santos et al., 2012). In absence or reduced content of $SO_2$, the wine phenolic chemistry changes during winemaking and storage.

Phenolic compounds, present in grape must and wine, are directly related to wine quality attributes such as color, flavor, astringency and aging attitude (Li et al., 2011), and have antioxidant properties (Rice-Evans et al., 1996), but they are susceptible to oxidation and to cause browning (Gómez et al., 1995). In particular, white wine contains lower levels of polyphenols than red wine and mainly hydroxycinnamic acids, thus it is susceptible to the oxidative browning and losses of varietal aroma (Oliveira et al., 2011). The browning of wine can be classified into enzymatic browning (almost entirely in must grape) and non-enzymatic browning (both grape and wine, but prevails in wine):

- Enymatic browning. The enzymatic browning occurs in presence of atmospheric oxygen and some oxidoreductases enzymes. The main oxidoreductases responsible for browning during grape processing are polyphenoloxidase (PPO), Cu-containing enzymes, namely tyrosinase and laccase, and peroxidase (POD) which is a Fe-containing enzyme (Li et al., 2008; Oliveira et al., 2011). Tyrosinase or catechol oxidase can catalyze the oxidation of monophenols and o-diphenols (Singleton, 1987; Li et all., 2008), whereas laccase can oxidize some substrates, especially 1,2- and 1,4-dihydroxyphenene (Li et al., 2008). In grape must, enzymatic browning is associated with the content of hydroxylcinnamates such as caffeoyltartaric acid (caftaric acid) and ρ-coumaroyltartaric acid (coutaric acid) and is promoted by flavanols (Cheynier et al., 1986). Caftaric acid or ρ-coumaric acid is oxidized by PPO to produce caffeoyltartaric acid o-quinones, which are powerful oxidants and able to oxidize other compounds in wine causing great changes to color intensity and tonality (Figure 4) (Robards et al., 1999; Li et al., 2008). Furthermore, the available glutathione quickly reacts with the caffeoyltartaric acid o-quinones forming a colorless compound 2-S-gluthathionyl caftaric acid (grape reaction product, GRP), which is not a suitable substrate for further oxidation (Cheynier et al., 1986). Indeed, the formation of GRP is believed to limit the must browning. On the other hand, $SO_2$ inhibits tyrosinase and prevent the production of GRP with high browning potential, and GRP is also to be oxidized by laccase.

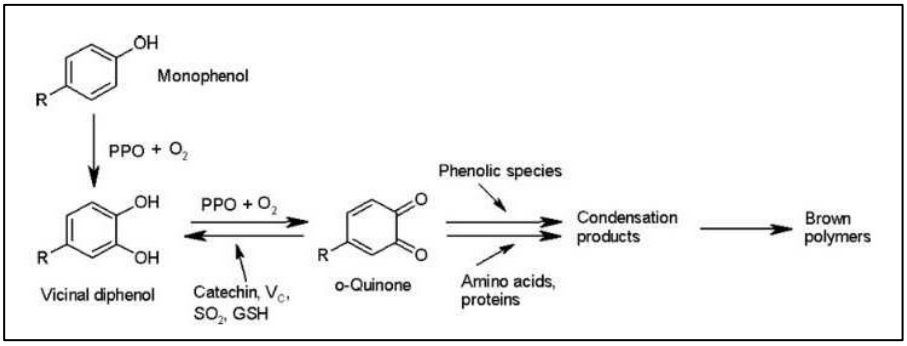


**Figure 4.** Enzymatic browning process in grape must (Li et al., 2008).

- Non enzymatic browning is favored by the oxidation of polyphenols containing an ortho-dihydroxybenzene or 1,2,3-trihydroxybenzene moieties, such as (+)-catechin /(-)-epicatechin, gallocatechin, gallic acid and its esters, and caffeic acid (Singleton, 1987; Singleton, 2000; Danilewicz, 2003; Li et al., 2008; Oliveira et al., 2011). These compounds are oxidized to semiquinone radicals and benzoquinones, while oxygen is reduced to hydrogen peroxide in presence of transition metal ions (Oliveira et al., 2008). Thus,

quinones can combine with nucleophilic compounds, such as phenols, thiols and amines, and the produced dimers or polymers may rearrange their structure through an enol-like conversion reaction to form new dihydroxybenzene moieties (Figure 5) (Li et al., 2008). Furthermore, the oxidation of the regenerated product brings in an acceleration of the polymerization process (Boulton et al., 2001; Zhai et al., 2001). The hydrogen peroxide in presence of $Fe^{2+}$ generates hydroxyl radicals (Fenton reaction), which can oxidize any organic molecule found in wine (Waterhouse et al., 2006), such as ethanol, tartaric acid, glycerol, sugars ad organic acids (Danilewicz, 2003; Oliveira et al., 2011). Thus, ethanol and tartaric acid could be oxidized by Fenton reaction to form acetaldehyde and glyoxylic acid (Li et al., 2008). Moreover, the oxidation of tartaric acid can produce dihydroxyfumaric, which react with (+)- catechin in a wine-like solution; the formed yellow pigments were identified as xanthylium cations (Clark, 2008; Oliveira et al., 2011). These pigments are potentially contributors to white wine browning and lead to an increase of absorption in the 400-500 nm region of the visible spectrum (Martinez and Wintaker, 1995).

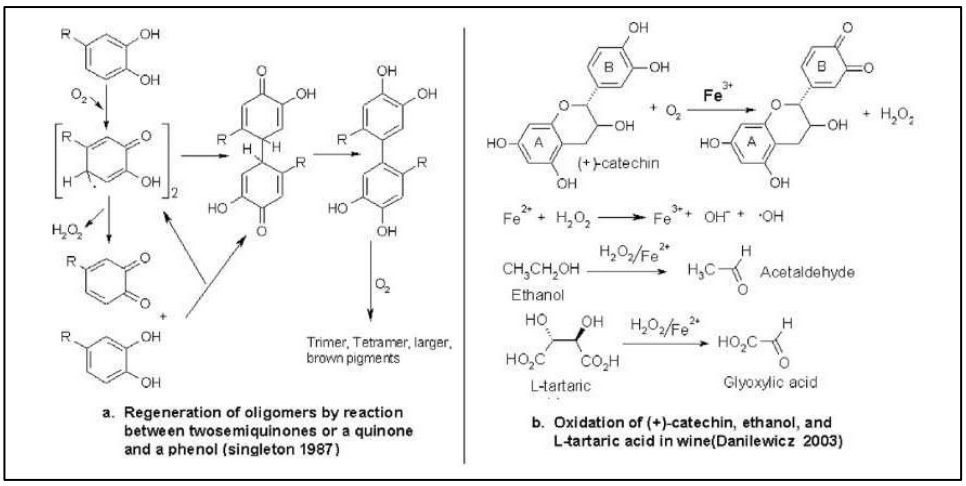


**Figure 5.** Non-enzymatic oxidation reactions in wine (Li et al., 2008).

### *1.2.4 Thermovinification*

Red wines are a rich source of different phenolic compounds, which are responsible of the sensorial characteristic of wines such as color, astringency and aging ability, and can exhibit antioxidant properties (Benítez et al., 2002). The final composition of phenolic compounds in wines depends on various factors such as phenolic content in grapes, the extraction parameters, winemaking technology and the reactions that occur in wine aging (Paixao et al., 2007). It is know that some winemaking techniques as thermovinification can increase phenolic content in wines. Furthermore, there is a crescent interest in the world wine market of red wines with fruity

characteristics and old techniques developed some decades ago such as thermovinification, are becoming more widespread (Geffroy et al., 2015). Thermovinification is a winemaking process consisting of heating grapes between 60 and 80 °C during the pre-fermentation step for a length of time varying from 30 min to 24 hours. Heating helps to destroy laccase activity on botrytized grapes and assists in the extraction of grape anthocyanins (Sacchi et al., 2005) and polysaccharides (Doco et al., 2007), responsible for color and roundness in wine, respectively, and, to a lesser extent, tannins (Girard et al., 1997). Therefore, for wines that are to be consumed young, thermovinification, may help to enhance the color (Clarke and Bakker, 2004; Margalit, 2004), the total phenolic content, the antioxidant potential and the ester formation. However, the low tannin/anthocyanin ratio in thermovinified wines can lead to color instability, thus exogenous tannins are often added.

### 1.3 Dairy-based fermentation: kefir

Dairy-based fermented products of various animal species are probably the most common fermented foods worldwide. Yoghurt is very familiar to consumers than other fermented milk products such as kefir. However, the latter product is most popular in some regions of the world and nowadays it is increasingly popular in Japan, the USA, European countries due to various bioactive components that can provide humans with unique health benefits (Farnworth, 1999; Otles and Cagindi, 2003).

Kefir is a refreshing, self-carbonated and slight foamy fermented milk drink with creamy consistency and sour, acidic and mildly alcoholic flavor, which is believed to have originated in the Caucasian mountains (Farnworth, 2005; Altay et al., 2013). The word kefir means “good feeling” originates from Turkish word “keyif” and linked to overall sense when consumed (Güzel-Seydim et al., 2000) and also is noted with several names such as kephir, kefer, kiaphur, knapon, kepi and kippi (Sakar, 2007). The chemical and nutritional composition is variable because depends of fat content of milk, technological process and kefir grains composition (Otles and Cagindi, 2003). Table 1 show the chemical composition of kefir with some important quality parameters concentration (fat, proteins, lactic acid and ethanol).

**Table 1.** Chemical composition and nutritional value of kefir (Otles and Cagindi, 2003).

| **Components** | **100 g** | **Components** | **100 g** | **Components** | **100 g** |
|---|---|---|---|---|---|
| Energy | 65 kcal | Mineral content (g) | | Vitamins (mg) | |
| Fat (%) | 3.5 | Calcium | 0.12 | A | 0.06 |
| Protein (%) | 3.3 | Phosphor | 0.10 | Carotene | 0.02 |
| Lactose (%) | 4.0 | Magnesium | 12 | $B_1$ | 0.04 |
| Water | 87.5 | Potassium | 0.15 | $B_2$ | 0.17 |
| Milk acid (g) | 0.8 | Sodium | 0.05 | $B_6$ | 0.05 |
| Ethyl alcohol (g) | 0.9 | Chloride | 0.10 | $B_{12}$ | 0.5 |
| Cholesterol (g) | 1 | Trace elements | | Niacin | 0.09 |
| Essential amino acids (g) | | Iron (mg) | 0.05 | C | 1 |
| Tryptophan | 0.05 | Copper (µg) | 12 | D | 0.08 |
| Phenilalanin+Tyrosine | 0.35 | Molybdenum (µg) | 5.5 | E | 0.11 |
| Leucine | 0.34 | Manganese (µg) | 5 | | |
| Isoleucine | 0.21 | Zinc (mg) | 0.36 | | |
| Threonine | 0.17 | | | | |
| Methionine+Cystine | 0.12 | | | | |
| Lysine | 0.27 | | | | |
| Valine | 0.22 | | | | |

Lactic acid, $CO_2$ and alcohol are the major products formed during fermentation by lactic acid bacteria (LAB) and yeasts (Otles and Cagindi, 2003). L (+)-lactic acid is the most abundant organic acid in kefir and is derived about 25% of the original lactose in milk (Arslan, 2015). The alcohol content in kefir is dependent on the type of yeasts in the starter cultures or kefir grains, and the extent of the ripening period (Wszolek et al., 2006). $CO_2$ content is between 0.85-1.05 g/L for kefir produced from kefir grains (Beshkova et al., 2002) and about 1.7 g/L for kefir obtained from purified cultures (Gobbetti et al., 1990). However, the $CO_2$ content produced after packaging presents some practical problems, since yeasts continue to grow after packaging and, thus strong or flexible package must be used to contain the volume of gas produced (Kwak et al., 1996). Kefir is a good source of vitamins, minerals and essential amino acids, and is a good diet for lactose intolerant individuals (Otles and Cagindi, 2003). Typical taste and aroma of kefir, which do not receive a high positive taste rating when people tasting first time kefir (Farnworth, 2005), depends on the metabolism of LAB and yeasts, which produce some compounds during fermentation (Beshkova et al., 2003). In particular, diacetyl, acetaldehyde and acetoin, which are aromatic compounds, impact to kefir flavour and during storage acetaldehyde increase in concentration whereas acetoin decreases (Güzel-Sydim et al., 2000a, 2000b).

*1.3.1 Kefir production*

Kefir can be made from any type of milk (cows, ewes, goats, buffalos) and also from substitutes such as soy, rice and coconut drinks but typically, cow milk is used (Irigoyen et al., 2005; Otles and Cagindi, 2003). In addition, milk can be pasteurized, unpasteurized, whole fat, low fat, skim and no fat (Otles and Cagindi). Traditionally, kefir making requires cow's milk inoculation directly with grains or mother cultures prepared from kefir grains, which is a complex symbiotic community of lactic acid and acetic bacteria (about 83-90%) and yeasts (about 10-17%) embedded with casein and complex sugars by a polysaccharide matrix, known as kefiran (Prado et al., 2015; Baschali et al., 2017). Industrial production, on the other hand, uses selected starter cultures to standardize the commercial kefir production and preserve its desirable properties (Beshkova et al., 2002). The quality and organoleptic characteristic of kefir can be affect from several variables, such as grain-milk ratio, incubation temperature and time, final pH, washing grains and storage (Guzel-Seydim et al., 2005; Irigoyen et al., 2005). The microorganisms present in the grains carry out the lactose fermentation producing an acidification of the product (pH 4.8-4.5 and sometimes more less) and slight alcoholic fermentation (alcoholic content is usually < 2%, w/v), which both contributes to the characteristic kefir acidic and prickly taste and yeasty flavour. Figure 6 show the two methods for kefir process (Otles and Cagindi, 2003; Guzel-Seydim et al., 2010):

- Traditional process: milk is directly inoculated with 2-10% (generally 5%) kefir grains after pasteurization for few minutes and cooling to 20-25°C. After fermentation, 18-24 h at 20-25°C, the grains are removed from milk with a sterile sieve, dried at room temperature and then kept at cold storage for the next inoculation. Kefir is stored at 4 °C for a time and then is ready for consumption (Otles and Cagindi, 2003). In some cases, mother culture is obtained from a percolate of kefir grains and can be used as inoculum in fresh milk (Farnworth, 2005).
- Industrial process: well-defined and lyophilized starter cultures containing LAB and yeast are used for inoculation in milk. After homogenization of milk to 8% dry matter, milk is pasteurized at 90-95°C for 5-10 minutes and then is cooled at 18-20°C. Commercial starter cultures (2-8%) are added to milk and time fermentation is about 18-24 h. The coagulum is separated by pomp and packaged. Maturation step is made at 12-14°C or 3-10°C for 24 h and kefir is stored at 4°C (Otles and Cagindi, 2003).

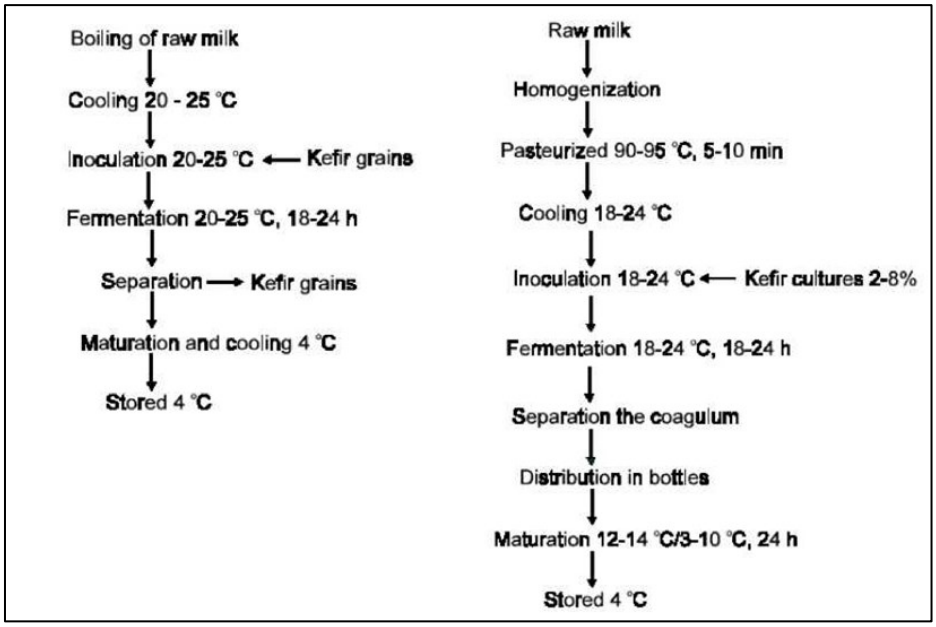


**Figure 6.** The traditional (left) and industrial (right) processes of kefir (Otles and Cagindi, 2003).

*1.3.2 Microbiological characteristics of kefir*

The microbial population found in kefir grains has been used as an example of a symbiotic community, which has made difficult identification and study of microorganism within kefir grains (Farnworth, 2005). Kefir grains (Figure 7) are small with irregular shape and variable size (3-35 mm), which look like small cauliflower florets or gelatinous particles varying of white to yellow-white in color (Otles and Cagindi, 2003; Arslan, 2015). In the outer layer of kefir grains was reported the prevalence of rod-shaped LAB, whereas yeasts at the core and a balance of bacteria and yeasts in the intermediate zone (Sarkar, 2008). Grains are kept viable by transferring them into fresh milk and consenting them to grow for about 20 hours, but washing grains in water reduce the viability (Farnworth, 2005). Initially grains are very small but increase during fermentation and storage at 4°C for three months, changing their microbiological profile compared to the fresh one (Pintado et al., 1996). The microbial population found in kefir grains contain many microorganism (e.g. *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter*, *Streptococcus*, *Enterococcus*, *Bacillus*, *Saccharomyces*, *Candida*, *Kluyveromyces*, *Torulaspora*, *Brettanomyces* and *Issatchenkia*) and depends on many factors such as the

**Figure 7. Kefir grain (Arslan, 2015).**

primary origin of the grains, the ratio kefir grains/milk and of species each other, and method of cultivation of grains and the properties of substrates (Guzel-Seydim et al., 2005; Witthuhn et al., 2005). Generally, the number of LAB are higher ($10^8$-$10^9$) than yeasts ($10^5$-$10^6$) and acetic acid bacteria ($10^5$-$10^6$) in kefir grains, though fermentation conditions can affect this pattern (Garrote et al., 2001). The ratio of LAB to yeasts in kefir grains was detected as 1000:1 (Guzel-Seydim et al., 2005). The *Lactobacillus* spp. was the most encountered microorganism in kefir grains (Witthuln et al., 2005), but *Lactococcus lactis* subsp. *lactis* and *S. thermophilus* are predominant species in kefir grains (53-65%) and kefir (74-78%) (Simova et al., 2002). In addition to LAB, acetic acid bacteria such as *Acetobacter lovaniensis* (8.91%) (Magalhaes et al., 2011) and *Acetobacter syzygii* (Kesmen and Kacmaz, 2011) were found in kefir. Finally, lactose and non-lactose assimilating yeasts are very important because are responsible of the production of $CO_2$ and ethanol during kefir fermentation and provide unique, slightly effervescent and mildly alcoholic taste to kefir and release essential growth nutrients such as vitamins and amino acids (Beshkova et al., 2002).

### *1.3.3 Bioactive components and health benefits of kefir*

A wide variety of health benefits have been attributed to the consumption of kefir (Hafez et al., 2014). In according to FAO/WHO (2001) definition, which report probiotics are “live microorganism that, when administrate in adequate amounts, confer a health benefit on the host”, kefir is considered a potentially natural probiotic product as contain live microorganism, which are beneficial to health (Salminen et al., 1998). Probiotic foods have some possible sources of bioactive ingredients (Figure 8): microorganism themselves, metabolites of microorganism produced during fermentation (antiobiotics or battericides, which inhibit pathogenic microorganisms) and breakdown products of food matrix, such as peptides, may be impart several beneficial effects (Farnworth, 2005).

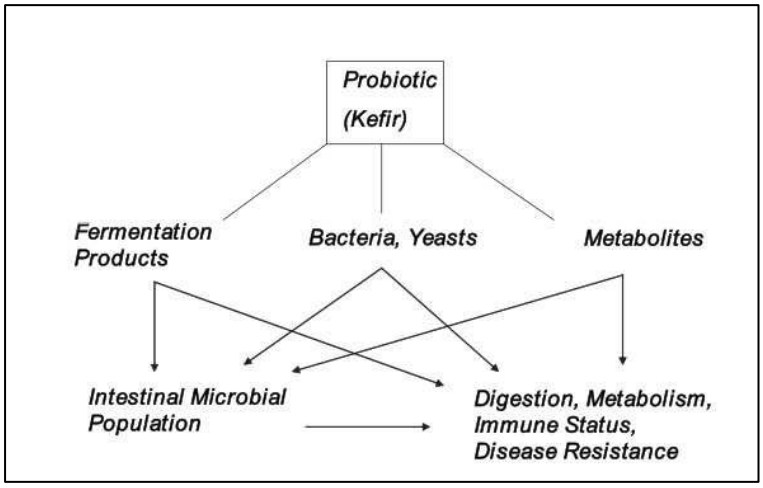


**Figure 8.** Probiotic effects on metabolism and health (Farnworth, 2005).

Several probiotic LAB was isolated from kefir such as *Lactobacillus acidophilus*, *L. helveticus*, *L. casei*, *Pediococcus dextrinicus*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactococcus cremoris*, and *Lactococcus lactis* (Sabir et al., 2010) and in particular, *L. acidophilus* and *L. kefiranofaciens* had the best probiotic characteristics tested within the *Lactobacillus* spp. (Santos et al., 2003).

It is possible to consider the bioactive properties of kefir linked to two principal compounds: exopolysaccharides and bioactive peptides (Farnworth, 2005; Hafeez et al., 2014; Prado et al., 2015). In kefir grains has been found a unique polysaccharide called kefiran produced by *Lactobacillus kefiranofaciens* (Kooiman, 1968), which contains D-glucose and D-galactose in a ratio 1:1 (Farnworth, 2005). Exopolysaccharide have several physicochemical, rheological and biological properties in kefir (Mitsue et al., 1999; Prado et al., 2015).

Bioactive peptides have been defined as specific protein fragments with 2-20 amino acids residues, which are inactive within the sequences of parent proteins and can be released from different way, such as enzymatic hydrolysis by endogenus or digestive enzymes or by fermentation trought proteolytic system of microorganisms (Korhonen, 2009). Indeed, microorganisms possess proteinase and peptidases that are able to hydrolyze the proteins and release peptides and amino acids (Hafez et al., 2014; Pessione and Cirrincione, 2016). Many LAB are auxotrophic for several amino acids and have to degrade milk proteins into peptides and amino acid during fermentation for their growth (Juillard et al., 1995). Some LAB, including *Lactococcus* and *Lactobacillus*, have a complex and sophisticated proteolytic system that is able to hydrolyze caseins and/or whey proteins to peptides containing 4-20 amino acids by cell wall protease or Cell Envelope Proteinase (CEP) (Laan and Konings, 1989). Oligopeptides are taken up by the oligopeptide transport system (Opp) and peptides are

degraded into amino acids by intracellular peptidases (Kunji, 1996). Two general specificity classes of proteinases are reported for *Lactococcus*: PI-type preferentially hydrolyzing β-casein and lesser extent κ-casein, whereas PIII-type capable of hydrolyzing $\alpha_{s1}$-, β- and κ-casein (Kunji, 1996). Kefir contains a large number of peptides and the majority of kefir peptides have molecular weight of ≤ 5000 Da (Farnworth, 2005). Several studies, have shown that milk peptides released during kefir fermentation were examined for homology with milk peptides know to have several bioactivies such as antimicrobial, anticarcinogenic, cholesterol lowering, improving lactose tolerance, antioxidant, immune system, gastrointestinal system and reducing blood pressure (Figure 9) (Furukawa et al., 1990; Liu et al., 2005; Liu et al., 2006; Hafeez et al., 2014; Tamang et al., 2016). For example, Quiros et al. (2005) have found that two low molecular mass of peptides (PYVRYL and LVYPFTGPIPN) shown high ACE-inhibitory properties.

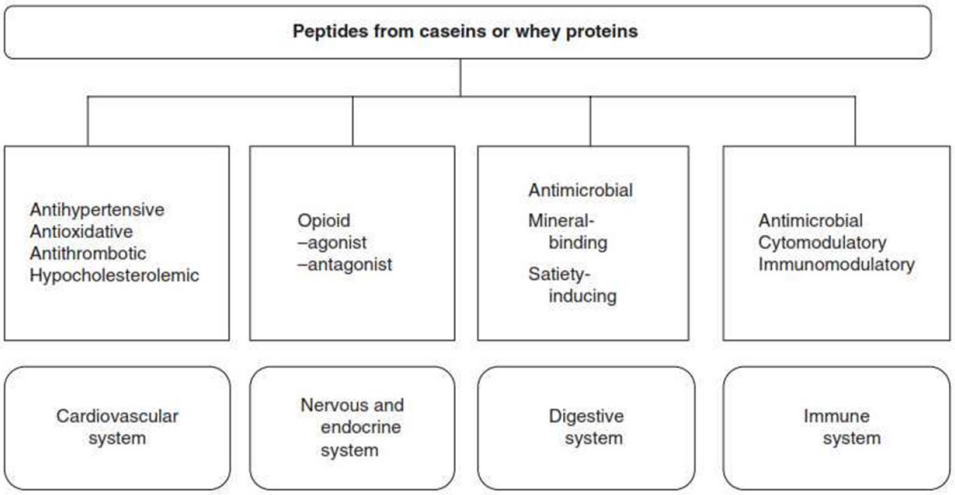


**Figure 9.** Physiological functionality of milk-derived bioactive peptides (Korhonen, 2009).

### *1.3.4 Casein phosphopeptides and their analysis*

The first study on casein phosphopeptides (CPP) dates back to 1950 concerning the physiological importance of CPP on vitamin D independent bone calcification in rachitic infants (Mellander, 1950). The caseino-phosphopeptides (CPPs), a subgroup of bioactive peptides, are strongly phosphorylated peptides obtained by gastrointestinal digestion after the consumption of dairy product (Meisel et al., 2003), by tryptic hydrolysis of endogenus enzyme (Baum et al., 2013) or by enzymes derived from microorganism present in milk (Lund et al., 2004). Some health promoting effect include the ability to bind and solubilize macroelements such as Ca, Mg, Fe along with trace elements such as Zn, Ba, Cr, Ni, Co and Se; enhance the

minerals absorption in the gastrointestinal tract (FitzGerald, 1998), tooth enamel remineralization in the oral cavity and buffering of plaque pH (Nongonierma and FitzGerald, 2012). In particular, the negative charges of phosphate groups and side chains make caseino-phospeptides resistant to the gastrointestinal enzymatic digestion and thus suitable as carriers for metal ions (FitzGerald, 1998).

The casein represent 80% of total milk proteins and can be subdivided in four types: $\alpha_{s1}$-casein (34%), β-casein (25%), κ-casein (9%), $\alpha_{s2}$-casein (8%), which exhibit different level of phosphorylation dependent on casein variants: bovine $\alpha_{s1}$-casein can have eight to nine and $\alpha_{s2}$-casein eleven to thirteen phosphate groups, β-casein contains five phosphorylation sites and κ-casein has one or two serine-bound phosphates (Berliz et al., 2009). There are a direct relationship between the phosphorylation degree and mineral binding properties: $\alpha_{s2}$-casein> $\alpha_{s1}$-casein>β-casein>κ-casein (Kitts, 1994). The available serine and/or potentially phosphorylatable threonine residues are not phosphorylated in the caseins and the incorporation of phosphate groups is linked to the casein kinase activity in the mammary gland (Bingham et al., 1974). In addition, it has been suggested that phosphorylation occur in specific amino acid sequence S/T-X-A, when X represent any amino acids residue, but P, and A an acidic residue such as pS, E or D, although several factors, such as secondary structure of caseins, insufficient available pool of kinase or the charge and hydrophilicity of environmental can affect the phosphorylation degree of caseins (Mercier, 1981).

Caseino-phosphopeptides share a highly polar acidic motif "pSpSpSEE" known to be responsible for bioactivity such as mineral binding activity, which plays also an important role in calcium absorption and bioavailability (Ferraretto et al., 2003; Perego et al., 2013; Tsuchita et al., 2001). Moreover, calcium dissolution by phosphopeptides is due to the capability of phosphoserine residues to combine with calcium forming amorphous $Ca_3(PO4)_2$ nanoclusters (Ferraretto et al., 2003). In fact, dephosphorylated peptides do not bind calcium (Berrocal et al., 1989). Besides the presence of the typical cluster sequence, other factors are important for mineral binding, as the total negative charge, the total number of amino acids, the phosphorylation degree and the amino acid composition around the phosphorylated region (Cross et al., 2005; Zong et al., 2012). In fact, the known bioactive β-casein (f1-25) phosphopeptide is more active in allowing mineral uptake by human tumor cells HT29 than the bioactive $\alpha_{s1}$-casein (f59-79) phosphopeptide (Ferraretto et al., 2003), despite the S/T rich $\alpha_{s1}$-casein generally generates peptides more active in mineral binding than β- and κ-casein (Gagnaire et al., 1996). Indeed, specific secondary structure motifs and the aggregation degree of caseino-phosphopeptides in presence of divalent cations are required for a correct mineral

absorption (Ferraretto et al., 2003). Moreover, calcium availability on meal composition and complex interactions between different foods ingested (Pessione and Cirrincione, 2016).

Mass spectrometry analysis, which has long been used in the proteomic study, is now the preferred technique for mapping post-translational modifications in proteins. Phosphopeptides analysis is exacting because the negative charge on phosphate group result in a low ionization efficiency, especially with multi phosphorylated peptides (Liao et al., 1994; Janek et al., 2001), and they can be suppressed by non-phosphorylated peptides in complex peptide matrix (Raska et al., 2002). Furthermore, the low abundance of phosphorylated peptides in a complex mixture makes the exclusive characterization of phosphopeptides difficult. Therefore, several approaches have been adopted to overcome these problems before mass analysis. In particular, phosphopeptides enrichment techniques is grouped in two methods based on chemical or affinity methods:

- Chemical modification include several methods such as carbodiimide condensation coupled with bead fishing, β-elimination coupled with a Michael addition, oxidation-reduction condensation, a-diazo substituted resin and carbodiimide condensation using a dendrimer (Fíla and Honys, 2012).
- Enrichment based on affinity use immobilized metal affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) based on various metal oxide materials, such as titanium dioxide ($TiO_2$), $ZrO_2$, $Fe_2O_3$ and $Al_2O_3$ adsorbents for the selective isolation of phosphoproteins (Fíla and Honys, 2012).

Recently, selective enrichment with hydroxyapatite was developed (Mamone et al., 2010) which achieves the recovery of caseino-phosphopeptides from complex peptides mixtures (Pinto et al., 2010). Hydroxyapaptite, which have a chemical formula $Ca_{10}(PO_4)_6(OH)_2$, comprise both groups positively charged of calcium ions (C-sites) and negatively charged oxygen atoms associated with phosphates (P-sites) (Kawasaki, 1978). Furthermore, phosphoryl groups on proteins interact more strongly with $Ca^{2+}$ (C-sites) than carboxyl groups present on the proteins (Kawasaki, 1991). Thus, the more phosphates are present on a biomolecule, the more dominating is the $Ca^{2+}$ site binding, and the tighter the binding is of the phosphorylated form to the matrix (Mamone et al., 2010). The hydroxyapatite enrichment methods was applied to food matrix, such as milk, by Pinto et al. (2010) and consists of immobilizes casein phosphopeptides on hydroxyapaptite microgranules though phosphate-containing buffer while no-phosphorylated peptides are removed using a washing buffers (Pinto et al., 2010).

*1.3.5 Alternative non-dairy kefir drink*

Milk kefir drink is widely consumed all over the world, but this beverage is limited for vegan and dairy-product allergic consumers. Thus, an alternative non-dairy kefir drink is through use and adaptation of non-dairy substrates (Figure 10), such as fruits (apple, strawberries, pears, grapes), some vegetables (ginger, carrots, fennel, soybean) and molasses (honey, piloncillo) and this beverage is known as water kefir or sugary kefir drink (Fiorda et al., 2017).

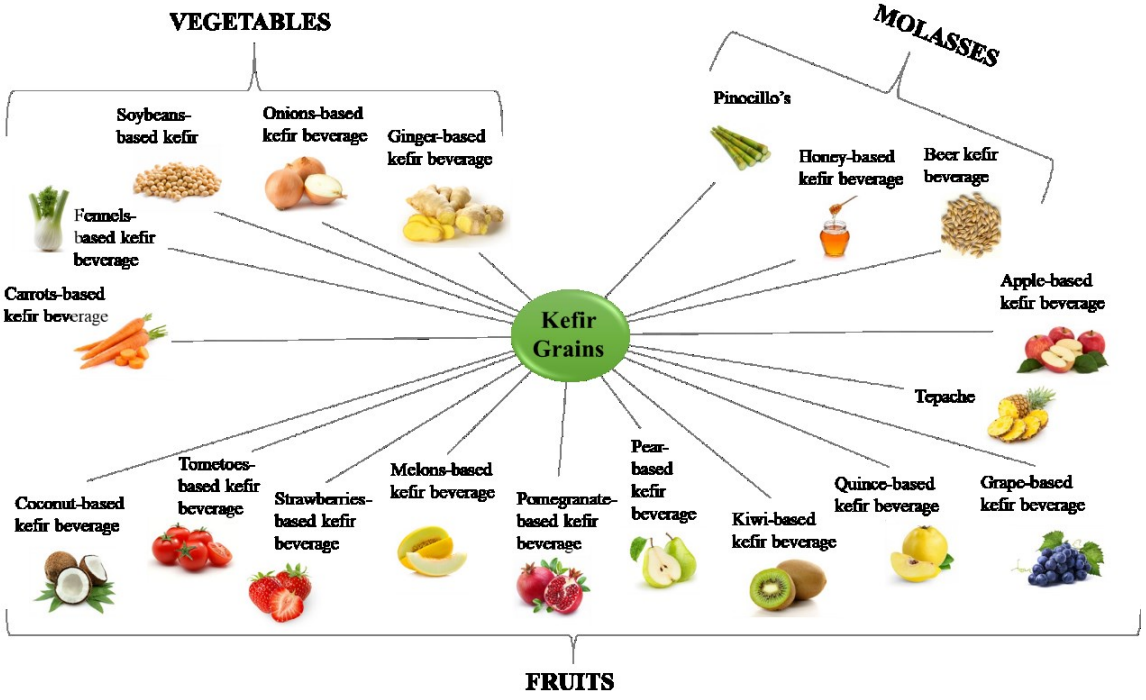


**Figure 10.** Different non-dairy products from sugary kefir fermentation (Fiorda et al., 2017).

Soy-based foods attracted a lot of interest in the worldwide due to their positive effect on health as was demonstrated among Asian populations due to to their large soy intake (Singh et al., 2014; Sanjukta and Rai, 2016). Indeed, soybean and its derivatives are good source important components to health benefits, such as proteins, essential fatty acids, oligosaccharides, isoflavones and fiber (Liu, 1997). Soy proteins have high nutritional value, around equal to animal protein of high biological value, and excellent functional properties as are potential source of bioactive peptides (Singh et al., 2014).

The major storage proteins are Glycinin (11S globulin), which is composed of five major subunits (G1, G2, G3, G4 and G5), and β-conglycinin (7S globulin) with four subunits (α, α', β and γ), which both of them constitute 65-85% of the total soy proteins and they are the

precursors of the most of the isolated bioactive peptides (Yang et al., 2000; Vasconcellos et al., 2014). The minor proteins in soybean comprise trypsin inhibitors, lectins, lipoxygenases, sucrose binding proteins, urease, which are recognized bioactive and allergenic, and other low abundance proteins (Herman et al., 2014). Moreover, lunasin is peptide isolated from soybean cotyledon and has been reported to have anti-inflammatory, anticancer and antioxidant activity (Hernandez-Ledesma et al., 2009a; Hernandez-Ledesma et al., 2009b). Soybean or soy-based foods derived bioactive peptides are well known for potential health benefits such as immunomodulatory, antihypertensive, antioxidative, hypocholesterolemic, antiobesity and anticancer (Singh et al., 2014).

Soybean can be utilized as unfermented, such as soybean, soy powder, soybean oil, soy milk etc., or fermented products, such as miso, natto, soy cheese, soy yogurt, soy milk kefir and others. An example of an alternative to non-dairy kefir is soy drink kefir, which is a fermented product can be obtained by inoculation of kefir grains or selected culture into soy drink. Fermentation is a good process to improve both the digestibility of soybean proteins by reducing proteinase and trypsin inhibitors ad indigestible oligosaccharides (raffinose and stachyose) and the objectionable beany flavor and taste (Egounlety and Aworh, 2003). In addition, the proteolytic enzyme microorganism during fermentation hydrolyzes the proteins into peptides and amino acids, increasing bioactive peptides in soy-based fermentation foods (Donkor et al., 2005; Tsai et al., 2006; Ibe et al., 2009; Singh and Vij, 2017).

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# CHAPTER 2

## AIM OF THE STUDY

Fermentation is an olden form of food preservation, which often improves the quality of foods in relation to the nutritional content and potential health-promoting effects. On the other hand, food processing affects, widely, the composition of fermented foods.

The general aim of the present work was the study of influence of several technological parameters on the composition of two fermented-foods: wine and kefir.

In the first part, the work was focused on the study of the investigation of several factors on the quality of white and red wines. The first aim was the study of the evolution of a white wine without added sulfites, during 15 months storage, at different light and temperature conditions, compared with a control stored with sulfur dioxide. The main oenological parameters and phenolic and volatile profiles were considered. Then, thermovinification was investigated with the aim to evaluate the influence of thechnology on the ochratoxin-A concentration. To achieve this goal, a new method for the determination of OTA in wine using a microextraction by packed sorbent (MEPS) combined with high performance liquid chromatography (HPLC) combined with fluorescence detection (FLD) detection was developed and validated.

In the second part, the aim of the present work was the study of the influence of some technological parameters on the production of phosphopeptides in kefir. To achieve this goal, first, multiphosphorylated peptides in kefir were identified using a selective enrichment with hydroxyapatite and subsequent enzymatic dephosphorylation, followed by liquid chromatography coupled to mass spectrometry analysis. Then, a comprehensive study of microbiological and phosphopeptide profiles of milk kefir prepared with traditional kefir grains, according to different technological conditions, was carried on. Finally, the characterization of microbiological and peptide profiles of kefir prepared with kefir grains from soy-drink was performed.

# CHAPTER 3

## EVOLUTION OF PHENOLIC AND VOLATILE COMPOUNDS DURING BOTTLE STORAGE OF WHITE WINE WITHOUT ADDED SULFITE.

### 3.1 Abstract

The reduced content of $SO_2$ in wines without added sulfites affects the wine phenolic chemistry, volatile profile, together with the main oenological attributes during storage. However, little is reported about the bottle evolution of wine during storage of white wines without added sulfites or other antioxidants. Therefore, the aim of this work was to study the evolution of the main oenological parameters, the phenolic, and the volatile profiles of a white wine without added sulfites, during 15 months storage at different light and temperature conditions, compared with a control stored with sulfur dioxide. Dark storage at 12 °C (D12-S, D12-NS) and 30 °C (D30-S, D30-NS) were compared to investigate the temperature effect, meanwhile uncontrolled temperature and light condition (UTL-S, UTL-NS) were selected to simulate improper storage conditions. Volatile acidity (VA), absorbance at 420 nm (Abs420), $SO_2$, and total phenols (TP), revealed significant differences. VA slightly increased at 15 months, above all in UTL-NS and D30-NS wines, which showed significant higher values than the S wine stored at the optimal condition (in the dark at 12 °C). Total $SO_2$ and free $SO_2$ decreased with time in S wine samples, with the exception of D12-S wine, due to its action as antioxidant. The Abs 420 values increased up to the end of storage (15 months), faster in NS than in S wines. In no-sulfite added wines, the increase in 420 nm was faster in the first six months and for samples D30-NS and UTL-NS. Also, at 15 months, sample stored at the constant temperature of 30°C was the most browned, followed by UTL-NS and D12-NS. Samples stored with sulfites were not significantly different at 15 storage months. The trans forms of hydroxycinnamic esters decreased in samples without added sulfites, whereas, interestingly, the cis forms significantly increased. Also caffeic acid derivatives increased. As concerns storage condition factor, the sample UTL showed the lowest content of the trans forms of hydroxycinnamic esters, followed by the sample D30, suggesting that the combination of

improper conditions of light and temperature favor hydrolysis reactions more than the effect due to the only high temperature, although the UTL sample was not constantly subjected to high temperature as the D30. The storage without sulfites accelerated the hydrolysis of acetate esters which had lower values down to 54%, as in the case of isoamyl acetate, than S wines. However, it did not affect the most of ethyl esters (i.e. ethyl hexanoate, ethyl octanoate, and ethyl decanoate) whose content remained almost the same between S and NS wines. Finally, it is worth noting the great increase, over than 100%, of furfural, 5-methyl-2-furancarboxaldehyde, and benzaldehyde contents in NS wines. In conclusion, results demonstrated that the presence of sulfites helps the typical aroma of young wines; however, even more important seems to be the proper storage in the dark, at low temperature, as improper storage conditions favored aroma degradation regardless sulfites addition.

### 3.2 Introduction

Wine is a complex matrix whose composition continuously changes during storage depending on many factors such as, temperature, oxygen content, light exposition, position of bottles, and storage time. Generally, for white wine, bottle storage can affect the color and aroma, as well as the phenolic composition, eventually deteriorating its overall quality and marketability (Kallithraka et al., 2009).

This is mainly due to the oxidative reactions that happen during storage; in particular, after fermentation young white wines, rich in hydroxycinnamic acids, as such or esterified with tartaric acid (i.e. caffeic acid and caftaric acid, respectively) (Cejudo-Bastante et al., 2010 Myers and Singleton, 1979), are very susceptible to the non-enzymatic oxidative browning which occurs in absence of active polyphenol oxidase and is favored by the presence of transition metal ions, light, and high temperature (Simpson, 1982¸ Danilewicz et al., 2006). The quinones formed from hydroxycinnamates oxidation are unstable and may undergo further reactions to produce yellow-brown pigments (Singleton, 1987; Cheynier and da Silva, 1991; Kader et al., 1999; Oliveira et al., 2011). These reactions have been widely investigated in model solutions by HPLC-UV-vis and HPLC-MS for structural identification (Cilliers and Singleton, 1991; Fulcrand et al., 1994; Tazaki et al., 2001; Antolovich et al., 2004; Pati et al., 2006; Sonni et al., 2011) and tentatively identified in wine (Pati et al., 2014). Aside from changes involving phenolics, oxidative processes can also led to the loss of some characteristic aroma compounds (i.e. fruity esters) and the appearance of atypical ones associated with wine deterioration (i.e. woody aroma) (Lambropoulos and Roussis, 2007; Hernanz et al., 2009).

Generally, sulphur dioxide ($SO_2$) is added, in the form of sulfite salts, in order to protect

wine thanks to its antimicrobial and antioxidant activities, inhibiting, for instance, the enzymatic and non-enzymatic browning during production and storage (Boroski et., 2017). However, in the last years, there is growing interest in winemaking without added sulfites that is becoming increasingly feasible due to technological improvements (Lustrato et al., 2006; Sonni et al., 2009; Santos et al., 2012). Indeed, although negative health effects have not been definitely demonstrated (Vally and Thompson, 2003), the perception that sulfites may cause health problems (i.e migraines and headaches) appears to be common (Costanigro et al., 2014). Alternatively, a reductive winemaking can be carried out for obtaining wines without added sulphur dioxide (Antonelli et al., 2010), which consists of the maceration of the must of white grapes in a low-oxygen atmosphere obtained through the addition of carbon dioxide, nitrogen, argon or their mixture, in order to protect wine from oxidation (Di Lecce et al., 2011).

Browning and oxidation reactions in white wines during storage are of both technological and nutritional significance because of their influence on wine organoleptic characters and antioxidant status. Moreover, the reduced content of $SO_2$ in wine (due only to the naturally produced amount by yeasts during wine fermentation) certainly affects the wine phenolic chemistry, volatile profile, together with the main oenological attributes during storage (Pati et al., 2014; Boroski et al., 2017). However, even though there are several investigations regarding changes to wines which have been subjected to accelerated browning or conventional storage conditions (Cejudo-Bastante et al., 2010; Ferreira-Lima et al., 2013; Kallithraka et al., 2009; Hernanz et al., 2009; Recamales et al., 2006) or to the replacement of $SO_2$ with other antioxidants (Raposo et al., 2016; Boroski et aal., 2017; Sonni et al., 2011), little is reported about the bottle evolution of wine during storage of white wines without added sulfites or other antioxidants.

Therefore, the aim of this work was to study the evolution of the main oenological parameters, the phenolic, and the volatile profiles of a white wine without added sulfites, during 15 months storage at different light and temperature conditions, compared with a control stored with sulfur dioxide.

## 3.3. Material and methods

### *3.3.1 Wine samples*

Frascati Superiore white wine (30 hL), made from cv. Malvasia of Lazio (70%) and Trebbiano (30%) grapes (*Vitis vinifera* L.) harvested at the optimal technological maturity (21 °Brix) was produced in Capodarco winery (Grottaferrata–Rome in central Italy), following a

reductive winemaking, as described in Pati et al. (2014). To minimize air contact and oxidation, all juice and wine transfers, during the whole winemaking process until bottling, were made by previous saturation of pipes and tanks with carbon dioxide.

Briefly, after early morning picking, the grapes were quickly and carefully transported in 25 kg plastic bins to the winery. The mass was gently pressed with a dry ice (15 mm pellets) covering (3 kg /100 kg of grape) and the free run juice was quickly clarified via nitrogen flotation using pectolytic enzyme preparation (3 mL /100 L) and gelatin (15 mL /100 L), as flocculants. Clear juice was then moved to temperature-controlled stainless steel tank under carbon dioxide blanket and, after thiamine (0.5 mg/L) and ammonium phosphate (0.1 g/L) supplementation, was added with activated (12 hours before) *S. cerevisiae* starter (Lalvin D254 Yseo, Lallemand Italia) culture (0.2 g/L) for alcoholic fermentation. Fermentation, carried out at 16 °C, lasted about 3 weeks with a nearly complete consumption of the reducing sugars (5 g/L). One week after the end of alcoholic fermentation, the wine was separated from the yeast and grape lees, and racked to clean tank to be further settled using a complex plant protein-based fining allergen free agent (0.35 g/L), and granular sodium bentonite (0.3 g/L). After cold stabilization, the wine was racked and filtered, previous saturation of pipes and tanks with carbon dioxide, at 3 months from vintage. Then, wine bottling was performed under isobaric condition using stoppers (DIAM Bouchage, Ceret, France) with low permeability (0.15 $cm^3$/giorno); in one hundred bottles, metabisulfite (160 mg/L) was added before filling. Afterwards, from bottling up to 15 storage months, 33% of sulfite added (S) and no sulfite added (NS) wine bottles were stored at 30 °C, in the dark (D30-S, D30-NS), 33% of S and NS wine bottles were stored at uncontrolled conditions of temperature and light (UTL-S, UTL-NS) simulating an improper storage, and 33% of S and NS bottles were stored at 12 °C, in the dark (D12-S, D12-NS), as control. All bottles were arranged lying down to keep the technical cork stopper moist. The main oenological analyses of wine samples were performed during the storage time at bottling (time 0), and after 6 and 15 months; volatile and phenolic analyses were performed at bottling and after 15 months.

### *3.3.2 Chemicals and analysis of the main oenological parameters*

Formic acid and HPLC grade water were purchased from J.T. Baker (Deventer - Holland). LC-MS grade solvent acetonitrile was purchased from Riedel-de Haën (Steinheim-Germany). (+)-Catechin, (-)-epicatechin, *trans*-caffeic acid, p-coumaric acid, and ferulic acid were purchased from Extrasynthese (Genay, France). *Trans*-caftaric was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of individual phenolic compounds were

prepared in methanol (1 g $L^{-1}$). The working solutions were prepared by dilution of respective stock solutions and kept in the dark under refrigerated temperature (4 °C).

The main oenological parameters were determined according to European Regulation (EEC No. 2676/1990) on five bottle samples, for each wine. Total phenol concentration was determined using the Folin–Ciocalteau assay. Results were expressed as mg/L catechin equivalents.

Spectrophotometric Measurement. The absorbance at 420 nm of the wines was measured using a 10-mm-path-length cuvette against the blank (Milli-Q water) by a Lambda 25 spectrophotometer (PerkineElmer, USA).

*3.3.3 Phenolic derivatives determination by HPLC-DAD-MS/MS.*

Separation and identification of phenolic derivatives were executed as previously described by Ragone, et al. (2015), with some modifications. Briefly, a HPLC 1100 (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with a diode array detector (model G1315B DAD system) and a XCT-trap Plus mass detector (model G2447A) coupled with a pneumatic nebulizer-assisted electrospray LC-MS interface was used. After filtration through 0.45 μm pore size regenerated cellulose filters (VWR International, USA), wine samples were directly injected onto a reversed stationary phase column, Luna C18 (150 x 2 mm i.d., particle size 3 μm, Phenomenex, USA). Mobile phase consisted of water/formic acid (99:1, v/v) (solvent A) and acetonitrile (solvent B) and the gradient program was as follow: 0 min, 2% B; 10 min, 13% B; 27 min, 18% B, followed by washing and re-equilibrating the column. The column temperature was not controlled, while the flow rate and sample injection volume were 0.2 mL/min and 3 μL, respectively. UV-vis detection wavelengths were set at 330, for cinnamic acids and esters, and 280 nm, for flavanols; spectrophotometric spectra were registered from 250 to 450 nm.

Negative electrospray mode (ESI) was used for ionization of molecules, with capillary voltage at 4000 V and skimmer voltage at 40 V. The nebulizer pressure was 30 psi and the nitrogen flow rate was 8 L/min. Temperature of drying gas was 350 °C. In the full scan mode, the monitored mass range was from *m/z* 100 to 1200 at a scan speed of 13000 Da/s. MS/MS analysis was performed by using helium as the collision gas at a pressure of 4.6 x $10^{-6}$ mbar. Fragmentation spectra were obtained with an isolation width of 4.0 *m/z* for precursor ions and a fragmentation amplitude of 0.8 V. Tentative compound identification was based on comparison with retention times and UV-Vis and MS spectra of standards and results reported in Pati et al. (2014).

Quantitative determinations were achieved applying the standard external calibration method, considering the peak area data. The calibration curves were built by injecting the standard solutions at five different concentrations in the range of 0.1–30 mg/L for epicatechin, caffeic, *p*-coumaric, and ferulic acid, and in the range of 10–80 mg/L for caftaric acid. Fertaric acid was determined as mg/L ferulic acid, and coutaric acid as mg/L *p*-coumaric acid equivalents. The coefficients of determination ($R^2$) for the calibration curves were all higher than 0.99.

*3.3.4. Volatile determination by GC-MS.*

Volatile compounds were extracted using a solid-phase extraction (SPE) procedure. A Vac Elut 20 station equipment from Varian (Palo Alto, USA) was used. The extraction was performed according to Lòpez et al. (2002), with some modifications. LiChrolut EN resins prepacked in 200-mg cartridges (Merck, Darmstadt, Germany) were rinsed with 4 mL of dichloromethane/hexane (2:3), 4 mL of methanol and, finally, 4 mL of a water–ethanol mixture (12 %, v/v); 50 mL of wine, containing 1 ppm of 2-methyl-pentanol as internal standard, was passed through the SPE cartridge at around 2 mL/min. Afterwards, the sorbent was dried by letting air to pass through it. The analytes were recovered by elution with 1.3 mL of dichloromethane/hexane (2:3). 1μL sample was injected onto a gas-chromatograph (GC 6890N) coupled to a mass selective detector (MS 5973) and equipped with a HP-INNOWAX capillary column (60 m x 0.25 mm i.d, 0.25 μm film thickness, J&W Scientific Inc., Folsom, USA). The carrier gas was helium at a flow rate of 1 mL/min. The injection was made in the splitless mode and the injector temperature was 250 °C. The column oven temperature was initially held at 40 °C, and then, it was programmed to 230 °C at 2.5 °C/min, with a final holding time of 20 min. Spectra were recorded in the electron impact mode (EI, 70 eV) in a range of 30–500 *m/z* at 3.2 scans/s. A solvent delay time of 10 min was used to avoid overloading the mass spectrometer with solvent. Sixty-one compounds were identified by comparing their retention times and mass spectra with those of pure compounds, when commercially available, analysed under the same conditions. Otherwise, comparison of MS fragmentation patterns with those included in the National Institute for Standards and Technology database (NIST 02, p>80) were used to determine tentative identification. The semi-quantitative analysis of individual compounds was performed using peak areas normilized to that of the internal standard. Peak areas were calculated from selected ions based on relative abundance, stability and resolution from co-elutors.

*3.3.5. Statistical analysis*

All data were statistically analyzed using the STATISTICA 7.0 software package (StatSoft Inc., Tulxa, OK). Factorial ANOVA followed by a Tukey post hoc test with a 95 % confidence level were applied.

## 3.4 Results and discussion

The study of the evolution in bottle of white wine with (S) and without sulfites (NS), under several experimental conditions, was carried out over 15 months. Dark storage at 12 °C (D12-S, D12-NS) and 30 °C (D30-S, D30-NS) were compared to investigate the temperature effect, meanwhile uncontrolled temperature and light condition (UTL-S, UTL-NS) were selected to simulate improper storage conditions. Within the main oenological parameters, the evolution of parameters which showed changes according to the different experimental conditions, at time 0, 6, and 15 storage months are reported in Table 1.

**Table 1.** The main oenological parameters in no sulfite-added wine (NSW) and in sulfite-added wine (SW).

| **Oenological parameters** | D12-S[a] | UTL-S[a] | D30-S[a] | D12-NS[a] | UTL-NS[a] | D30-NS[a] |
|---|---|---|---|---|---|---|
| **Volatile acidity (g/L acetic acid)** | | | | | | |
| t = 0 | 0.36Aa | 0.36Aa | 0.36Aa | 0.36Aa | 0.36Aa | 0.36Aa |
| t = 6 | 0.40Aa | 0.42Ba | 0.42Ba | 0.38Aa | 0.38Aa | 0.40Aa |
| t = 15 | 0.40Aa | 0.44Bab | 0.48Cb | 0.46Bab | 0.52Bb | 0.54Bb |
| **Total $SO_2$ (mg/L)** | | | | | | |
| t = 0 | 80Aa | 80Ba | 80Ba | 10 | 10 | 10 |
| t = 6 | 79Aa | 79Ba | 78Ba | nd | nd | nd |
| t = 15 | 83Ac | 70Ab | 58Aa | nd | nd | nd |
| **Free $SO_2$ (mg/L)** | | | | | | |
| t = 0 | 68Aa | 68Ba | 68Ca | 10 | 10 | 10 |
| t = 6 | 67Ab | 56Aa | 56Ba | nd | nd | nd |
| t = 15 | 64Ac | 57Ab | 45Aa | nd | nd | nd |
| **Total phenols (mg/L catechin)** | | | | | | |
| t = 0 | 250Aa | 250Aa | 250Aa | 250Aa | 250Aa | 250Aa |
| t = 6 | 245Aa | 237Aa | 238Aa | 256Aa | 230Aa | 237Aa |
| t = 15 | 260Aa | 255Aa | 253Aa | 248Aa | 212Bb | 220Bb |

[a]Means in the same column followed by different capital letters differ significantly (One-way variance analysis, $p<0.05$) according to time factor. Means in the same row followed by different small letters differ significantly (One-way variance analysis, $p<0.05$) according to the storage condition.

The mean values of the other oenological parameters were: pH, 3.4; alcohol concentration, 12.9; reduced dry extract, 24; total acidity, 5.1 g/L as tartaric acid, which were not statistically different among samples at 0, 6 and 15 storage months. Volatile acidity (VA), absorbance at 420 nm (Abs420), $SO_2$, and total phenols (TP), revealed significant differences. The low values for VA (< 0.6 g/L), throughout the storage time for all samples, suggested the lack of acetic fermentation, which could be responsible for wine quality degradation. VA slightly increased at 15 months, above all in UTL-NS and D30-NS wines, which showed significant higher values than the S wine stored at the optimal condition (in the dark at 12 °C).

Obviously, $SO_2$ contents resulted different between NS and S, at each sampling time, as in NS samples yeasts naturally produced 10 mg/L of $SO_2$ and, in S, $SO_2$ was added before bottle filling as preservative. Total $SO_2$ and free $SO_2$ decreased with time in S wine samples, with the exception of D12-S wine, due to its action as antioxidant. The unchanged values of $SO_2$ in D12-S wine revealed a low extent of oxidation in wine stored at the optimal condition. Before storage, the mean value of TP content was equal to 250 mg/L catechin equivalents. During the storage in the dark, at 12 °C, no variation in the TP was observed (Table 1). Results are in accordance with Garaguso and Nardini (2015), which observed a total phenol content of organic wines without sulfites addition comparable to that of conventional wines. No significantly changes from the initial total phenolic concentration was also observed by other authors (Kallithraka et al., 2009) immediately after an accelerated browning test (12 days at 55 °C). Differently, in the case of D30-NS and UTL-NS, TP content significantly decreased in the last 9 months. A decline in total phenols was also observed by Recamales et al. (2006) in white wine with added sulfites subjected to daily and seasonal temperature variations. The reductive conditions of winemaking applied in the present study are likely to have slowed down oxidation reactions, thus, the loss of TP was detectable only in NS wines, which were not protected by sulfur dioxide.

The absorbance at 420 nm (A420) is considered to be an indicator of the degree of browning of white wines during storage (Mayén et al., 1997) and its evolution is reported in Figure 1. A420 values increased up to the end of storage (15 months), faster in NS than in S wines. In no-sulfite added wines, the increase in 420 nm was faster in the first six months and for samples D30-NS and UTL-NS. Also, at 15 months, sample stored at the constant temperature of 30°C was the most browned, followed by UTL-NS and D12-NS. Samples stored with sulfites were not significantly different at 15 storage months.

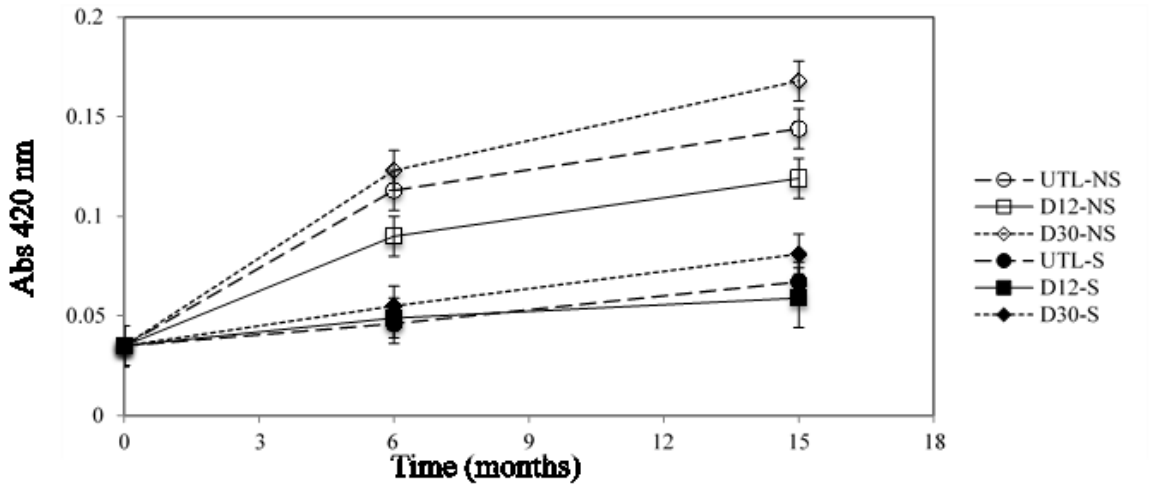


**Figure 1.** Absorbance values at 420 nm. Control at 12 °C, in the dark, with (D12-S) and without (D12-NS) added sulfites; wine sample stored at 30 °C, in the dark, with (D30-S) and without (D30-NS) added sulfites; wine sample stored at uncontrolled conditions of temperature and light, with (UTL-S) and without (UTL-NS) added sulfites.

As for the evolution of the main phenolic and volatile compounds, in order to simplify data discussion, first a comparison between wine sample at bottling (t = 0) and D12-S at 15 months was undertaken to highlight the evolution of Frascati Superiore wine elaborated with reductive winemaking and stored under ideal condition. Then, a comparison among the wines stored under the different conditions of added sulfites, temperature and light at 15 months was carried out to point out the composition differences as affected by storage conditions.

*3.4.1 Phenolic compounds in white wine stored under ideal conditions with added sulfites*

Detection of hydroxycinnamic acids and esters, caffeic acid derivatives and the flavanols (+)-catechin and (-)-catechin in Frascati Superiore wines, with added sulfites (D12-S), in the dark, at 12 °C , was carried out according to Pati et al. (2014). Peak number has been assigned in accordance with elution order in the chromatogram (Figure 2).

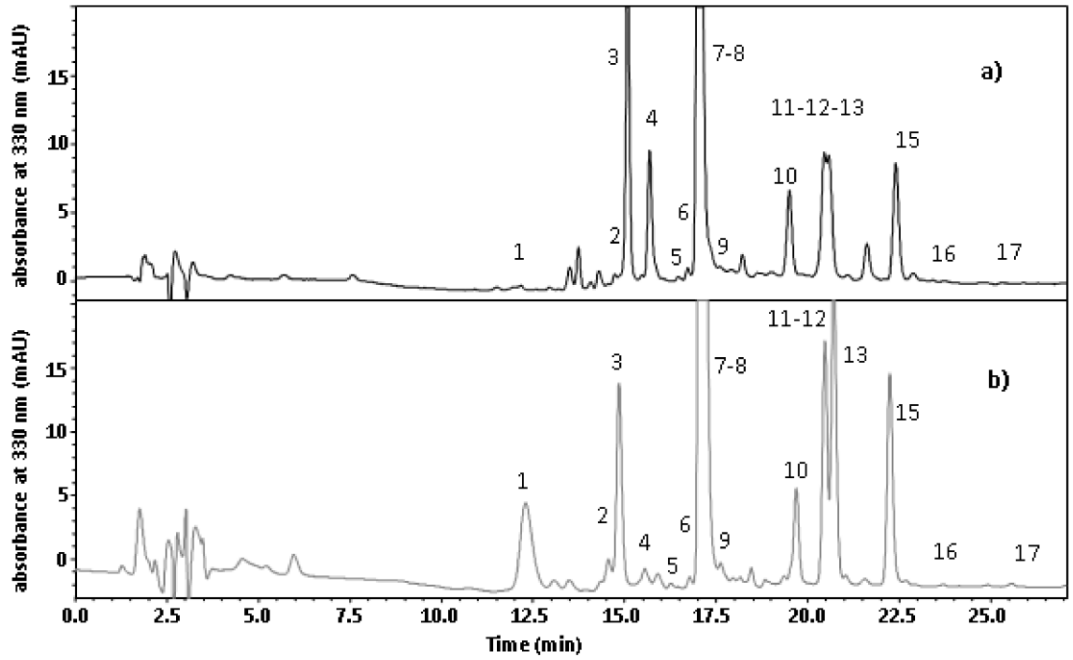


**Figure 2.** HPLC-DAD trace of sulfite added wine (D12-S) at 0 (a) and 15 (b) storage months in dark at 12°C. Peaks 14 is detected at 280 nm.

Legend of peaks: 1, GRP hydrolysis product; 2, hydroxy-caffeic acid dimer isomer I; 3, 2-S-glutathionyl-caffeoyltartaric acid; 4, cis-caftaric acid; 5, hydroxy-caffeic acid dimer isomer II; 6, acid 3-(4',5'-*o*-quinone-caffeoyl) caffeic; 7, *trans*-caftaric acid; 8, hydroxy-caffeic acid dimer isomer III; 9, *cis*-coutaric acid; 10, *trans*-coutaric acid; 11, caffeic-(*o*-quinone-caffeic)-ether; 12, *trans*-caffeic acid; 13, *cis*-fertaric acid; 14, epicatechin; 15, *trans*-fertaric acid; 16, *p*-coumaric acid; 17, ferulic acid.

The predominant hydroxycinnamic acids and esters were cis- and trans-caftaric (peaks 4 and 7), followed by cis- and trans-coutaric (peaks 9 and 10), cis- and trans-fertaric (peaks 13 and 15), trans-caffeic (peak 12), ferulic (peak 17), and p-coumaric acid (peak 16). As regards the non-hydroxycinnamate phenolics, (-)-epicatechin (peak 14) was the most abundant. These values are within the range reported for white wines from other researchers (Mayén et al., 1997; Recamales et al., 2006; Sioumis et al., 2006). As far as hydroxycinnamic esters are concerned, there was a significant decrease with storage time in trans-caftaric acid and in cis-caftaric, cis-coumaric and cis-fertaric acids, whereas trans-coutaric and trans-fertaric acid concentrations remained unchanged. On the other hand, an increase of the caffeic and ferulic acids concentrations (Table 2) was observed, which may be related to the hydrolysis of the esterified forms (caftaric and ferulic, respectively) of these acids (di Lecce et al., 2013). Conversely, no change was observed for coumaric acid. Moreover, it is worth noting that also (-)-epicatechin

followed a similar trend showing a significant decrease with time, which may be ascribed to the oxidation reactions forming o-quinones and leading to browning. Indeed, Hernanz et al., (2009) also observed a marked reduction in the concentration of catechin and caftaric acid during the storage of white wines, and related this finding to the susceptibility of these compounds to browning because of the presence of two orto-OH groups linked to a benzenic ring in their structure.

Peak 3 was attributed to 2-S-glutathionylcaftaric acid (GRP) according to previous results (Pati et al. 2014). GRP is formed in the must from the reaction of glutathione with o-quinone produced by the oxidation of caftaric acid. According to Singleton et al. (1985), the GRP is not able to further react with PPO, avoiding the formation of brown polymers and consequently preventing browning. Although reductive conditions were used during winemaking, GRP was observed in wines, thus demonstrating that quinones of caftaric acid however formed even if the presence of oxygen was avoided as much as possible. GRP decreased with storage even still present at 15 months, likely due to the gradual hydrolysis during wine aging, in accordance with other authors (Cejudo-Bastante, Perez Coello, & Hermosin-Gutierrez, 2010).

Peak 1 could be ascribed to a GRP hydrolysis product on the basis of its elution order and UV-vis spectrum, very similar to that of GRP, in agreement with what Cejudo-Bastante et al. (2010) reported about 12-months aged white wines. The content of this GRP hydrolysis product increased with time, supporting the hypothesis that derives from hydrolysis processes.

**Table 2.** Concentrations (expressed in mg/L) of hydroxycinnamates derivatives in Frascati Superiore wines after 15 months as affected by sulfites and storage conditions. Main effects and significant interactions are shown.

| | Sulfites | | *p* | Storage conditions | | | *p* | Sulfites x Storage conditions | | | | | | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compounds** | **S** | **NS** | | **D12** | **D30** | **UTL** | | **D12-S** | **D12-NS** | **D30-S** | **D30-NS** | **UTL-S** | **UTL-NS** | |
| *Hydroxycinnamic acids and flavanols* | | | | | | | | | | | | | | |
| GRP hydrolysis product[3] | 83a | 95a | ns | 144a | 72b | 51b | *** | 151a | 138a | 84b | 60b | 14.3c | 88b | *** |
| 2-S-glutathionyl-caffeoyltartaric acid[3] | 160a | 150a | ns | 173a | 172a | 119b | *** | 190a | 156b | 176a,b | 169a,b | 114c | 125c | * |
| *cis*-caftaric acid[2] | 0.429b | 1.326a | *** | 0.72b | 0.50c | 1.41a | *** | 0.128d | 1.31b | 0.06d | 0.95c | 1.10b,c | 1.72a | * |
| *trans*-caftaric acid | 36.75a | 33.97b | *** | 37.13a | 35.18b | 33.78c | *** | 39.1a | 35.1c | 36.6b | 33.8e | 34.5d | 33.0f | *** |
| *cis*-coutaric acid[2] | 0.821b | 1.005a | *** | 0.98a | 0.82b | 0.94a | *** | 0.899c | 1.05a | 0.58d | 1.07a | 0.98b | 0.90c | *** |
| *trans*-coutaric acid | 2.24a | 1.80b | *** | 1.98b | 2.24a | 1.86c | *** | 2.22b | 1.73e | 2.70a | 1.80d,e | 1.82c,d | 1.89c | *** |
| *trans*-caffeic acid | 3.36a | 3.32a | ns | 3.03c | 3.80a | 3.20b | *** | 3.02c | 3.03c | 3.81a | 3.80a | 3.26b | 3.13c | ns |
| *cis*-fertaric acid[2] | 0.38a | 0.43a | ns | 0.398a | 0.443a | 0.394a | ns | 0.42a,b | 0.37b,c | 0.45a,b | 0.44a,b | 0.28c | 0.51a | ** |
| epicatechin[1] | 8.09b | 17.50a | *** | 6.34c | 10.6b | 21.4a | *** | 3.5d | 18b | 2.0d | 11c | 19b | 24a | * |
| *trans*-fertaric acid | 1.91a | 1.47b | *** | 1.88a | 1.86a | 1.32b | *** | 2.196a | 1.57d | 2.08b | 1.64c | 1.45e | 1.21f | *** |
| *p*-coumaric acid | 0.31a | 0.26b | ns | 0.265b | 0.392a | 0.208c | ns | 0.28c | 0.24c | 0.42a | 0.36b | 0.23c,d | 0.18d | ns |
| ferulic acid | 0.48a | 0.35b | *** | 0.43b | 0.45a | 0.36c | *** | 0.51a | 0.35d | 0.53a | 0.38c | 0.42b | 0.31e | ** |
| *Oxidation products* | | | | | | | | | | | | | | |
| hydroxy-caffeic acid dimer isomer[3] | 267b | 707a | *** | 233c | 457b | 770a | *** | 18d | 449b,c | 300c | 614b | 482b,c | 1058a | ns |
| hydroxy-caffeic acid dimer isomer[3] | 417b | 1334a | *** | 196c | 866b | 1564a | *** | 54c | 338c | 547c | 1185b | 649b,c | 2480a | ** |
| acid 3-(4',5'-*o*-quinone-caffeoyl) caffeic[3] | 214b | 950a | *** | 95b | 968a | 684a | *** | 22c | 168b,c | 580b | 1356a | 39c | 1328a | ** |
| hydroxy-caffeic acid dimer isomer[3] | 142a | 490b | ** | 969b | 315a,b | 538a | * | 45b | 148b | 288b | 342b | 94b | 981a | * |
| caffeic-(*o*-quinone-caffeic)-ether[3] | 86 | 135 | ns | 18b | 198a | 116a,b | * | 0.2b | 36a,b | 186a,b | 210a | 71a,b | 161a,b | ns |

[1]Detection at 280 nm; [2]cis forms are quantified using trans forms as standards; [3]relative quantification using MS peak area x $10^{-3}$ as no standard was available.
D12, samples stored in the dark, at 12°C; D30, sample stored in the dark, at 30°C; UTL, samples stored under uncontrolled light and temperature conditions.
*, p≤0.05; **, p≤0.01; ***, p≤0.001; ns, p>0.05.

*3.4.2 Effect of sulfites, temperature and uncontrolled conditions of temperature and light, during storage, on the phenolic profile of white wine*

The evolution of the main phenolics compounds in white wine with and without added sulfites were investigated under dark condition at 12°C (D12-S and D12-NS) and 30 °C (D30-S and D30-NS), and with uncontrolled temperature and light condition (UTL-S and UTL-NS). Chromatograms in the Figure 3 show, as an example, the phenolic profiles of D30-S and D30-NS, at the end of storage (15 months).

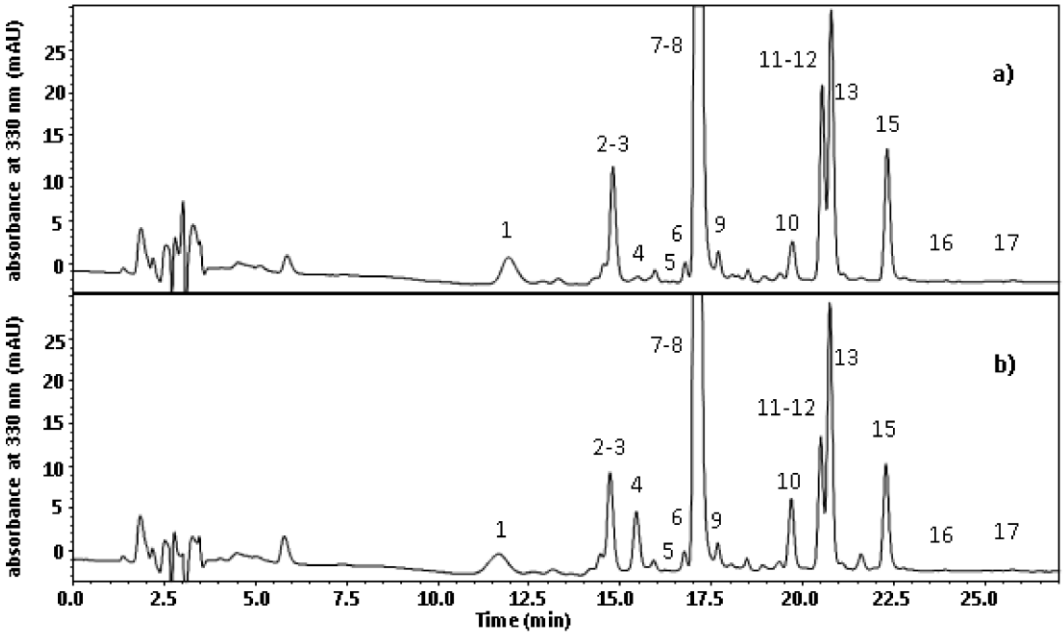


**Figure 3.** HPLC-DAD traces, at 330 nm, of a) sulfite added wine (D30-S) and b) without sulfite added wine (D30-NS) at 15 months storage in dark at 30°C.

Legend of peaks: 1, GRP hydrolysis product; 2, hydroxy-caffeic acid dimer isomer I; 3, 2-S-glutathionyl-caffeoyltartaric acid; 4, cis-caftaric acid; 5, hydroxy-caffeic acid dimer isomer II; 6, acid 3-(4',5'-*o*-quinone-caffeoyl) caffeic; 7, *trans*-caftaric acid; 8, hydroxy-caffeic acid dimer isomer III; 9, *cis*-coutaric acid; 10, *trans*-coutaric acid; 11, caffeic-(*o*-quinone-caffeic)-ether; 12, *trans*-caffeic acid; 13, *cis*-fertaric acid; 14, epicatechin; 15, *trans*-fertaric acid; 16, *p*-coumaric acid; 17, ferulic acid. Peak 14 was detected at 280 nm.

According to a two-factorial ANOVA (added sulfites vs storage conditions) all the tested variables (phenolic compounds) were significantly affected from the investigated factors ($p < 0.05$) and all interactions among them resulted significant. This indicates that the effect of sulfite addition on each of the dependent variables is different for each of the three storage conditions. ANOVAs results are reported in Table 2. The trans forms of hydroxycinnamic esters decreased in samples without added sulfites, whereas, interestingly, the cis forms significantly

increased. The absence of sulfites, and thus conditions favoring oxidation, seems to have favored the presence of cis forms, especially for caftaric acid. In addition, caffeic acid derivatives increased. Finally, the lack of sulfites did not affect GRP and peak 1 contents supporting the importance of the role of GRP. Indeed, this compound, once formed, does not take part in oxidation reactions but only in hydrolysis thus acting as reserve of caftaric acid.

As concerns storage condition factor, the sample UTL showed the lowest content of the trans forms of hydroxycinnamic esters, followed by the sample D30, suggesting that the combination of improper conditions of light and temperature favor hydrolysis reactions more than the effect due to the only high temperature, although the UTL sample was not constantly subjected to high temperature as the D30. According to Recamales et al. (2006), factors such as light and temperature might contribute to the degradation of some individual phenolic compounds such catechin and caftaric acid.

Epicatechin, cis forms of hydroxycinnamic esters, hydroxycinnamic acids (particularly trans-caffeic acid), the oxidized caffeic acid dimer isomer and hydroxy-caffeic acid dimer increased with an improper storage. Hydrolysis of hydroxycinnamic esters seems to be favored by improper storage with the highest production of hydroxycinnamic acids, which are substrate for oxidation/condensation reactions involved in the formation of dark different compounds. Also hydrolysis of GRP was favored by uncontrolled temperature and light conditions more than a constant high temperature and dark condition.

### *3.4.3 Volatile Profile of white wine as affected by $SO_2$, temperature and uncontrolled conditions of temperature and light, during storage*

Sixty-one volatile compounds were detected in all wines during storage and were grouped into different classes, namely esters (the most abundant class with 26 compounds), alcohols (18), acids (9), and ketones and aldehydes (8); attribution is reported in Table 3, with peak number assigned in accordance with elution order in the chromatogram. All the compounds were identified initially and after 15 months of bottle storage. As for phenolic compounds, the evolution of the main volatile compounds in the white wine was investigated by comparing the profile of D12-S, D12-NS, UTL-S, UTL-NS, D30-S, and D30-NS at 15 months.

**Table 3.** Peak areas normalized for internal standard area of volatile compounds in Frascati Superiore wines after 15 months as affected by sulfites and storage conditions. Main effects and significant interactions are shown. *, p≤0.05; **, p≤0.01; ***, p≤0.001; ns, p>0.05.

| | | Sulfites | | *p* | Storage conditions | | | *p* | Sulfites x Storage conditions | | | | | | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | Compounds | S | NS | | D12 | D30 | UTL | | D12-S | D12-NS | D30-S | D30-NS | UTL-S | UTL-NS | |
| | *Esters* | | | | | | | | | | | | | | |
| 1 | Ethyl butanoate | 0.10 | 0.08 | ns | 0.043b | 0.143a | 0.086a,b | * | 0.029b | 0.057b | 0.198a | 0.087b | 0.071b | 0.102b | ns |
| 2 | Ethyl 2-methyl- butanoate | 0.0037 | 0.0044 | ns | 0.0036 | 0.0049 | 0.0036 | ns | 0.0026b | 0.0046a,b | 0.0059a | 0.0039a,b | 0.0025b | 0.0048a,b | ns |
| 4 | Isoamyl acetate | 0.24a | 0.11b | *** | 0.30a | 0.097b | 0.13b | *** | 0.44a | 0.17b | 0.15b | 0.044c | 0.14b | 0.12b,c | ** |
| 6 | Ethyl 2-(E)-butenoate | 0.0031 | 0.0026 | ns | 0.0030 | 0.0032 | 0.0024 | ns | 0.0030a,b | 0.0030a,b | 0.0043a | 0.0021b | 0.0020b | 0.0028a,b | * |
| 8 | Ethyl hexanoate | 0.26 | 0.25 | ns | 0.26 | 0.25 | 0.26 | ns | 0.28a | 0.25b,c | 0.27a,b | 0.22d | 0.23c,d | 0.29a | ** |
| 11 | Ethyl lactate | 0.58b | 0.68a | * | 0.56b | 0.64a,b | 0.70a | * | 0.54b | 0.58b | 0.65b | 0.62b | 0.55b | 0.85a | ** |
| 16 | Ethyl 2-hydroxy-3-methyl-butanoate | 0.0049 | 0.0061 | ns | 0.0043b | 0.0063a | 0.0060a | * | 0.0037c | 0.0048b,c | 0.0068a,b | 0.0058a,b,c | 0.0042c | 0.0077a | * |
| 17 | Ethyl octanoate | 0.193 | 0.187 | ns | 0.22a | 0.17c | 0.19b | *** | 0.24a | 0.19b,c | 0.187c | 0.150d | 0.155d | 0.22a,b | *** |
| 21 | Ethyl 3-hydroxy- butanoate | 0.024 | 0.025 | | 0.024 | 0.023 | 0.027 | | ns | | ns | | | ns | ns |
| 27 | Isoamyl lactate | 0.00095 | 0.00093 | ns | 0.00093 | 0.00095 | 0.00093 | ns | 0.00088a,b | 0.00098a,b | 0.0013a | 0.00061b | 0.00067b | 0.0012a | ** |
| 28 | 3-methyl-butyl metoxyacetate | 0.018 | 0.019 | ns | 0.017 | 0.019 | 0.020 | ns | 0.016b | 0.017b | 0.021a | 0.017b | 0.016b | 0.023a | ** |
| 30 | Diethyl malonate | 0.0049 | 0.0052 | ns | 0.0043c | 0.0057a | 0.0051b | *** | 0.0041c | 0.0045b,c | 0.0063a | 0.0050b | 0.0042c | 0.0061a | *** |
| 32 | Ethyl 2-furancarboxylate | 0.0056b | 0.0095a | *** | 0.0046b | 0.0092a | 0.0089a | *** | 0.0032e | 0.0060d | 0.0080c | 0.010b | 0.0057d | 0.012a | *** |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 35 | Ethyl-methyl succinate | 0.0049b | 0.0059a | * | 0.0043b | 0.0059a | 0.0061a | * | 0.0038c | 0.0048b,c | 0.0061a,b | 0.0057a,b | 0.0049b,c | 0.0073a | ns |
| 36 | Ethyl decanoate | 0.015 | 0.014 | ns | 0.020a | 0.011c | 0.014b | *** | 0.024a | 0.016b,c | 0.012c,d | 0.0094d | 0.010d | 0.017b | *** |
| 37 | Diethyl 2-(E)-fumarate | 0.0038b | 0.0055a | ns | 0.0036b | 0.0050a | 0.0052a | ns | 0.0016b | 0.0057a | 0.0079a | 0.0022b | 0.0018b | 0.0086a | *** |
| 40 | Diethyl succinate | 1.91b | 2.18a | *** | 1.74c | 2.34a | 2.05b | *** | 1.85b | 1.64d | 2.33a | 2.34a | 1.75c | 2.36a | *** |
| 44 | Diethyl glutarate | 0.0070 | 0.0088 | ns | 0.0054b | 0.0099a | 0.0083a | * | 0.0042c | 0.0067b,c | 0.011a | 0.0088a,b | 0.0058b,c | 0.011a | * |
| 45 | Ethyl benzeneacetate | 0.0028 | 0.0032 | ns | 0.0025b | 0.0034a | 0.0031a | * | 0.0022d | 0.0027b,c,d | 0.0037a | 0.0032a,b,c | 0.0026c,d | 0.0036a,b | ns |
| 46 | 2-phenyl-ethyl acetate | 0.032a | 0.026b | ns | 0.030a | 0.023b | 0.034a | * | 0.035a | 0.025a,b | 0.026a,b | 0.019b | 0.033a | 0.035a | ns |
| 51 | Diethyl succinate | 0.0066 | 0.0086 | ns | 0.012a | 0.0063b | 0.0050b | *** | 0.0077b | 0.015a | 0.0098b | 0.0029c | 0.0025c | 0.0076b | *** |
| 53 | Diethyl malate | 1.07b | 1.59a | ** | 0.92b | 1.55a | 1.53a | ** | 0.64c | 1.20b,c | 1.58a,b | 1.52a,b | 0.99b,c | 2.06a | ns |
| 55 | Butanedioic acid, diethyl ester | 0.06 | 0.08 | ns | 0.058b | 0.076a | 0.078a | ns | 0.055b | 0.062b | 0.085a,b | 0.066b | 0.044b | 0.11a | * |
| 56 | Ethyl 5-Oxotetrahydrofuran-2-carboxylate | 0.14b | 0.18a | *** | 0.12c | 0.16b | 0.19a | *** | 0.12c | 0.13c | 0.17b | 0.15b,c | 0.13c | 0.25a | *** |
| 60 | Diethyl tartrate | 0.039 | 0.052 | ns | 0.035c | 0.056a | 0.047b | ns | 0.0270 | 0.0428 | 0.0597 | 0.0530 | 0.0317 | 0.0622 | ns |
| 61 | Ethyl hydrogen succinate | 3.2b | 3.8a | ** | 3.0b | 3.6a,b | 3.8a | * | 2.88b | 3.17b | 3.42b | 3.77a,b | 3.17b | 4.45a | ns |
| | *Alcohols* | | | | | | | | | | | | | | |
| 3 | 2-methyl-1-propanol | 0.76 | 0.84 | * | 0.74b | 0.76b | 0.89a | ** | 0.74b | 0.75b | 0.79b | 0.72b | 0.74b | 1.04a | ** |
| 5 | 1-butanol | 0.010 | 0.011 | * | 0.0084b | 0.012a | 0.012a | ** | 0.0068b | 0.010a | 0.011a | 0.012a | 0.0117a | 0.011a | ns |
| 7 | 2-methyl-1-butanol/3-methyl-1-butanol | 7.5 | 8.6 | *** | 7.5b | 7.9b | 8.8a | *** | 7.52b | 7.59b | 7.61b | 8.14b | 7.50b | 10.2a | *** |
| 9 | 1-pentanol | 0.0074b | 0.011a | * | 0.0069b | 0.0078b | 0.013a | * | 0.0062b | 0.0076b | 0.0076b | 0.0079b | 0.0087b | 0.018a | ns |
| 12 | 1-hexanol | 0.19 | 0.20 | ns | 0.20 | 0.19 | 0.20 | ns | 0.21a,b | 0.19b,c | 0.21a,b | 0.18b,c | 0.16c | 0.24a | *** |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | 3-hexen-1-ol | 0.0044 | 0.0048 | *** | 0.0047 | 0.0042 | 0.0050 | *** | 0.0047b | 0.0047b | 0.0043b,c | 0.0041c | 0.0043b,c | 0.0057a | *** |
| 14 | 3-ethoxy-1-propanol | 0.0080 | 0.0079 | ns | 0.0081 | 0.0080 | 0.0077 | ns | 0.0090a | 0.0072a,b | 0.0085a | 0.0075a,b | 0.0065b | 0.0090a | ** |
| 15 | 3-(Z)-hexen-1-ol | 0.0065 | 0.0064 | ns | 0.0064 | 0.0064 | 0.0066 | ns | 0.0070a | 0.0058b | 0.0071a | 0.0058b | 0.0055b | 0.0077a | *** |
| 19 | 1-heptanol | 0.0026 | 0.0028 | ns | 0.0026 | 0.0026 | 0.0029 | ns | 0.0029b | 0.0023c | 0.0027b,c | 0.0025b,c | 0.0022c | 0.0036a | *** |
| 25 | 2,3-butanediol isomer 1 | 0.22b | 0.28a | * | 0.21 | 0.29 | 0.26 | ns | 0.23b,c | 0.19b,c | 0.16c | 0.41a | 0.27b | 0.25b,c | *** |
| 31 | 2,3-butanediol isomer 2 | 0.026b | 0.044a | ** | 0.027b | 0.047a | 0.031b | ** | 0.029b | 0.024b | 0.016b | 0.078a | 0.032b | 0.029b | *** |
| 41 | p-menth-1-en-8-ol | 0.0029 | 0.0034 | ns | 0.0027 | 0.0033 | 0.0036 | ns | 0.0023b | 0.0032a,b | 0.0038a,b | 0.0028a,b | 0.0028a,b | 0.0043a | ns |
| 42 | 3-(methylthio)-1-propanol | 0.021 | 0.020 | ns | 0.022 | 0.019 | 0.021 | ns | 0.023a | 0.020a,b | 0.022a | 0.015b | 0.018a,b | 0.024a | * |
| 48 | 2-methoxy-phenol | 0.010b | 0.014a | * | 0.014 | 0.010 | 0.012 | ns | 0.0082b | 0.019a | 0.016a | 0.0043b | 0.0058b | 0.018a | *** |
| 49 | Benzyl alcohol | 0.0108 | 0.012 | ns | 0.010 | 0.011 | 0.012 | ns | 0.0076c | 0.014a | 0.013a | 0.010b | 0.012a,b | 0.012a,b | * |
| 50 | Phenylethyl alcohol | 3.5 | 3.7 | *** | 3.5 | 3.5 | 3.7 | ** | 3.53b | 3.51b | 3.51b | 3.49b | 3.37b | 4.10a | *** |
| 58 | 2,6-dimethoxy-phenol | 0.026b | 0.032a | * | 0.027 | 0.029 | 0.031 | ns | 0.021b | 0.033a | 0.037a | 0.021b | 0.019b | 0.043a | *** |
| | *Acids* | | | | | | | | | | | | | | |
| 18 | Acetic acid | 0.38b | 0.49a | ** | 0.32 | 0.42 | 0.57 | *** | 0.29c | 0.36c | 0.35c | 0.49b | 0.51b | 0.63a | ns |
| 24 | Propanoic acid | 0.0064b | 0.0077a | ** | 0.0065 | 0.0066 | 0.0081 | ** | 0.0060b | 0.0070b | 0.0070b | 0.0063b | 0.0062b | 0.0099a | ** |
| 26 | 2-methyl-propanoic acid | 0.044 | 0.048 | ** | 0.043 | 0.042 | 0.052 | *** | 0.046b | 0.040c | 0.044b,c | 0.040c | 0.040c | 0.063a | *** |
| 33 | Butanoic acid | 0.084 | 0.094 | ** | 0.081 | 0.085 | 0.099 | *** | 0.085b,c | 0.077c | 0.089b | 0.082b,c | 0.077c | 0.12a | *** |
| 38 | 3-methyl-butanoic acid | 0.060 | 0.065 | ** | 0.063 | 0.064 | 0.061 | ns | 0.068b | 0.059c | 0.063b,c | 0.064b,c | 0.049d | 0.073a | *** |
| 39 | 2-methyl-butanoic acid | 0.037b | 0.045a | *** | 0.036 | 0.035 | 0.053 | *** | 0.037b | 0.035b | 0.036b | 0.033b | 0.038b | 0.067a | *** |
| 47 | Hexanoic acid | 0.86 | 0.93 | *** | 0.85 | 0.88 | 0.95 | *** | 0.83d | 0.87b,c | 0.90b | 0.86c,d | 0.84c,d | 1.06a | *** |
| 54 | Octanoic acid | 1.03 | 1.08 | *** | 1.05 | 10 | 1.11 | *** | 1.05b | 1.055b | 1.03b,c | 0.96d | 0.99c,d | 1.22a | *** |
| 59 | Decanoic acid | 0.12 | 0.12 | ns | 0.12 | 0.10 | 0.13 | ** | 0.13b | 0.11c | 0.11c | 0.093d | 0.11c | 0.15a | *** |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Ketones and aldheydes* | | | | | | | | | | | | | |
| 10 | 3-hydroxy-2-butanone, | 0.018 | 0.018 | ns | 0.017 | 0.018 | 0.017 | ns | 0.020a,b | 0.015b,c | 0.019a,b | 0.016b,c | 0.013c | 0.022a | ** |
| 20 | Furfural | 0.0096b | 0.035a | *** | 0.0043b | 0.030a | 0.032a | *** | 0.00088e | 0.0077d | 0.020c | 0.041b | 0.0080d | 0.055a | *** |
| 22 | Benzaldehyde | 0.0022b | 0.0064a | *** | 0.0020b | 0.0035b | 0.0074a | ** | 0.0013c | 0.0027b,c | 0.0012c | 0.0058b | 0.0040b,c | 0.011a | ns |
| 23 | dihydro-2-methyl-3(2H)-thiophenone | 0.0080 | 0.0081 | ns | 0.0074 | 0.0084 | 0.0085 | * | 0.0077b | 0.0070b | 0.0097a | 0.0071b | 0.0066b | 0.010a | *** |
| 29 | 5-methyl-2-furancarboxaldehyde, | 0.00064b | 0.0015a | *** | 0.00047b | 0.0014a | 0.0012a | ** | 0.00013c | 0.00080b,c | 0.0013a,b | 0.0016a | 0.00051c | 0.0020a | ns |
| 34 | Butyrolactone | 0.026 | 0.028 | ** | 0.026 | 0.026 | 0.028 | * | 0.027b,c | 0.025c | 0.028b | 0.024c,d | 0.021d | 0.036a | *** |
| 43 | 4-hydroxy-2-butanone | 0.018a | 0.0089b | *** | 0.023a | 0.0095b | 0.0080b | *** | 0.028a | 0.018b | 0.011c | 0.0079c | 0.016b | 0.00011d | ** |
| 52 | 2H-Pyran-2,6(3H)-dione | 0.031b | 0.043a | *** | 0.029b | 0.038a | 0.044a | ** | 0.032c | 0.026c | 0.030c | 0.046b | 0.031c | 0.057a | *** |

As concerns the influence of time storage on the aroma evolution of wine, considering what happened in D12-S as reference, it can be noted that changes in volatile concentration were similar to those reported in the literature (Coetzee et al., 2016). Indeed, isoamyl acetate and 2-phenylethyl acetate significantly decreased during the 15 months of storage, due to the hydrolysis of the ester to acetic acid and the corresponding alcohol, causing the known decline in fruity sensory attributes common for young wines. Moreover, ethyl lactate and diethyl succinate concentrations, reported as the main discriminant variables for white wines with various ages, significantly increased. No other change in volatile composition was observed.

Regarding the comparison of wines stored at the different conditions, a two-factorial ANOVA (added sulfites vs storage conditions) showed that the tested variables were significantly affected from the investigated factors ($p < 0.05$) and interactions among them resulted highly significant (Added Sulfites, F= 2.9E+08; storage conditions, F=2.0E+17; ASxSC, F=5.0E+7) (Table 3).

The storage without sulfites accelerated the hydrolysis of acetate esters, which had lower values down to 54%, as in the case of isoamyl acetate, than S wines. However, it did not affect the most of ethyl esters (i.e. ethyl hexanoate, ethyl octanoate, and ethyl decanoate) whose content remained almost the same between S and NS wines; on the contrary, the concentration of ethyl 2-furancarboxilate and ethyl 5-oxotetrahydrofuran-2-carboxilate as well as the main dioic acid esters increased up to 48%, as for diethyl malate, in NS wines (Table 3). Diethyl succinate concentrations were reported to increase in Sauvignon blanc wine during gradual progressive oxidation (Coetzee et al., 2016), described with notes as " fruity", "floral", and "brandy", arising from the transformation of lactic and succinic acids to form ethyl lactate and diethyl succinate during maturation.

Because of the ethyl esters give a large contribute in defining the fruity aroma of white wines, these findings are very interesting and can be attributed to the protective effect of the reductive winemaking chosen, which quickly reduced the oxygen availability, allowing to preserve the amounts of esters in wines during storage at low temperature (Boroski et al., 2017).

Furthermore, the absence of added sulfites facilitated the increase of alcohols, and diols, such as 2,3-butanediol, as well as 2-methyl-butanoic acid, propanoic acid, and acetic acid, probably derived from partial acetic fermentation during storage (Table 3). Then it is worth noting the great increase, over than 100%, of furfural, 5-methyl-2-furancarboxaldehyde, and benzaldehyde contents in NS wines, which is in line with literature reports because these aldehydes have been suggested to originate from the degradation of carbohydrates during bottle

wine aging (Coetzee et al. 2016). Finally, 2H-Pyran-2,6(3H)-dione was found to increase and 4-hydroxy-2-butanone was found to decrease.

As concerns storage conditions factor, the improper storage (UTL and D30) caused significant changes in volatile profiles. In detail, both UTL and D30 samples showed a dramatic decrease in acetates (from 25% to 68%) and some ethyl esters (i.e. ethyl octanoate, ethyl decanoate, and diethyl succinate, down to 57%) together with 4-hydroxy-2-butanone, showing a level lower than 60% respect to D12 wines (Table 3). On the contrary, the other investigated esters, including mainly dioic esters, increased in UTL and B30 conditions; even ethyl butyrate and ethyl 2-furancarboxylate resulted almost twice more concentrated than in D12 samples. An increase in alcohols and acids was observed mainly for UTL sample, suggesting that the uncontrolled storage conditions of both temperature and light caused a greater volatile imbalance, although the UTL sample was not constantly subjected to high temperature as the D30. In particular, acetic acid concentration increased more in UTL conditions than in D30, likely due to favored hydrolysis conditions. Finally, furfural, 5-methyl-2-furancarboxaldehyde, benzaldehyde and 2H-Pyran-2,6(3H)-dione concentrations were affected by storage conditions increasing in UTL and D30 samples.

As for the interaction of the factors added sulfites x storage condition, in the case of ideal storage 8/26 esters, 6/17 alcohols, 4/9 acids and 2/8 ketones and aldehydes concentrations changed when the sample was stored without added sulfites. In the case of constant storage at 30°C, in the dark, 11/26 esters, 7/17 alcohols, 4/9 acids and 4/8 ketones and aldehydes concentrations changed when the sample was stored without added sulfites; however, 17/26 esters, 2/17 alcohols, 1/9 acids and 5/8 ketones and aldehydes concentrations changed just for the temperature increase. In the case of uncontrolled light and temperature storage, 19/26 esters, 11/17 alcohols, 9/9 acids and 8/8 ketones and aldehydes concentrations changed when the sample was stored without added sulfites, whereas 18/26 esters, 4/17 alcohols, 5/9 acids and 5/8 ketones and aldehydes concentrations changed just for the storing conditions.

### 3.5 Conclusion

In conclusion, although the applied technology involved a reduced contact between oxygen and must in all the steps of winemaking in order to preserve quality attributes, the improper storage, primarily, and the absence of sulfites, secondarily, caused reactions of oxidation. Epicatechin, cis forms of hydroxycinnamic esters, hydroxycinnamic acids (particularly trans-caffeic acid), the oxidized caffeic acid dimer isomer and hydroxy-caffeic acid dimer increased with an improper storage. Hydrolysis of hydroxycinnamic esters and GRP seems to be favored

by improper storage with the highest production of hydroxycinnamic acids, which are substrate for oxidation/condensation reactions involved in the formation of dark different compounds. Moreover, the improper storage, primarily, and the absence of sulfites, secondarily, caused reactions of aroma oxidation, with the formation of oxidation marker compounds at 15 month storage. Results demonstrated that the presence of sulfites helps the typical aroma of young wines; however, even more important seems to be the proper storage in the dark, at low temperature, as improper storage conditions favored aroma degradation regardless sulfite addition.

**Author contributions**

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# CHAPTER 4

## THERMOVINIFICATION PROCESS. DETERMINATION OF OCHRATOXIN A CONCENTRATION AS AFFECTED BY PROCESSING CONDITIONS. RAPID AND AUTOMATABLE DETERMINATION OF OCHRATOXIN A IN WINE BASED ON MICROEXTRACTION BY PACKED SORBENT FOLLOWED BY HPLC-FLD.

### 4.1 Abstract

The influence of termovinification processing on ochratoxin A (OTA) content in red wines was studied. Two heating treatments were investigated i) heating at 60-65°C for 2 h and ii) heating at 80-85°C for 30 min, both treatments experimented with and without added tannins. To achieve this goal, a rapid and automatable method for the determination of OTA in wine using a microextraction by packed C18 sorbent followed by high performance liquid chromatography with fluorescence detection was developed and validated for a successful application in the context of wine production. Important experimental parameters, such as sample and eluent volumes, extraction mode, draw and dispense speeds, number of eluent passes up and down through the stationary phase, were optimized. The validation included the comparison of the sensitivities related to solvent-matched, matrix-matched and standard addition calibrations and the participation to a proficiency test in an inter-laboratory circuit. Matrix effects were also investigated. Accuracies relevant to real samples were estimated to range between 76 and 100% at 0.2 μg/L, and between 84 and 108% at 1.0 μg/L, in compliance with the EU Regulation 401/2006; the limits of detection and quantification were of 0.08 and 0.24 μg/L, respectively, i.e. much lower than the maximum level currently permitted for OTA in the European Union (2.0 μg/kg, corresponding to ca 2.0 μg/L). Sixty different wines produced in the Foggia (Italy) area were analyzed for their OTA content using the developed method and none of them was found to overcome the maximum permitted limit.

## 4.2 Introduction

Thermovinification is a winemaking process consisting of heating grapes between 60 and 80 °C during the pre-fermentation step for a length of time varying from 30 min to 24 h. When heating is limited to a short time period (<1 h), this technique is known as ‘‘thermovinification’’; when the heating is extended over a longer period, up to 24 h, it is known as pre-fermentation heat treatment (Escudier et al., 2008). Heating helps to destroy laccase activity on botrytized grapes and assists in the extraction of grape anthocyanins (Sacchi et al., 2005) and polysaccharides (Doco, et al., 2007), responsible for color and roundness in wine, respectively, and, to a lesser extent, tannins (Girard, et al., 1997). Therefore, for wines that are to be consumed young, thermovinification may help to enhance the color (Clarke and Bakker, 2004; Margalit, 2004), the total phenolic content, the antioxidant potential and the ester formation. However, the low tannin/anthocyanin ratio in thermovinified wines can lead to color instability, thus exogenous tannins are often added. While phenolic composition, color and aroma of thermovinified wines have been largely investigated (Fisher et al., 2000; Atanacković et al., 2012; Darra et al., 2016; ), scarce attention has been paid to safety aspects of wine, such as the presence of ochratoxin A (OTA).

The mycotoxin OTA, chemically known as N-{[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl]-carbonyl}-3-phenyl-L-alanine, was classified in 1993 as a possible human carcinogen, in the group 2 B, by International Agency for Research on Cancer (IARC, 1993). Its immunosuppressive, teratogenic, carcinogenic and mutagenic properties were widely reported by the European Food Safety Authority (EFSA) in 2006; in particular, the EFSA Scientific Panel on Contaminants in the Food Chain established an OTA Tolerable Weekly Intake of 120 ng/kg body weight (EFSA, 2006). The highest OTA levels in wines are usually found in the Mediterranean area, frequently in Spain, southern France and Italy (Otteneder and Majerus, 2000; Battiliani, et al., 2006; Brera et al., 2008). The presence of OTA in wine grapes is generally attributed to *Aspergillus carbonarius* and *Aspergillus niger* (Bau et al., 2005). OTA occurrence in wines is due both to the fungal growth on grapes and to extraction during winemaking, therefore its concentration depends on various factors, such as climatic conditions, mycoflora composition, grape cultivation and winemaking techniques (Delage et al., 2003). A maximum limit of 2.0 µg/kg in wine is recommended by the European Union for a safe intake according to the Regulation (EC) No 1881/2006 (2006).

The main analytical methods for OTA determination in wine are based on reversed-phased high performance liquid chromatography (RP-HPLC) combined with fluorescence detection (FLD) (Battilani et al., 2004; Aresta et al, 2006), often following a clean-up step, such as solid-

phase extraction (SPE) or immunoaffinity clean-up (IAC) (Visconti et al., 1999; Hernández et al., 2006). The latter method is recommended by the official International Organization of Vine and Wine (OIV) (Resolucìon OENO 16/2001, 2001). Due to the complexity of such procedures, usually time-consuming and requiring expert operators, especially for sample preparation, the development of miniaturized and automatized analytical methods, hopefully requiring a reduced use of solvents and a limited involvement of expert operators, would be highly desirable for a high-throughput analysis of wines by analytical laboratories, including those directly related to wineries. Microextraction by packed sorbent (MEPS) can be defined as a miniaturization of the conventional solid phase extraction (SPE), using reduced sample and solvent volumes (μL volumes) and easily interfaced to LC and GC systems to provide a completely automated method (Altun et al., 2004; Abdel-Rehim, 2010). The time to prepare and inject samples is reduced from hours to minutes; additionally, the cartridge can be reused about 100 times. Although a method based on the extraction by a molecularly imprinted polymer packed into a syringe needle has been reported for the analysis of ochratoxin A in red wine (Wei et al., 2007), a MEPS approach based on commercially available products for the analysis of this mycotoxin in wine has been never explored so far.

Therefore, the aim of the present study was to determine OTA concentration in thermovinified wines as affected by processing. Two heating treatments were investigated: i) heating at 60-65°C for 2 h and ii) heating at 80-85°C for 30 min, both treatments experimented with and without added tannins. To achieve this goal, a new, simple, fast and accurate method for the determination of OTA in wine using a MEPS extraction combined with HPLC-FLD detection was developed and validated. Besides the parameters generally considered for method validation, such as linearity, LOD, LOQ, precision and accuracy, the method performance was evaluated also in terms of easiness and rapidity, i.e., highly desirable parameters for a successful application in the context of wine production.

## 4.3 Materials and methods

### *4.3.1 Materials*

The OTA standard was purchased from Sigma (Sigma–Aldrich, St. Louis, MO, USA). A stock solution (5 g/L) was prepared in HPLC gradient grade methanol (Sigma–Aldrich); intermediate standard solutions (500 μg/L, 100 μg/L and 50 μg/L) were obtained by diluting the stock one in HPLC gradient grade methanol; all standards were stored at -20°C in the dark. Seven working standard solutions (0.1-3.0 μg/L) were prepared daily, in duplicate, by dilution

in 2% aqueous acetic acid/ethanol (88:12, v/v). Water used in this work was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC gradient grade), acetic acid (analytical quality), ethanol (99% purity), sodium chloride (NaCl), polyethylene glycol (PEG 8000) and sodium hydrogencarbonate (NaHCO3) were obtained from Sigma–Aldrich.

*4.3.2 Wine samples used for the optimization of OTA determination method*

Sixty different wines, with alcoholic grade ranging between 11% and 14%, elaborated from grapes Montepulciano, Merlot, Cabernet, Syrah, Nero di Troia, Chardonnay, Falanghina, Bombino, Fiano, cultivated in Foggia territory (Italy) and provided by Teanum (San Severo, Foggia, Italy) and La Marchesa (Lucera, Foggia, Italy), were analyzed during this study. Among them only a rosé wine was found to be virtually free of OTA (i.e., it contained OTA levels well below the limits of detections of the applied method) and was then used as a blank sample. The OTA reference material, having an assigned concentration value of 3.35 µg/kg and a -2<z<2 z score range corresponding to a 1.88-4.82 µg/kg interval (RM, T17128QC), and the proficiency test material, with an assigned concentration value of 2.34 µg/kg and a range for the -2<z<2 z score corresponding to a 1.31-3.37 µg/kg interval (PTM, 17143), were obtained from Fapas (Fera Science Ltd, York, UK). Both the RM and the PTM were white wines.

*4.3.3 Samples used for thermovinification and winemaking procedure*

Six wines were elaborated from grapes of the variety cv. Merlot, cultivated in San Severo territory (Apulia region, Southern Italy), in the Teanum winery. About 300 Kg of grapes, previously destemmed, were added with and $SO_2$ (8 mL/100kg) and subjected to the following winemaking technologies. The crushed grapes were heated, added or not added with 20 g/hL of sixtan oenological tannins and pressed by the use of a decanter. Four samples were obtained: long-heated sample obtained heating the crushed grapes on 60-65 °C for 2-3-hour (LH), short-heated sample obtained heating the crushed grapes on 80-85 °C for 30 min (SH), long-heated sample with added oenological tannins (ATLH), short-heated sample with added oenological tannins (ATSH). Afterwards, the must was cooled at 20 °C, and inoculated with Saccharomyces cerevisiae yeasts (796 Maurivin Awri, Istituto di Ricerca Vitivinicola Australiano, 20 g/hL) to begin the alcoholic fermentation. Fermentation, carried out at 20 °C, lasted 14 day. Then the young wine was separated and it was added 80-90 mg/L of $SO_2$ after completion of malolactic

fermentation. At the end, wines were racked into dark green Bordeaux bottles later filtration. A conventional winemaking with (ATC) and without added tannins (C) was also carried out.

All the winemaking processes were performed in duplicate. Wines were analyzed, in duplicate, both after alcoholic fermentation and 3-months aging.

### *4.3.4 Optimization of the MEPS-based method on standard OTA solutions*

During the present investigation MEPS was performed using the Barrel Insert and Needle Assembly (BIN) provided by SGE Analytical Science (Milton Keynes, UK), characterized by a 8 µL barrel volume, packed with 4 mg of C18 sorbent material (particle size 45 µm, pore size 60 Å). The BIN was always mounted on a 100 µL eVol® MEPSTM hand-held automated analytical syringe, also manufactured by SGE. Before each extraction the sorbent phase was conditioned using 50 µL of acetonitrile, 50 µL of methanol and 50 µL of a 2% aqueous acetic acid/ethanol mixture (88:12, v/v). The sample volume subjected to the loading procedure (Vs), the eluent volume (Ve) and the influence of OTA concentration were evaluated with the aim of maximizing the OTA recovery, changing one variable at a time. Multiple 50 µL aliquots were drawn through the BIN when sample volumes higher than 50 µL were loaded. Additionally, the two different loading approaches available with the described MEPS device were compared during this study, namely the "draw-eject" mode, consisting in a sequence of aspirations and injections cycles in the same sample vial, and the "extract-discard" mode, consisting in a similar cycle sequence, the only difference being that the drawn sample is discarded into the waste each time, in the second case. Besides the loading mode, the speed adopted during the extract/discard or draw/inject procedures, for which three values were available (level-1, 3.33 µL/s; level-2, 7.14 µL/s; level-3, 16.67 µL/s), was optimized preliminarily on a OTA standard solution (concentration 0.5 µg/L). Further details on the optimization procedure and on the application of the MEPS-based method to wine samples will be provided in the Results and Discussion section.

### *4.3.5 Comparative experiments on wine samples: sample preparation by Solid-Phase Extraction (SPE), Immunoaffinity cleanup (IAC) and MEPS*

For the sake of comparison the OTA concentration was determined in a naturally OTA-containing wine sample using a SPE, a IAC or a MEPS procedure for the extraction, all followed by HPLC-FLD analysis under the same conditions. A standard addition approach was adopted for calibration purposes in all cases; in particular, wine aliquots (50 mL) were spiked

with OTA at different concentration levels, ranging from 0 to 3.0 μg/L, with two replicates for each level. Standard addition volumes were such to leave the wine sample volume virtually unchanged.

SPE purification. OTA extraction was performed using Bond Elut C18 (500 mg) cartridges (Varian, Harbor City, USA) and a vacuum manifold (Varian), as reported and validated by Hernàndez et al. (2006), with some modifications. The cartridge was first conditioned with 4 mL of acetonitrile and 4 mL of methanol, then it was equilibrated with 4 mL of 2% aqueous acetic acid/ethanol (88:12, v/v). 10 mL of spiked wine, diluted with 10 mL of 2% aqueous acetic acid, were passed through the C18 cartridge. The cartridge was then washed with 2 mL of 2% aqueous acetic acid and 2 mL of methanol/2% aqueous acetic acid (40/60, v/v), before being air-dried. Finally, OTA elution was carried out with 2 mL of acetonitrile. The eluted extract was injected into the HPLC system.

IAC purification. OTA was extracted according to the method reported by Visconti et al. (1999), which has become the official method adopted by OIV, as well as by the Association of Official Analytical Chemists (AOAC International). In particular, a 10 mL volume of spiked wine was diluted with 10 mL of a water solution containing PEG (1%) and NaHCO3 (5%), mixed and filtered through a cellulose filter Whatman grade-1 (Maidstone, England). A 10 mL volume of diluted and filtered wine (equivalent to 5 mL of the original wine) was cleaned up through an OTA CLEAN™ (LCtech GmbH, Dorfen-Germany) immunoaffinity column (3 mL volume, wide bore). The column was washed with 5 mL of a solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%), followed by 5 mL milliQ water. OTA was eluted with 2 mL methanol and collected in a clean glass vial.

MEPS purification. Each sample of spiked wine was divided into two sample subsets: diluted 1:4 and 1:2 (v/v) with 2% aqueous acetic acid; they were then subjected to the optimized MEPS procedure, as described in the Results and discussion section.

All the extracts were analyzed by the HPLC-FLD method described in the following section.

### *4.3.6 HPLC-FLD analysis*

Chromatographic analysis was performed by an Agilent (Palo Alto, USA) chromatographic system, including a model G1311A pump, a model G1329B autosampler, a Zorbax SB-C18 column (100 mm × 4.6 mm i.d., 1.8 μm packing) and a model G1321A fluorescence detector. The excitation and emission wavelengths adopted for fluorescence detection were 333 and 460 nm, respectively. The elution was carried out at a flow rate of 0.6 mL/min using a binary

gradient based on water containing 2% acetic acid (solvent A) and acetonitrile (solvent B). The gradient was run at ambient temperature as follows: (1) from 50% to 75% B in 7 min, followed by washing and re-equilibrating the column. The injection volume was 20 μL. Under these conditions, OTA was eluted after 5.3-5.5 min.

### *4.3.7 Method validation*

Method validation for OTA quantification in wines implied the assessment of selectivity and linearity and the determination of LOD and LOQ, precision (expressed as relative standard deviation - RSD), accuracy, matrix effect (expressed as signal suppression/enhancement - SSE%). The performance characteristics on wines were established using a blank wine spiked with OTA, the RM and the PTM.

Selectivity was assessed by the analysis of several fortified wines, to ensure the absence of chromatographic interferences. Linearity and linear range were first evaluated in standard solutions, through a calibration curve constructed by plotting OTA peak area vs OTA concentrations, ranging from 0.02 to 3.0 μg/L. The analysis at each concentration was performed in triplicate. Detection and quantification limits (LOD and LOQ respectively) in standard solutions were calculated using the regression line parameters, as follows: LOD = 3.3 σ/b and LOQ = 10 σ/b, where σ is the intercept standard deviation and b the slope.

In order to evaluate matrix effects, a matrix-matched calibration was performed using aliquots of the already cited OTA-free rosé wine purposely spiked with different OTA concentrations. As a result, linearity was found to occur between 0.09 and 3.0 μg/L (correlation coefficient 0.9988). Once the slopes relevant to standard and matrix-matched calibration lines were known, the signal suppression/enhancement (SSE%) was calculated as SSE% = ($slope_{spiked\ wine}$ / $slope_{standard\ solution}$) × 100. The precision of the whole method was evaluated in terms of repeatability (intra-day precision) and reproducibility (inter-day precision), expressed as percent relative standard deviation (% RSD), both for standard solutions and for spiked wine samples. Repeatability was assessed by the application of the whole procedure to the same sample, on the same day and by the same analyst (eight experimental replicates performed on a 0.5 μg/L standard solution or on the OTA-free rosé wine spiked at 0.5 μg/L, adopted as representative of a real sample). Inter-day precision was evaluated with a similar procedure, by analyzing the same wine sample on different days (eight experimental replicates in eight days).

## 4.4 Results and discussion

### 4.4.1 *Optimization of the MEPS procedure on OTA standard solutions*

In the first stage of MEPS method development, some parameters were evaluated with the aim of maximizing the recovery. The recovery (R) was calculated using the following formula: $Area_{MEPS}/ (F_{conc} \times Area_{HPLC\text{-}FLD})$, where $Area_{MEPS}$ represents the peak area for OTA as obtained by HPLC-FLD analysis after the MEPS procedure, $Area_{HPLC\text{-}FLD}$ is the peak area obtained using HPLC-FLD directly on the OTA standard solution and $F_{conc}$ is the concentration factor, expressed as the $V_s$ to $V_e$ ratio. The influence of three key factors, namely, the sample ($V_s$) and eluent ($V_e$) volumes and the OTA concentration ($C_{OTA}$) was evaluated changing one variable at a time and the main results are shown in Figure 1. At this stage, the "extract-discard" mode, operated at 3.33 µL/min, was used, since a previous investigation had suggested this to be the most efficient approach (Quinto et al., 2014).

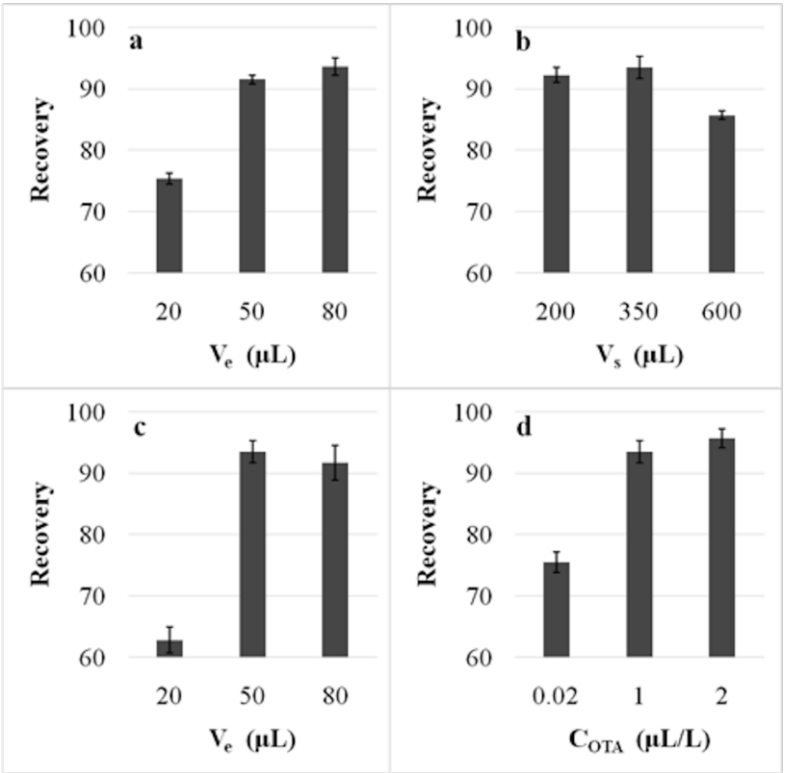


**Figure 1.** Effect of elution volume ($V_e$), sample volume ($V_s$) and OTA concentration ($C_{OTA}$) on the OTA recovery provided by the MEPS procedure. a)-c) $V_e$ at constant $V_s$ (a, $V_s$ = 100 µL; c, $V_s$ = 350 µL) and at $C_{OTA}$ = 1 µg/L; b) $V_s$, at $V_e$ = 50 µL and $C_{OTA}$ = 1 µg/L; d) $C_{OTA}$ at $V_s$ = 350 µL and $V_e$ = 50 µL.

As MEPS is the miniaturization of SPE, we started from typical SPE conditions as the initial parameters to be scaled down. Thus, a$V_s$ of 100 µL and a $V_e$ of 20 µL (concentration

factor as for SPE) were first adopted for a 1.0 μg/L OTA solution and a 75 % recovery was obtained (see Figure 1a), likely because the elution volume was a limiting factor. Indeed, the recovery was increased to 92% upon increasing $V_e$ to 50 μL, whereas no significant variation was observed after a further increase of $V_e$ to 80 μL (see Figure 1a). Since the best concentration factor obtained with the described Vs and $V_e$ values ($F_{conc}$ 2) could be not suitable for wines containing very low OTA concentrations, an increase of Vs was attempted, keeping $V_e$ at 50 μL, to reach good recoveries for higher $F_{conc}$ values. As shown in Figure 1b, a recovery higher than 90% was obtained also for $V_s$ = 350 μL and $V_e$ = 50 μL, thus for $F_{conc}$ = 7; on the other hand, a further increase of the sample volume, up to 600 μL, corresponding to $F_{conc}$ = 12, led to a significant recovery decrease. This result can be explained with the combination of two phenomena: the saturation of the extraction phase in the BIN and a partial elution of OTA extracted in the first stage of sample loading, due to the prolonged withdrawal of sample.

After fixing $V_s$ as 350 μL, the influence of the elution volume was checked again, using two further values for $V_e$, namely 20 and 80 μL (Figure 1c). A $V_e$ = 50 μL was found to be already able to provide a good recovery. Finally, after choosing 350 and 50 μL, respectively, as the best values for $V_s$ and $V_e$, the evolution of the recovery with OTA concentration was investigated by considering two further values, namely 0.02 and 2.0 μg/L; although the recovery was significantly lower for the lowest concentration, as shown in Figure 1d, the values retrieved for the recovery were generally satisfactory over the investigated concentration range, as required by Regulation (EC) No 401/2006 (2006).

Among further experimental factors related to the MEPS procedure, those defined as “draw speed” and “dispense speed” were evaluated on the 1.0 μg/L OTA standard solution and the best recovery was achieved by keeping both speeds at their lowest value (3.33 μL/s). This result is likely related to the longer time available for the interaction between OTA and the sorbent phase when lower drawing and dispense speeds are adopted. The “extract-discard” mode was also compared to the “draw-eject” during a specific test and was found to provide a better recovery (88 vs 64 %, expressed as mean values obtained from three replicates), in accordance with Quinto et al. (2014), thus it was adopted during the subsequent steps of method optimization.

Finally, a slight improvement (5%) was observed by increasing the number of eluent passes up and down through the BIN from 1 to 2, thus two elution cycles were adopted when the method was applied.

*4.4.2 Application of the MEPS-based method to wine samples: evaluation of matrix interference*

Starting from the parameter values optimized on OTA standard solutions the MEPS-based method was applied to OTA-containing wine samples. In this case, after preliminary experiments based on the cited C18 BIN mounted on an eVol® autosampler (SGE), the method was transferred to the MEPS sample preparative workstation HT4000A (HTA Scientific Instruments, Brescia, Italy), in order to achieve automation of the analysis.

As described in Figure 2, after washing and conditioning the BIN, wine analysis was started by loading 350 µL (7 × 50 µL) of each sample through the syringe and the C18 sorbent phase at a speed of 3.33 µL/s (level-1 speed).

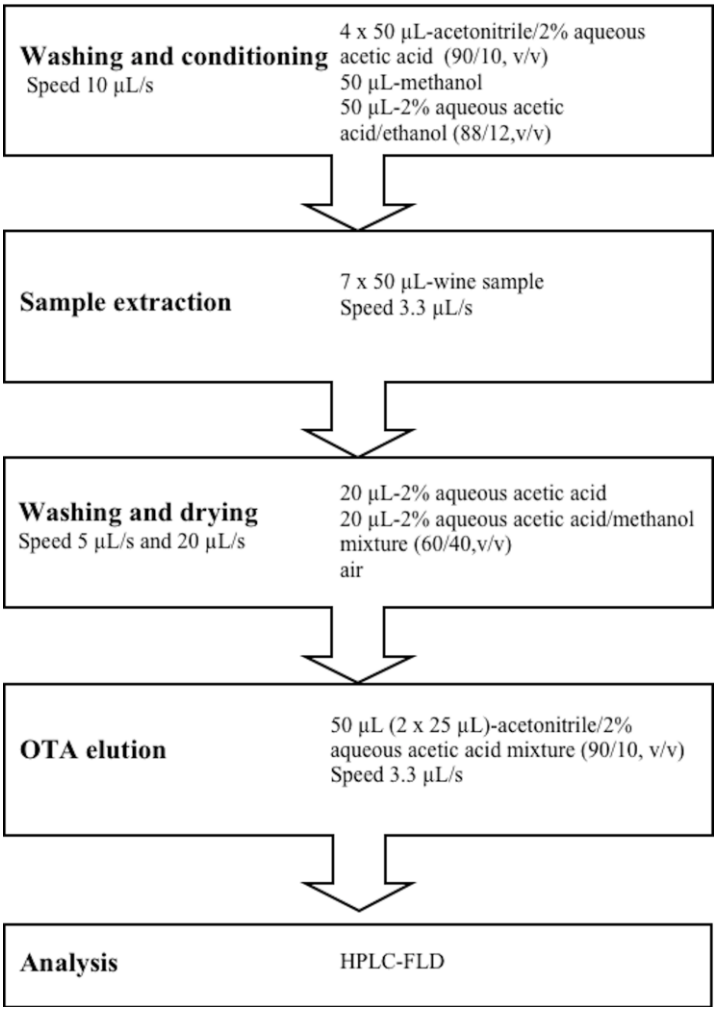


**Figure 2.** Schematic representation of the MEPS-based method developed for OTA determination in wine.

The sorbent bed was then washed first with 20 µL of 2% aqueous acetic acid and then with the same volume of a 2% aqueous acetic acid/methanol mixture (60/40 v/v), to remove eventual

interferences, and dried. The adsorbed analyte was subsequently eluted with 50 μL (2 × 25 μL) of acetonitrile/2% aqueous acetic acid (90/10, v/v), which was pulled/pushed through the syringe twice, at the speed of 3.33 μL/s. In view of subsequent analyses, the BIN was washed with 50 μL-acetonitrile/2% aqueous acetic acid (90/10, v/v) for three times after each extraction. To control memory effects blank samples were also randomly extracted on a previously washed BIN and the eluent was analyzed by HPLC-FLD, under the same conditions adopted for real samples. As a result, no significant memory effect was observed. Indeed, the same sorbent could be used reliably for more than 100 subsequent wine extractions during the present work.

Before undertaking the systematic application of the MEPS-based method to wine samples an evaluation of eventual interference effects due to the wine matrix was performed. At this aim the only wine found to be virtually free of OTA (a rosé wine, see the Experimental section) was used as a blank matrix and was spiked with 0.5 μg/L OTA, thus obtaining a matrix-matched standard solution of the micotoxyn. An aliquot of the spiked wine was first injected directly, without any dilution, into the HPLC-FLD system. The resulting OTA peak, shown in Figure 3 (trace a), was found to be almost symmetric (symmetry, S, 0.88), with a full width at half height peak (FWHH) equal to 0.094 min. On the other hand, the low peak height (H, $4.6 \times 10^{-3}$) suggested the presence of suppression effects due to interfering compounds, although it is not possible to establish if such effects arose from a fluorescence quenching, a chemical interference or both. Another aliquot of the same OTA-spiked blank wine was subjected, undiluted, to MEPS extraction followed by HPLC-FLD analysis, as described before. The resulting OTA peak (see trace c in Figure 3), although significantly higher, as expected, due to the preconcentration associated to the MEPS procedure, was found to be asymmetrical and wide (S 1.43, FWHH 0.23 min, H 7.4 x $10^{-2}$). When the extract obtained from the MEPS procedure performed on the same wine previously diluted 1:2 with 2% aqueous acetic acid/ethanol (88/12, v/v) was analyzed by HPLC-FLD the OTA peak (see trace b in Figure 3) appeared symmetrical but still significantly larger than the peak obtained after wine direct analysis (S 1.09, FWHH 0.18 min). It is worth noting that the OTA peak enlargement seems to be related to the MEPS procedure itself, rather than to an effect of wine matrix; indeed, the enlargement occurred also when OTA standard solutions were involved, as clearly inferred by the comparison of traces d and e in Figure 3. The phenomenon could then be due to the higher amount of OTA injected into the HPLC column when the MEPS procedure is performed.

As far as peak height is concerned, a value higher by almost an order of magnitude, compared to that retrieved for OTA after direct HPLC-FLD analysis of the wine sample, was observed in trace b (H 3.9 x $10^{-2}$). Since the final preconcentration factor inherent to the

optimized MEPS procedure on a 1:2 diluted wine is actually equal to 3.5 (i.e., the ratio between the MEPS preconcentration factor and the wine dilution factor), the almost ten-fold improvement observed in peak height, with respect to direct injection of OTA, might be related to an enhancement in OTA fluorescence, achieved by reducing the incidence of matrix interferences. Consequently, the drawback of peak enlargement is clearly overcome by the advantage in terms of sensitivity provided by the MEPS procedure. A final feature observed in Figure 3 deserves a comment. Indeed, the retention time observed for OTA when a wine sample was involved was systematically, although only slightly, lower than that observed on standard solutions of the mycotoxin. This peculiar effect could be due to interactions of the OTA molecule with one, or more, wine matrix components, a process that does not seem to impair the fluorescence yield but is able to influence the interaction of OTA with the C18 stationary phase.

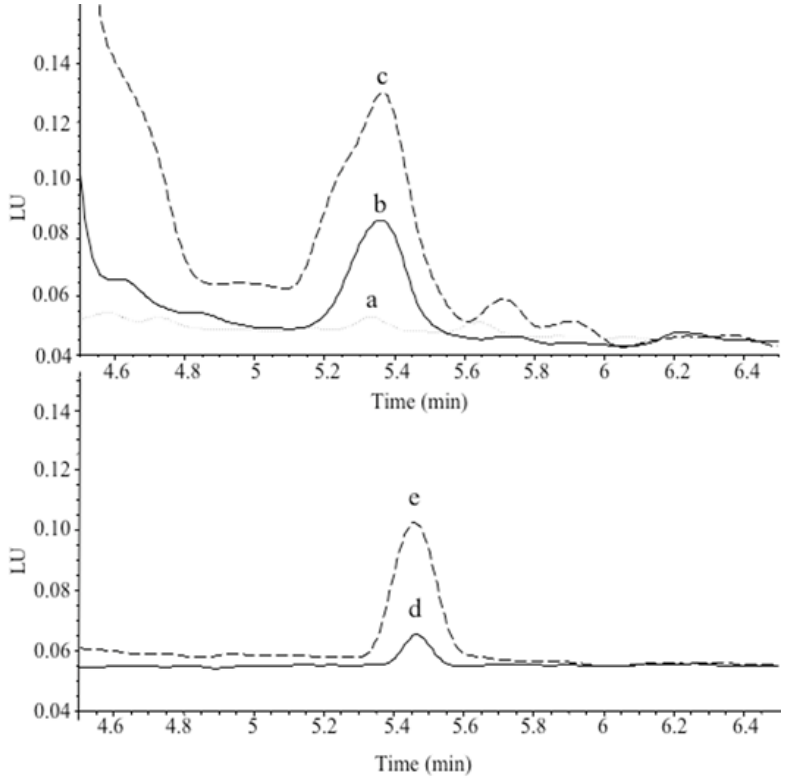


**Figure 3.** Effect of wine matrix and of the MEPS procedure on the characteristic of the OTA chromatographic peak. a) Undiluted 0.5 µg/L spiked wine without previous MEPS extraction; b) MEPS extract on the same wine after 1:2 dilution or c) undiluted; a 0.2 µg/L standard solution d) and e) after MEPS extraction.

As a result of the experiments now described, a 1:2 (v/v) dilution of the wine samples seemed to provide the best compromise between fluorescence signal intensity and peak width.

Actually, the peak enlargement due to the MEPS procedure did not represent a relevant problem during the analysis of wine samples; indeed, a comparison of the chromatograms obtained for unspiked and OTA-spiked wines, carried out for ten different wine samples, showed no interfering peaks apparently overlapping with the OTA one.

*4.4.3 Study of method reliability. Comparison of the results obtained using SPE, IAC and MEPS for the OTA extraction from a red wine sample*

The reliability of MEPS extraction was evaluated by comparison with the well-established SPE (Hernàndez et al., 2006) and IAC techniques (Visconti, et al., 1999), the latter being also recommended by the International Organization of Vine and Wine (OIV). In particular, OTA concentration was determined in a naturally OTA-containing red wine sample by SPE-HPLC/FLD, IAC-HPLC/FLD and MEPS-HPLC/FLD, using a standard addition method, in order to account for matrix effects. It is worth noting that two dilution factors (1:2, 1:4) were adopted in the case of the MEPS-HPLC/FLD method, for the sake of performance comparison. Indeed, as the positive effect of wine dilution was assessed during the experiments described in the 3.2 section, a 1:4 dilution was also considered to evaluate the occurrence of eventual signal improvements (in spite of the higher dilution of the matrix). The extrapolated OTA concentrations, along with standard deviations and 95% confidence interval widths, are reported in Table 1.

**Table 1**. Comparison between the results obtained during a standard addition-based determination of OTA in a test wine sample using different clean-up methods. $x_E$ is the OTA concentration, retrieved as intercept of the standard addition line on the axis reporting added concentrations; $s_{xE}$ and $s_{xE} \times t_{(0.975)}$ represent its standard deviation and the width of its 95% confidence interval, respectively.

| | $x_E$ (µg/L) | $s_{xE}$(µg/L) | $s_{xE} \times t_{(0.975)}$ (µg/L) |
|---|---|---|---|
| SPE-HPLC/FLD | 0.64 | 0.11 | 0.31 |
| IAC-HPLC/FLD | 0.66 | 0.03 | 0.09 |
| MEPS (1:4)-HPLC/FLD | 0.64 | 0.05 | 0.14 |
| MEPS (1:2)-HPLC/FLD | 0.63 | 0.08 | 0.21 |

According to t-test results (95% confidence level), the OTA concentration values obtained by the MEPS procedure on the differently diluted wines were not statistically different and were comparable with those resulting from the SPE and IAC procedures. As far as precision is concerned, the MEPS procedure appeared similar to the IAC one, especially when the 1:4 diluted wine was considered, whereas SPE was clearly characterized by a worse reproducibility.

The 1:4 dilution of wine before MEPS extraction might then be useful to guarantee a good precision also in the case of wines whose OTA content is relatively high (thus enabling the use of a higher dilution factor), yet the preliminary 1:2 dilution of wine was considered as the usual approach during the present study, thus it was introduced in the automatized MEPS procedure in all cases.

It is worth noting that the comparison with the well-established SPE and IAC procedures was done using a red wine sample to understand if the MEPS procedure could be applied also to wine matrices much more complex than those represented by white wines, especially due to the presence of pigments. Moreover, the choice of a naturally OTA-contaminated red wine for the test was due to the fact that neither a red wine-based reference material nor a OTA-free red wine (that could be subsequently spiked to generate a real sample with a known OTA concentration) were available. Nonetheless, the successful comparison obtained with respect to SPE and IAC approaches, whose accuracy is well established, suggested that the MEPS-based one has a good accuracy even when red wine matrices are concerned. The accuracy of the MEPS-based standard addition approach, following a 1:2 dilution of the original wine sample, could be directly assessed on a white wine using the reference material (RM) cited in the experimental section. Indeed, the OTA concentration in the RM was found to be 3.22 ± 0.12 µg/L (95% confidence interval), a value in accordance with the certified one (3.35 µg/kg, corresponding to 3.33 µg/L considering a wine density of 0.9946 g/mL).

### *3.4.4 Validation of MEPS-HPLC/FLD method for OTA determination: comparison of the use of different calibration curves*

Quantitative data obtained for OTA-spiked wine samples during the comparison test described in section 3.3 were very promising in terms of linearity of the developed MEPS-based method, yet they were obtained using a standard addition approach, that it is certainly complex and time-consuming, thus it is not the most practical one, especially if several real samples have to be analyzed at a time. Further tests were then made to verify whether an external calibration could be used reliably for quantitation purposes.

In particular, the MEPS-HPLC/FLD method was applied, under identical conditions, to eight OTA standard solutions in 2% aqueous acetic acid/ethanol (88:12, v/v), with concentrations ranging between 0.02 and 3.0 µg/L, and to as many samples obtained from the already cited OTA-free rosé wine spiked with OTA at the same concentrations. The solutions were analyzed in triplicate and the corresponding average responses were plotted against OTA concentrations, thus enabling a direct comparison between a solvent-matched and a matrix-

matched calibration. The comparison provided excellent results, as emphasized in Table 2, where the main calibration parameters, namely, linear range, linearity (R), regression equation, LOD and LOQ were reported.

**Table 2**. Values obtained for the main calibration and performance parameters of the proposed MEPS-HPLC/FLD method when applied to OTA solvent-matched and matrix-matched standard solutions. Note that the matrix-matched calibration was achieved using as matrix a rosé wine virtually free from OTA. Precision values were estimated from replicated analyses at a 0.5 µg/L OTA concentration.

| **Parameter** | **Solvent matched calibration** | **Matrix matched calibration** |
|---|---|---|
| ***Linear range*** | 0.09-3.0 µg/L | 0.09-3.0 µg/L |
| ***Linearity (R)*** | 0.9991 | 0.9988 |
| ***Regression equation*** | y = 0.812 x + 0.019 | y = 0.784 x – 0.010 |
| **slope standard error** | 0.014 | 0.015 |
| **intercept standard error** | 0.020 | 0.022 |
| ***Limit of detection (LOD)*** | 0.08 µg/L | 0.09 µg/L |
| ***Limit of quantification (LOQ)*** | 0.24 µg/L | 0.28 µg/L |
| ***Precision – $RSD_{intra\text{-}day}$ (%, n= 8)*** | 3.8 | 4.5 |
| ***Precision – $RSD_{inter\text{-}day}$ (%, n=8 )*** | 7.6 | 8.2 |

In particular, the 95% confidence intervals of the respective slopes: 0.81 ± 0.03 and 0.78 ± 0.04 LU min L/µg (where LU represents the luminescence units) were clearly overlapped, indicating no significant signal suppression or enhancement, i.e., a SSE% close to 100%. Moreover, the intercepts of the regression lines were not statistically different from zero (at a 95% confidence level) in both cases, thus indicating the absence of a response due to an interferent eventually present either in the solvent or in the wine matrix. The method showed also promising quantitative performances, as both LOQs were remarkably lower than the maximum level permitted in the European Union (2.0 µg/kg, which corresponds to as many µg/L, if a wine density closed to unity is assumed) for the OTA concentration in wines.

Interestingly, the SSE% was evaluated also after comparing the calibrations lines obtained for the same set of solvent- and matrix-matched standards but without applying the MEPS procedure as a preliminary step. The resulting value, 20%, was dramatically low, thus confirming the precious role of MEPS in removing wine matrix interferents that can lead to a significant suppression of the OTA response.

Turning back to the calibrations involving the MEPS step, one could argue that a single successful comparison between solvent- and matrix-matched calibrations does not guarantee that the solvent-matched calibration can be used as a general approach to the quantification of OTA in every possible wine, since wines could be potentially very different in terms of matrix interference. Since further wines virtually free from OTA were difficult to find, the evaluation of matrix effects could be extended only by using standard addition calibrations, which were applied to ten wines, (two for each of the following varieties: Nero di Troia, Cabernet, Merlot, Syrah and Montepulciano) naturally containing OTA levels detectable by the MEPS-based method. As a result, a good method linearity was always found over the explored concentration range, i.e. up to 1.2 µg/L (correlation coefficients of linear regressions ranging in the interval 0.985-0.999). Moreover, t-tests showed nine and seven slopes to be not significantly different from that related to matrix-matched and solvent-matched calibration, respectively, at 95% confidence. Accordingly, SSE% values ranging between 80 and 105% were obtained.

The results now described confirmed that the external calibration method could provide reliable results in a good percentage of cases, in spite of the matrix variability existing between different wines. Further checks of the good accuracy achievable with the external calibration were also made. The first check was based on the Reference Material sample, previously adopted for a standard addition-based determination. Even if using the external calibration an accuracy of 97 ± 2% (n = 3), expressed as the ratio between the experimentally determined concentration and the true (assigned) one, was obtained. Finally, the 10 wines already contaminated by OTA were adopted to evaluate the accuracy at those levels. In this case, the increase in OTA response observed when passing from the as such sample to samples resulting from additions of 0.2 and 1.0 µg/L was used to extrapolate the added concentration using the external calibration line; accuracies ranging between 76 and 100%, at 0.2 µg/L, and between 84 and 108%, at 1.0 µg/L, were obtained, resulting compliant with the Regulation (EC) No 401/2006 (2006). A final verification of the method accuracy was obtained through participation to a proficiency test (PT) in an inter-laboratory circuit, during which the sample cited as 17143 in the Experimental section, having an assigned OTA concentration of 2.34 µg/kg, was analyzed by the developed MEPS-HPLC/FLD method. As a result, a z-score of -0.8 was obtained by the MEPS-HPLC/FLD method (FAPAS report N. 17143); it is worth noting that a PT can be considered fit-for-purpose if the corresponding z-score lies within the range ± 2.

The method repeatability and reproducibility were finally assessed, according to the procedures described in section 2.6, also on the OTA-free rosé wine spiked with 0.5 µg/L of

mycotoxin, chosen as a representative sample for an OTA-contaminated wine. As reported in Table 2, values of 4.5% and 8.2% were found for the two parameters, thus being comparable to those obtained for a 0.5 µg/L OTA solution in solvent (3.8 and 7.6 %, respectively). Finally, the solvent-matched calibration, adopted for the determination of OTA concentrations in wines, was replicated four times at time intervals of seven days and the resulting slopes were not statistically different, as assessed through a t-test at 95% confidence level. This result showed the good robustness of the proposed method.

### *3.4.5 Evaluation of OTA concentration in several wines*

In the last stage of the work sixty different wines were selected for OTA determination, in order to show the method applicability. Then, thermovinified wines were analysed. This sample number could be easily managed using the configured tray of the automatic preparative station described in section 3.2, since it allowed the preparation of up to 88 samples in one batch. 15 minutes were required for each preparation; the subsequent chromatographic run had the same duration. The whole procedure could be further automatized by directly connecting the preparative station to the chromatographic system, allowing the use overnight, without the presence of any operator. The values obtained for OTA concentrations in the analyzed wines, each extrapolated using the solvent-matched calibration, are reported in Table 3. As apparent, all concentration values were found to be under the legal limit of 2.0 µg/kg (i.e. ca. 2.0 µg/L) and 55% of them were even below the limit of detection obtained for the solvent-matched calibration (0.08 µg/L). Thermovinification processing did not affect OTA concentration in wine.

**Table 3**. OTA concentration levels found in white, rosè and red wines, and in thermovinified wines. LH, long-heated sample; SH, short-heated sample; ATLH, long-heated sample with added tannins; ATSH, short-heated sample with added tannins; C, conventional wine; ATC, conventional wine with added tannins.

| Wine sample | OTA concentration (µg/L) | Wine sample | OTA concentration (µg/L) |
|---|---|---|---|
| #1 | < LOD | #31 | < LOD |
| #2 | 0.110± 0.008 | #32 | < LOD |
| #3 | < LOD | #33 | < LOD |
| #4 | 0.220± 0.021 | #34 | 0.110± 0.012 |
| #5 | 0.89± 0.05 | #35 | 0.270± 0.024 |
| #6 | 0.120± 0.008 | #36 | 0.080± 0.006 |

| | | | |
|---|---|---|---|
| #7 | 0.41± 0.04 | #37 | < LOD |
| #8 | 0.090± 0.007 | #38 | < LOD |
| #9 | 0.160± 0.009 | #39 | 0.080± 0.005 |
| #10 | 0.34± 0.03 | #40 | 0.62± 0.04 |
| #11 | 0.090± 0.006 | #41 | 1.24± 0.08 |
| #12 | 1.07± 0.06 | #42 | < LOD |
| #13 | < LOD | #43 | 0.090± 0.006 |
| #14 | < LOD | #44 | < LOD |
| #15 | < LOD | #45 | 0.140± 0.010 |
| #16 | 0.190± 0.016 | #46 | < LOD |
| #17 | 0.130± 0.009 | #47 | 0.210± 0.013 |
| #18 | < LOD | #48 | < LOD |
| #19 | < LOD | #49 | 0.110± 0.008 |
| #20 | < LOD | #50 | < LOD |
| #21 | 0.210± 0.020 | #51 | < LOD |
| #22 | < LOD | #52 | < LOD |
| #23 | 0.230± 0.022 | #53 | 0.140± 0.011 |
| #24 | < LOD | #54 | 0.080± 0.006 |
| #25 | < LOD | #55 | < LOD |
| #26 | 0.37± 0.03 | #56 | < LOD |
| #27 | < LOD | #57 | < LOD |
| #28 | < LOD | #58 | < LOD |
| #29 | < LOD | #59 | < LOD |
| #30 | < LOD | #60 | < LOD |
| C | 0.18 ± 0.02 | ATC | 0.115 ± 0.009 |
| LH | 0.118 ± 0.015 | ATLH | 0.111 ± 0.010 |
| SH | 0.121 ± 0.009 | ATSH | 0.172 ± 0.017 |

## 4.5 Conclusion

After an appropriate optimization of the operative parameters, MicroExtraction by Packed Sorbent (MEPS) based on a C18 phase proved to be a successful approach to the extraction of Ochratoxin A from wine matrices, preliminary to its determination based on HPLC separation with fluorescence detection. In particular, the remarkable removal of wine interferents achievable using MEPS enabled an accurate determination of the analyte in real samples even

using a solvent-matched calibration. This feature, along with the easiness, rapidity and possibility of automation make the proposed MEPS procedure a very promising, reliable alternative to consolidated analytical approaches like SPE or IAC, especially when a significant number of samples has to be analyzed in a relatively short time. The proposed method could then be successfully used for OTA monitoring and for risk-assessment purposes in the context of wine production. Thermovinification processing did not affect OTA concentration in wine.

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**Publication**

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# CHAPTER 5

## IDENTIFICATION OF MULTI-PHOSPHORYLATED PEPTIDES IN KEFIR BASED ON HYDROXYAPATITE ENRICHMENT FOLLOWED DEPHOSPHORYLATION STEP AND LC-ESI-MS/MS ANALYSIS

### 5.1 Abstract

Kefir consumption has been historically linked to health promoting effects, and caseino-phosphopeptides exert several physiological properties as ability to bind and solubilize bivalent metal ions such as calcium. In the present study, the profiling of multi-phosphopeptides in kefir was carried on and phospheptides were selectively enriched on hydroxyapatite and further analyzed with mass spectrometry analysis. Thus, 22 phosphopeptide sequences were identified in kefir and 9 of them showed the polar acidic motif pSpSpSEE, which is the most active in binding minerals.

### 5.2 Introduction

Kefir is traditionally produced by fermenting milk directly with grains, a complex mixture of microorganism, mainly yeasts, lactic acid and acetic bacteria, combined with casein and polysaccharides (Beshkova et al., 2002; Otles and Cagindi, 2003). Alternatively, mother cultures prepared from grains or commercial freeze-dried kefir starter culture can be used for the industrial production. Kefir consumption has been historically linked to health promoting effects like anticarcinogenic, antimicrobial and immunomodulatory activities (St-Onge et al., 2002; Guven et al., 2003; Liu et al., 2005), mainly attributed to the probiotic microflora (Farnworth, 2005) and to its metabolites (Leite et al., 2013). More recent, peptide profiling revealed sixteen peptides in kefir, deriving from milk proteins, for which bioactivity has been described before (Ebner et al., 2015).

Casein-phosphopeptides (CPPs) are a subgroup of mulitphosphorylated peptides, which can be endogenously present in milk (Baum et al., 2013), or released from precursor proteins by digestive enzymes (Meisel et al., 2003), or by microbial enzymes during fermentation

(Dallas et al., 2016; Jin et al. 2016). CPPs can efficiently bind and solubilize bivalent metal ions such as $Ca^{2+}$, thus enhancing the absorption of minerals in the gastrointestinal tract (FitzGerald, 1998), improving tooth enamel remineralization in the oral cavity and buffering of plaque pH (Nongonierma and FitzGerald, 2012). Casein-phosphopeptides can chelate up to 250 mg Ca/g having an even higher affinity for zinc, iron and copper (FitzGerald, 1998). Calcium-binding is strongly correlated with the presence of phosphorylated serine residues, which are necessary to enhance calcium absorption from small intestine (Sato et al. 1983). Dephosphorylated peptides in contrast do not bind calcium (Berrocal et al., 1989). Calcium dissolution by phosphopeptides is due to the capability of phosphoserine residues to bind to calcium forming amorphous $Ca_3(PO_4)_2$ nanoclusters (Ferraretto et al., 2003).

In particular, the negatively charged phosphate groups and side chains make casein-phosphopeptides more resistant to the gastrointestinal enzymatic digestion and thus suitable as carriers for metal ions (FitzGerald, 1998). It can be expected that fermentation during the production of kefir greatly enhance the formation of CPPs compared to unfermented milk.

Peptide profiling in milk and milk products can be efficiently achieved by liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS) or MALDI-TOF MS (Janek et al., 2001; Baum et al., 2013; Dallas et al., 2014; Sassi et al., 2015). However, multiphosphorylated peptides are negatively discriminating in a complex peptide matrix during mass spectrometry analysis, since the negative charge of the phosphate group leads to a low ionization efficiency (Loyet et al., 2005) and ion suppression by non-phosphorylated peptides (Raska et al., 2002). Among the 257 peptides previously identified in kefir for example, additionally to the non-phosphorylated sequences, only mono- or diphosphorylated sequences were detected (Ebner et al., 2015). However, multiphosphorylated peptides, in particular those containing the pSpSpSEE motive (pS = phosphoserine) show strongest calcium binding and are therefore of particular relevance for their bio-function (Nongonierma and Fitzgerald, 2012). Several approaches have been applied to enrich phosphopeptides using for example immobilized metal affinity chromatography (IMAC), metal oxide affinity chromatography (MOAC) using e.g. titanium dioxide ($TiO_2$), $ZrO_2$, $Fe_2O_3$ and $Al_2O_3$ (Kokubu et al., 2005; Thingholm et al., 2006; Zhou et al., 2007; Fang et al., 2012), or hydroxyapatite (Mamone et al., 2010). Hydroxyapatite enrichment was used in the present study prior to MS-based phosphopeptide profiling to improve the coverage of multiphosphorylated peptides.

Therefore, the goal of the present study was to identify multiphosphorylated peptides present in kefir.

## 5.3 Materials and methods

### *5.3.1 Materials and reagents*

Hydroxyapatite (CHT Ceramic Type I, 40 μm) was purchased from Bio-Rad (Munich, Germany). Formic acid (FA, LC-MS grade), potassium chloride (KCl), 85% ortho-phosphoric acid (PA), ammonium hydrogencarbonate, 4-chloro-α-cyanocinnamic acid (ClCCA) and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma (Sigma-Aldrich, Taufkirchen, Germany). Acetonitrile (ACN, LC-MS grade) was purchased from Honeywell Fluka (Seelze, Germany) and 1,4-dithiothreitol (DTT) from Carl Roth (Karlsruhe, Germany). Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and ammonia (25%) were from Acros Organics (Geel, Belgium) and urea from Merck Millipore (Darmstadt Germany). Alkaline phosphatase (AP, 1500 U, grade I) from calf intestine was purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The digestive juices contained all enzymes were purchased from Sigma-Aldrich, Taufkirchen, Germany, except for mucin (Roth, Karlsruhe, Germany). The peptide standard solution II was acquired by Bruker (Bremen, Germany).

Three different batches of kefir from the same brand were purchased from a local supermarket. The kefir was industrially produced from pasteurized and homogenized low-fat (1.5 %) organic cow milk using a complex culture of yeast and lactic acid bacteria.

### *5.3.2 Sample preparation*

Kefir sample was centrifuged at 3850 rpm and 4 °C for 30 min for the separation of caseins and other insoluble components. Then, the supernatant was filtered through a 0.22 μm sterile membrane filter (Roth, Karlsruhe, Germany) and the water soluble fraction was divided into 5 mL aliquots and frozen at -80°C until use. The aliquots were further filtered using centrifugal filter units with molecular weight 10 kDa cutoff (Merck, Darmstadt, Germany) at 12000 rpm and 4°C for 90 min and stored at -80°C until use.

### *5.3.3 Phosphopeptide enrichment by hydroxyapatite extraction*

The enrichment of phosphopeptides with hydroxyapatite was based on an established protocol by Pinto et al. (2012) with some modifications. Briefly, Briefly, 100 mg of hydroxyapatite were suspended in 1 mL of loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0) and left to be conditioned, for 15 min, in a thermomixer (Eppendorf, Harmburg, Germany), at 1000 rpm, room temperature. After centrifugation at 7500

rpm for 3 min, the supernatant was discarded and the hydroxyapatite was put in contact with 1 mL of thawed sample, for 15 min, in the thermomixer, at 1000 rpm, room temperature. This procedure was repeated successively with 1 mL of loading buffer, 1 mL of 50 mM Tris-HCl (pH 8), 1 mL of 20 mM Tris-HCl in 20% ACN (pH 8) and 1 mL of ultrapure water for washing. The still moist enriched hydroxyapatite was dried using a SpeedVac concentrator system (ThermoFisher Scientific, Dreieich, Germany) and subsequently frozen at -80°C. Hydroxyapatite enriched with phosphopetides (4 mg) was dissolved in 240 µL of a 5% phosphoric acid solution for solubilize phosphopeptides (Pinto et al., 2012) and subjected to StageTips extraction as described in Liu and Pischetsrieder (2017) with some modification. Briefly, to produce StageTips, three small layers of C18 Empore Disk (3M, Neuss, Germany) were assembled by a biopsy punch (1 mm diameter, KAI Medical, Solingen, Germany), placed in 2-200 µL epTIPS pipette tips (Eppendorf, Hamburg, Germany), and inserted into previously perforated 1.5 mL test tube (Eppendorf, Hamburg, Germany) for centrifugation operations. Fifty µL of 0.1% FA in ACN, followed by 50 µL of 0.1% FA aqueous solution, were loaded into the StageTips and spunt down by centrifugation at 5000 rpm, at 25°C for 1 min, as a conditioning step. Afterwards, 50 µL of sample was loaded to the StageTips (centrifugation at 7000 rpm, 5 min). After a washing step with 50 µl of 0.1% FA (centrifugation at 5000 rpm, 3 min), the collecting vessel was changed and the phosphopeptides were eluted with 10 µL of ACN/0.1% FA aqueous solution (60:40) (centrifugation at 5000 rpm, 3 min). Extraction was made in quadruplicate and the eluted samples were combined into a final volume of 40 µL.

### *5.3.4 Dephosphorylation of the peptide fraction*

Prior to dephosphorylation, the samples were dried in the SpeedVac system and reconstituted with 50 µL of 0.4 % ammonium bicarbonate solution (pH 9.0, adjusted with 25% ammonia). Afterwards, 1 µL of alkaline phosphatase was added and incubated at 37°C for 1 hour in a thermomixer at 550 rpm. After enzymatic hydrolysis, the sample was cooled with ice, purified once again by StageTips as described above and analyzed by UHPLC-ESI-MS/MS.

### *5.3.5 UHPLC-ESI-QTrap-MS/MS analysis*

UHPLC-ESI-QTrap-MS/MS analyses were performed on a Dionex Ultimate 3000 RS (ThermoFisher Scientific, Germering, Germany) system coupled to a 4000QTrap mass spectrometer (Sciex, Darmstadt, Germany) with ESI source. Chromatographic separation was achieved with a Waters Acquity BEH 300 C18 (100 mm x 2.1 mm, 1.7 µm) column (Waters,

Eschborn, Germany), at 30°C. The mobile phase was 0.1% formic acid aqueous solution (eluent A) and acetonitrile (eluent B) and the separation was conducted applying a gradient (-6.0-5.0 min 5 % B, 5.0-25.0 min 5-42.5 % B, 25.0-25.5 min 42.5-95 % B, 25.5-30.0 min 95 % B) with a flow rate of 0.3 mL/min. The sample volume was 20 µL. In order to determine the retention time and the charge state of the phosphopeptides previously detected by MALDI-TOF-MS, measurements were carried out in positive enhanced mass spectra (EMS) mode with the m/z ranges 400-1200 and 1100-2000. The ion source voltage was 5000 V and the declustering potential was 50 V. To obtain fragmentation spectra of phosphopeptides, tandem mass spectra were then acquired in the enhanced product ion (EPI) mode, with collision energies in the range 20-30 V and collision energy spread of 10 V. Nitrogen was used for collision induced dissociation. Spectra with insufficient fragmentation were acquired again with a collision energy of 40 V and a collision energy spread of 10 V. Analyst software version 1.6.3 was used for data acquisition and processing.

### *5.3.6 UHPLC-ESI-QTOF-MS analysis*

Phosphopeptides were analyzed by microUHPLC instrument (Ultimate 3200 RS, ThermoFIsher Scientific, Germering, Germany) interfaced with a ESI-QTOF mass spectrometer (6600 TripleTOF, Sciex, Darmstadt, Germany). Chromatographic separation was carried out on a YMC Triart C18 column (100 x 0.5 mm, 3 µm, equipped with a pre-column containing the same material; YMC Europe GmbH, Dinslaken, Germany) with a flow rate of 30 µL/min and 35°C column temperature, using 0.1% FA (eluent A) and ACN containing 0.1% FA (eluent B) and applying a gradient as follows (15.0 min 2% B, 5.0 min 2% B, 55.0 min 42.5% B, 55.5 min 95% B, 65.0 min 95% B). The injection volume was set to 4 µL. Ion spray voltage was set to 5200 V and declustering potential was set to 80 V. Nitrogen was used as the collision gas in the MS/MS experiments for peptide sequencing. Raw data were processed using PeakView® (version 2.2, Sciex, Darmstadt, Germany) and Protein Pilot™ (version 5.0, Sciex, Darmstadt, Germany).

### *5.3.7 Database search*

MS/MS spectra were searched against UniProt database (The UniProt Consortium, 2015) by mMass (Open Source Mass Spectrometry Tool, version 5.5, Niedermeyer and Strohalm, 2012) and Protein Pilot™ (Sciex, Foxer City, CA, USA) software for structure elucidation purposes. Due to the scarce presence of peptide sequences randomly generated by kefir

microflora in the online databases, the phosphopeptide sequences of known phosphorylated bovine proteins were manually searched to achieve an unambiguous identification. The amino acid sequences of phosphopeptides were obtained by de novo peptide sequencing approach. Firstly, a bovine milk peptide database was generated by an ion fragment calculator in mMass software (Niedermeyer and Strohalm, 2012) including precursor, position, and mass of all possible peptides derived from $\alpha_{s1}$- (P02662), $\alpha_{s2}$- (P02663), β- (P02666), and κ-casein (P02668), serum albumin (P02769), GlyCAM-1 (P80195) and osteopontin (P31096) without enzyme restriction. Peak picking was applied for signals with a signal to noise ratios of 4 or higher. The peptides were manually identified by comparing the m/z values of product ions determined in the experimental spectra with the theoretical m/z values of product ions of all possible sequences in the database showing the same m/z value of the precursor ion. Mass tolerance was set to 0.2 Da for precursor ion and 0.2 Da and 0.02 Da for product ion spectra.

## 5.4 Results and discussion

### *5.4.1 Identification of the phophopeptides by UHPLC-ESI-MS/MS*

The UHPLC-ESI-MS/MS was applied to detect the amino acid sequences of the detected phosphopeptides. For this purpose, enhanced product ion (EPI) mass spectra were recorded using the m/z values of desphosphopeptdes detected by MALDI-TOF-MS in a previous study as parent ion (Table 1). Therefore, 18 dephosphopeptide structures were determined by LC-ESI-QTrap-MS/MS analysis. The structures of 9 dephosphopeptides (3, 8, 10, 13, 17, 18, 19, 20, 21 in Table 1), for which the quality of the LC-ESI-QTrap-MS/MS product ion spectra was not sufficient, were identified by LC-ESI-QTOF-MS/MS analysis. MS/MS spectrum of the multi-phosphopeptide β-casein$_{7\text{-}37}$ is displayed in Figure 1, with the relevant sequence scheme showing the amino acid bond cleavage yielding to the experimentally detected fragments. As expected, y-ions residues predominated in the LC-ESI-QTOF-MS/MS spectrum compared to the b-ions residues (Steen and Mann, 2004). The multi-phopshopeptide 20 is a breakdown product of β-casein$_{7\text{-}37}$, which contains five phosphorylated serine units and the sequence motif pSpSpSEE, which is most active in binding minerals (Meisel and Frister, 1988).

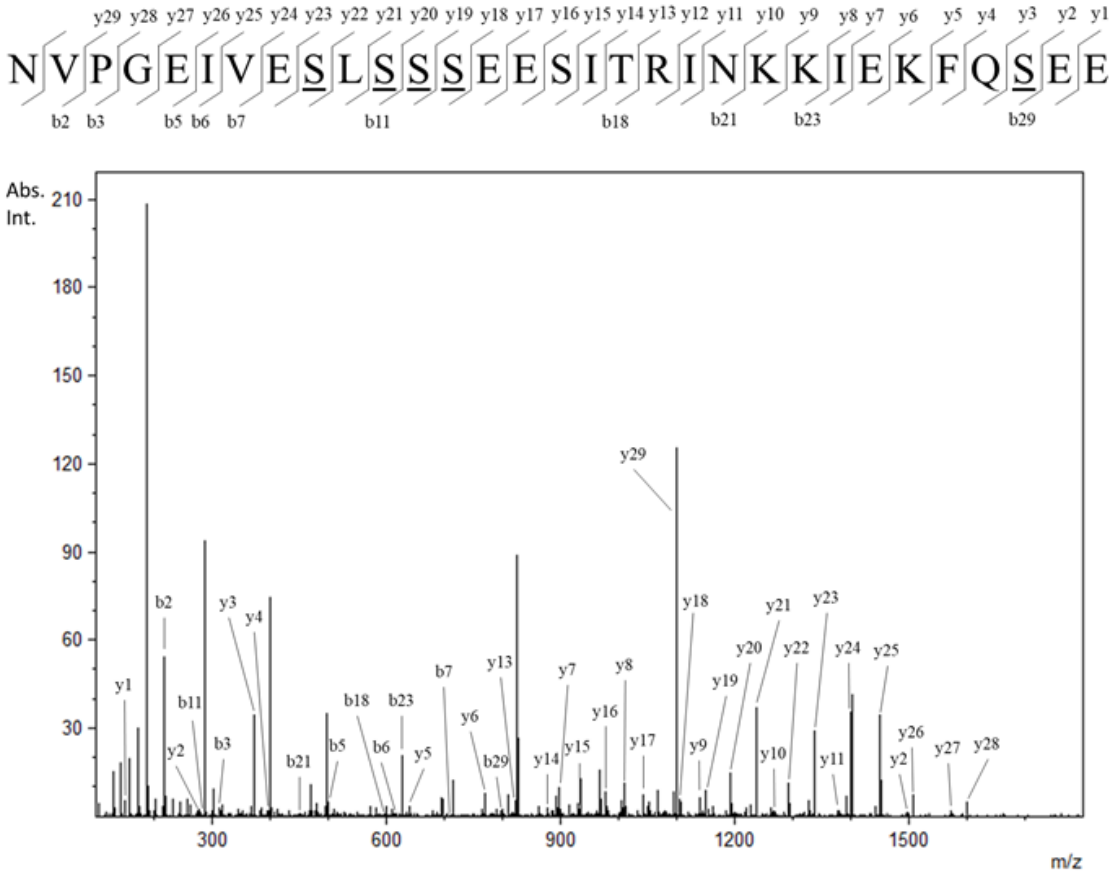


**Figure 1.** MS/MS spectrum of the multi-phosphopeptide with a precursor mass of 877.4 m/z (quadrupole charge). The b and y-ion series are single to triple charged. Phosphoserine residues are underlined.

Thus, a sequence structure could be attributed to each of 21 phosphopeptides previously detected by MALDI-TOF-MS (Table 1). In addition, chromatographic separation of the dephosphopeptides mixture allowed the detection of two different dephosphopeptides with the m/z 1461, eluting at different retention times, which could not be distinguished each other by MALDI-TOF-MS by analysis before and after dephosphorylation. Indeed, EPI mass spectra identified these dephosphopeptideds as β-casein$_{8\text{-}21}$ (9), revealed by MALDI-TOF-MS by dephosphorylation step, and GlyCAM-1$_{22\text{-}33}$ (22). In total, 22 phosphorylated peptides could be identified (Table 1). Finally, one peptide with an m/z 3161.6 and four putative phosphorylation sites was detected by MALDI TOF MS, but for which the structure could not be assigned, since the quality of UHPLC-ESI-MS and MS/MS spectra were not sufficient for structural elucidation.

**Table 1.** Identification of phosphopeptides in kefir. The sequences were then analyzed by subsequent enzymatic dephosphorylation by UHPLC-ESI-MS/MS. Phosphorylation degree was determined by MALDI-TOF-MS by analysis before and after dephosphorylation in a previous study. Sequences are shown in single letter code and peptides masses are given in Da. Phosphorylated serine residues known by literature are underlined (S) and cleveage sites are indicated by dots.

| Number | $[M+H]^+$ phosphorylated | $[M+H]^+$ dephosphorylated | Parent protein | Position | Sequence | Phosphorylation degree |
|---|---|---|---|---|---|---|
| 1 | 1216.6 | 1136.7 | β-Casein | 28-36 | N.KKIEKFQSE.E | 1 |
| 2 | 1217.5 | 1137.6 | β-Casein | 29-37 | K.KIEKFQSEE.Q | 1 |
| 3 | 1296.6 | 1136.5 | β-Casein | 10-20 | P.GEIVESLSSSE.E | 2 |
| 4 | 1340.6 | 1260.5 | GlyCam 1 | 32-42 | E.DLSKEPSISRE.D | 1 |
| 5 | 1345.6 | 1265.6 | β-Casein | 10-21 | P.GEIVESLSSSEE.S | 1 |
| 6 | 1393.8 | 1313.8 | GlyCam 1 | 43-53 | E.DLISKEQIVIR.S | 1 |
| 7 | 1505.9 | 1265.8 | β-Casein | 10-21 | P.GEIVESLSSSEE.S | 3 |
| 8 | 1684.7 | 1604.7 | $\alpha_{s1}$-Casein | 64-78 | E.SISSSEEIVPNSVEQ.K | 1 |
| 9 | 1702.1 | 1461.8 | β-Casein | 8-21 | N.VPGEIVESLSSSEE.S | 3 |
| 10 | 1728.8 | 1648.6 | β-Casein | 25-37 | T.RINKKIEKFQSEE.Q | 1 |
| 11 | 1781.9 | 1461.5 | β-Casein | 8-21 | N.VPGEIVESLSSSEE.S | 4 |
| 12 | 1895.6 | 1575.8 | β-Casein | 7-21 | L.NVPGEIVESLSSSEE.S | 4 |
| 13 | 2029.9 | 1950.0 | β-Casein | 22-37 | E.SITRINKKIEKFQSEE.Q | 1 |
| 14 | 2196.8 | 1876.9 | β-Casein | 7-24 | L.NVPGEIVESLSSSEESIT.R | 4 |
| 15 | 2579.7 | 2260.0 | β-Casein | 7-27 | L.NVPGEIVESLSSSEESITRIN.K | 4 |
| 16 | 2665.1 | 2345.2 | β-Casein | 1-21 | RELEELNVPGEIVESLSSSEE.S | 4 |
| 17 | 2989.1 | 2669.4 | β-Casein | 15-37 | E.SLSSSEESITRINKKIEKFQSEE.Q | 4 |
| 18 | 3068.9 | 2669.2 | β-Casein | 15-37 | E.SLSSSEESITRINKKIEKFQSEE.Q | 5 |
| 19 | 3808.4 | 3488.7 | $\alpha_{s2}$-Casein | 49-79 | N.EEEYSIGSSSEESAEVATEEVKITVDDKHYQ.K | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 20 | 3906.5 | 3506.6 | β-Casein | 7-37 | L.NVPGEIVESLSSSEESITRINKKIEKFQSEE.Q | 5 |
| 21 | 4635.9 | 4236.1 | β-Casein | 7-43 | L.NVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTED.E | 5 |
| 22 | 1541.8 | 1461.8 | GlyCam 1 | 22-33 | Q.FIRNLQISNEDL.S | 1 |

The mass range of the detected phosphopeptides was between 1216 and 4636 Da and 13 of the 22 identified peptides were multi-phosphorylated, ranging between two and five phosphoserine residues. These results confirm that hydroxyapatite enrichment is highly efficient for the analysis of multi-phosphorylated peptides (Mamone et al., 2010). As shown in Table 1, also partially phosphorylated peptides were present in kefir, such as the fragment GlyCam $1_{32-42}$1P (P=phosphate group). Additionally, β-casein$_{10-21}$1P and 3P, β-casein$_{10-20}$2P, β-casein$_{8-21}$3P, $\alpha_{s1}$-casein$_{64-78}$1P were present, all with four possible phosphorylation sites, and the β-casein$_{15-37}$4P with five possible phosphorylation sites. Non-phosphorylated or partially phosphorylated casein have been shown to be present in the mammary gland (Singh et al., 1967). In presence of cations such as $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, casein kinase catalyzes in the lactating mammary gland phosphorylation of partially phosphorylated caseins using ATP as a phosphate donor (Bingham et al., 1974). Furthermore, partial dephosphorylation of caseins is possibly due to the activity of alkaline phosphatase which are associated with a high somatic cell count (Piredda et al., 2000; Mauriello et al., 2007; Yang et al., 2011).

The identified phopshopeptides are breakdown products from the main milk proteins, such as $\alpha_{s1}$-casein (34% of the peptide fraction in milk), β-casein (25%), κ-casein (9%), $\alpha_{s2}$-casein (8%) and GlyCAM 1 (Figure 2). The latter protein is a small phosphoglycoprotein, also know as proteose peptone component 3 (PP3), lactophorin or bovine glycosylation-dependent adhesion molecule (GlyCAM1), which is the main component of proteose peptone fraction of bovine milk (approximately 25%) (Paquet, 1989).

GlyCAM 1 contains 135 amino acids and five phosphoserines ($S_{29}$, $S_{34}$, $S_{38}$, $S_{40}$ and $S_{46}$), as shown in Figure 3. Phosphopeptides found in kefir derived mainly by β-casein (77% of the identified phophopeptides), followed by Glycam 1 (14%) and $\alpha_{s1}$-casein and $\alpha_{s2}$-casein$_{49-79}$ both with one fragment (Table 1). These results confirmed β-casein as the preferential substrate for protein microbiological degradation during transformation of milk in kefir (Ebner et al., 2015; Dallas et al., 2016), because kefir microorganism proteolysis was protein specific and β-casein is more digested of microorganism than $\alpha_{s1}$-casein, though the latter is the most abundant milk protein (Ferreira et al., 2010; Dallas et al., 2016).

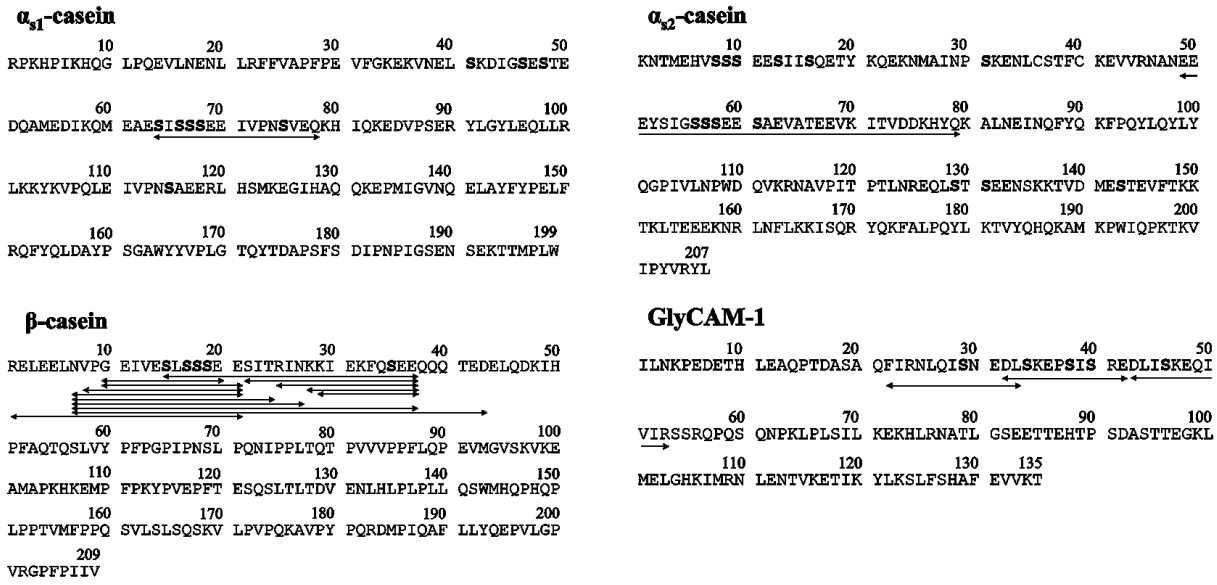


**Figure 2.** Amino acid sequence of milk parent proteins in single letter code. The released phosphopeptides (table 1) are indicated by arrows. Phosphoserine residue are marked in bold.

*5.4.2 Potential bioactivity of the identified kefir phosphopeptides*

Caseins exhibit different phosphorylation sites, namely 13 in $\alpha_{s2}$-casein, 9 in $\alpha_{s1}$-casein, 5 in β-casein and 2 in κ-casein, which often occur in clusters (Figure 2). In particular, the share a highly polar acidic motif pSpSpSEE of casein-phosphopeptides shows predominant bioactivity such as mineral binding, which is responsible for the remineralization of tooth enamel and calcium absorption and bioavailability (Ferraretto et al., 2003; Perego et al., 2013; Tsuchita et al., 2001). In our experiments, 9 peptide sequences, derived from breakdown of β-casein (8) and $\alpha_{s2}$-casein (1), showed the mineral binding motif (Figure 3). Besides the presence of the typical cluster sequence, other factors are important for mineral binding, as the total negative charge, the total number of amino acids, the phosphorylation degree and the amino acid composition around the phosphorylated region (Cross et al., 2005; Zong et al., 2012). In fact, β-casein$_{1\text{-}25}$ phosphopeptide is more active in promoting mineral uptake by human tumor cells HT29 than $\alpha_{s1}$-casein$_{59\text{-}79}$ phosphopeptide (Ferraretto et al., 2003), despite the Ser/Thr rich $\alpha_{s1}$-casein generally generates peptides with stronger n mineral binding than β- and κ-casein (Gagnaire et al., 1996). Indeed, specific secondary structure motifs and the aggregation degree of casein-phosphopeptides in presence of divalent cations are required for a correct mineral absorption (Ferraretto et al., 2003). Indeed, previous studies have demonstrated comformational changes to peptide backbone during calcium binding in β-casein$_{1\text{-}25}$ (loop-type structure of the residue $R_1$ to $E_4$, followed three β-turn of residues $V_8$ to $E_{11}$, $pS_{17}$ to $E_{20}$, $E_{21}$ to $T_{24}$) (Cross et

al., 2001) and in $\alpha_{s1}$-casein$_{59-79}$ phosphopeptides (loop-type structure of the residue $E_{61}$ to $pS_{67}$ and β-turn of residue $P_{73}$ to $V_{76}$) (Huq et al., 1995). The lack of typical loop-like structure (N-terminal region) in β-casein$_{1-25}$ failed to evoke calcium uptake despite the presence of the acidic motif, whereas the lack of the C-terminal residues preserved activity (Ferraretto et al., 2003). Thus, only phosphopeptide β-casein$_{1-21}$ found in kefir could be effectively enhance absorption of calcium in the intestine. Nevertheless, studies on the mineral absorption as favored by casein-phosphopeptides are controversial (Korhonen and Pihlanto, 2006; Miquel and Farré, 2007). Indeed, β-casein$_{29-41}$ have shown in vitro a positive effect on bone mineralization, although carrying only phosphorylated serine (Caroli et al., 2009) and in our results three phophopeptides (2, 4 and 5) show similar amino acids sequence

## 5.5 Conclusion

The present study combined two different mass spectrometry approaches to analyze, after the enrichment with hydroxyapatite and subsequent dephosphorylation step, the phosphopeptide profile in kefir. The phosphopeptides were identified by UHPLC-ESI-MS/MS analysis. Results showed that lactic acid bacteria hydrolyze mainly β-casein during the kefir processing and 9 peptide sequences displayed the mineral binding motif with potential beneficial physiological properties. Furthermore, the results indicate that phosphopeptides can be released form precursor proteins. Further studies however are required to determine the role of kefir-derived phosphopetides locally in the oral cavity or in the gastrointestinal tract for mineral absorption.

## Author contributions

This research work was carried out at the Department of Chemistry and Pharmacy, Friedrich-Alexander Universität Erlangen-Nürnberg, under the supervision of Prof. Dr. Monika Pischetsrieder.

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# CHAPTER 6

## MICROBIOLOGICAL CHARACTERISTICS, SENSORY AND PHOSPHOPEPTIDE PROFILES OF KEFIR: INFLUENCE OF THE PRODUCTION TECHNOLOGY

### 6.1 Abstract

Caseino-phosphopeptides, which can originate by the enzymatic action of microorganisms in fermented milk products, such as kefir, exert several physiological properties as capable to bind and solubilize bivalent metal ions such as calcium. The proteolytic activity of microorganisms is affected by kefir processing. Therefore, the influence of temperature, pH and the repeated use of grains on phosphopeptide profile in kefir prepared using kefir grains, was assessed. Phosphopeptide profile was characterized by LC-ESI-QTOF-MS/MS analysis after an enrichment step with hydroxyapatite, followed by enzymatic dephosphorylation. Thus, 93 phosphopeptides were identified, mainly derived from β-casein. Most phosphopeptides (88) showed the typical acid motif "SSSEE", crucial for exhibiting the bioactive properties of mineral binding. At lower pH (4.0) the proteolysis rate of microorganisms, increased and major hydrophilic phosphopeptides were produced. Finally, a reduced proteolysis rate was observed in kefir prepared with back-slopping approach, indicating a low reproducibility of proteolytic activity of kefir grains.

### 6.2 Introduction

Kefir is a refreshing, self-carbonated and slight foamy fermented milk drink with creamy consistency and sour, acidic and mildly alcoholic flavor, which is believed to have originated in the Caucasian mountains (Farnworth, 2005; Altay et al., 2013). Kefir making traditionally requires the inoculation of cow's milk directly with grains, a symbiotic community of lactic acid and acetic bacteria (about 83-90%) and yeasts (about 10-17%) embedded with casein and complex sugars in a polysaccharide matrix (Prado et al., 2015; Baschali et al., 2017), or with

mother cultures prepared from kefir grains. Industrial production, on the other hand, uses selected starter cultures to standardize the commercial kefir production and preserve its desirable properties (Beshkova et al., 2002).

Kefir grain microbial population includes many microorganisms (e.g. Lactobacillus, Lactococcus, Leuconostoc, Acetobacter, Streptococcus, Enterococcus, Saccharomyces, Candida, Kluyveromyces, Torulaspora, Brettanomyces and Issatchenkia) and depends on many factors, such as the origin of the grains and the storage condition (Farnworth, 2005; Garrote et al., 2001). Grain-milk ratio, incubation temperature and time, final pH, grain washing and storage affect the kefir quality (Guzel-Seydim et al., 2005; Irigoyen et al., 2005; Dimitreli and Antoniou, 2011).

Kefir is known as a probiotic product (Farnworth, 2005; Tamang et al., 2016) with several health-promoting properties such as immunological, antitumoral, hypocholesterolemic, antioxidant and antibacterial effects (Otles and Cagindi, 2003; Liu et al., 2005). Besides microorganisms themselves, several compounds in kefir may have bioactive properties, both metabolites released by microorganisms during fermentation and matrix breakdown products such as peptides (Farnworth, 2005). Recently, several bioactive peptides deriving from milk proteins and health promoting have been identified in kefir (Ebner et al., 2015; Dallas et al., 2016). Among the bioactive peptides, caseino-phosphopeptides (CPPs) are casein-derived phosphorylated peptides able to bind and solubilize minerals exerting several beneficial effects, such as the enhancement of minerals absorption in the gastrointestinal tract (FitzGerald, 1998), of tooth enamel remineralization in the oral cavity and buffering of plaque pH (Nongonierma and FitzGerald, 2012). CPPs are originated from milk caseins by: i) digestive enzymes (Meisel et al., 2003) ii) enzymes of microorganisms (Dallas et al., 2016) iii) endogenous enzymes of milk (Baum et al., 2013).

Single (MS) and tandem (MS/MS) mass spectrometry analysis, often integrated by liquid chromatography (LC), is a common technique in proteome research, but the LC-MS/MS analysis of phosphopeptides, especially multi-phosphorylated peptides, is challenging, due to their low concentration, compared to normal peptides, whose competition for ionization, when co-eluting with phosphorylated ones from the chromatographic column, can be a significant drawback. To overcome the suppressed or attenuated ionization of phosphopeptides caused by relatively high concentration of other peptides, several approaches have been proposed in the literature for the selective enrichment of phosphopeptides (Dunn et al., 2010; Leitner et al., 2011; Yue et al., 2015).

Hydroxyapatite (HA)-based affinity chromatography (Mamone et al., 2010) and solid phase extraction (SPE) based on ceramic HA (Pinto et al., 2010) have been also proposed to enhance the recovery of multi-phosphorylated peptides from complex peptide mixtures. As an application of the use of HA for phosphopeptide enrichment, a characterization of casein phosphopeptides in heated milk based on Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight mass spectrometry (MALDI-TOF-MS) was reported (Pinto et al., 2012). Actually, phosphopeptides, not previously enriched, have been characterized also in milk-related products implying milk fermentation. In particular, a total of 47 phosphopeptides were identified from yogurt (Jin et al., 2016), thus showing that fermented milk is a rich source of CPPs.

As far as kefir is concerned, previous MS-based studies have already evidenced the presence of phosphopeptides as part of the global ensemble of casein-related peptides present in the product (Ebner et al., 2015). More recently, the generation of bioactive peptides upon simulated gastro-intestinal digestion of kefir has been studied using MALDI-TOF-MS and LC-ESI-MS/MS (Liu Y. et al. 2017). A more systematic characterization of kefir phosphopeptides, focused also on the incidence of production technology on their abundance in the product, has not been pursued yet and will be the main goal of the present study, in conjunction with an assessment of the microbiological profile of the product.

## 6.3 Material and methods

### *6.3.1 Materials and reagents*

Hydroxyapatite (CHT Ceramic Type I, 40 µm) was purchased from Bio-Rad (Milan, Italy). Formic acid (FA, LC-MS grade), potassium chloride (KCl), 85% o-phosphoric acid (PA), ammonium hydrogen carbonate, sodium chloride (NaCl), cycloheximide and 1,4-dithiothreitol (DTT) were obtained from Sigma-Aldrich (Milan, Italy). Acetonitrile (ACN, LC-MS grade), water (LC-MS grade) and 25% ammonia solution were purchased from Merck (Vimodrone, Italy). Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) was from PanReac AppliChem (Cinisello Balsamo, Italy). Glucose was purchased from Biolife (Milan, Italy) and calcium carbonate (CaCO3) was obtained from Avantor (Arnhem, Netherlands). Alkaline phosphatase (AP, 1500 U, grade I) from calf intestine was purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany). Plate Count Agar (PCA), Man, Rogosa and Sharpe broth (MRS), WL Nutrient Agar, Yeast extract and Agar were obtained from Oxoid (Milan, Italy).

Pasteurized semi-skimmed cow's milk (1.5 % fat) was acquired from a local dairy supermarket. Kefir grains were purchased from Burumart Commerce S.L (Arrasate/Mondragón, Gipuzkoa, Spain) and consisted of *Lactobacillus Kefiranofaciens*, *Lactococcus lactis* ssp., and other lactic and acetic acid bacteria and yeasts as specified in the product.

### *6.3.2 Kefir production*

Kefir grains were activated by inoculation of the semi-skimmed cow's milk with 18 g/L of grains and incubation at 25°C for 24 h, in accordance with the manufacturer information. Grains were then separated from the milk with sterile sieve and added (2%, w/w) to a new 150 mL aliquot of semi-skimmed cow's milk. Kefir was manufactured, in triplicate, under the following conditions: i) control, at 25°C, pH 4.7 (Kc) and ii) at 18°C, pH 4.7 (K18) to investigate the influence of temperature, iii) at 18°C, pH 4.0 to assess the influence of pH (K18a), and iv) at 25°C, pH 4.7, using a back-slopping approach (grains from a 25°C fermentation were left at 4°C for 5 days and used for a second fermentation), (K25bs), to ascertain the influence of the repeated use of grains. A thermostatically controlled incubator was used. After fermentation, samples were left to mature at 4°C for 24 h before evaluation. During the incubation and maturation, kefir samples were kept open to recreate the aerobic conditions typical for kefir production. The pH was periodically measured and each sample manually agitated to favor the exchange of nutrients between grains and milk.

### *6.3.3 Microbiological analyses*

Microbiological analyses were performed on activation milk (M), activated kefir grains (G), kefir and grains after fermentation. Initially, the samples of kefir and the grains used for fermentation were diluted 1:10 with a sterile saline solution (0.9% NaCl) and homogenized through a Sterilmixer (Pbi Intl., Milan, Italy), at 4000 rpm for 5 min prior to plating. Then, serial dilutions of homogenized samples were executed and plated onto selective media. Total number of viable mesophilic microorganism was estimated on Plate Count Agar (PCA) by incubation at 30°C for 24 to 48 h. Lactic acid bacteria (LAB) counts were performed on MRS Agar, which was added with 0.17 g/L of cycloheximide to inhibit yeast growth, and incubated at 30°C under anaerobic conditions for 48 to 72 h. Acetic acid bacteria (AAB) count was carried out on GYC medium (Glucose 10%,Yeast extract 1%, Calcium carbonate 1.5%, Agar 1.5%,

w/v) in according to Gullo and Giudici (2008) with some modification, at 30°C for 24-48 h. Finally, yeasts count was performed on WL Nutrient Agar by incubation at 30°C for 24-48 h.

### *6.3.4 Extraction and dephosphorylation of phosphopeptides in kefir samples*

The first step of phosphopeptide extraction from each kefir sample was centrifugation at 3900 rpm, at 4 °C for 30 min, aimed at separating caseins and other insoluble components. Then, supernatant was filtered through a 0.22 µm sterile membrane filter (Roth, Karlsruhe, Germany) and was parceled into 10 mL aliquots, subsequently filtered, using centrifugal filter units with a 10 kDa molecular weight cutoff (Merck, Darmstadt, Germany) at 12000 rpm and 4°C for 90 min, for removal of all proteins, and stored at -80°C until use.

Phosphopeptides extraction was performed, in duplicate, by enrichment with hydroxyapatite in according to Pinto et al. (Pinto et al., 2012) with some modifications. Briefly, 100 mg of hydroxyapatite were suspended in 1 mL of loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0) and were conditioned, for 15 min at room temperature. Supernatant was discarded after centrifugation at 7500 rpm for 3 min, and the hydroxyapatite was left in contact with 1 mL of de-frozen kefir sample, for 15 min at room temperature. Then, the hydroxyapatite was washed with 1 mL of loading buffer, 1 mL of 50 mM Tris-HCl (pH 8), 1 mL of 20 mM Tris-HCl in 20% ACN (pH 8) and 1 mL of ultrapure water. The still moist enriched hydroxyapatite was dried using a SpeedVac concentrator system (Thermo Scientific, Waltham, MA USA). Afterwards, the dried hydroxyapatite (4 mg), enriched with phosphopeptides, was dissolved in 240 µL of a 5% phosphoric acid solution to solubilize phosphopeptides (Pinto et al., 2012) and subjected to StageTips extraction as described in Liu and Pischetsrieder (2017). For the preparation of a Stage (Stop and go extraction) Tips, three superimposed C18 Empore Disk (3M, Neuss, Germany) were punched by a biopsy punch (1 mm diameter, KAI Medical, Solingen, Germany). The resulting piece of disk material was transferred into a 2-250 µL epTIPS pipette tips (Mettler Toledo, Greifensee, Switzerland), that was then inserted into the previously perforated cap of a 1.5 mL test tube (Eppendorf, Hamburg, Germany). As a first step of phosphopeptides extraction, 50 µL of 0.1% formic acid (FA) in ACN, followed by 50 µL of 0.1% FA aqueous solution, were pipetted into the StageTips and spunt down by centrifugation at 5000 rpm, at 25°C for 1 min, as a conditioning step. Afterwards, 50 µL of the previously described solution of phosphopeptides in aqueous phosphoric acid were loaded into the StageTips and subjected to centrifugation at 7000 rpm for 5 min. After a washing step with 50 µL of 0.1% FA (centrifugation at 5000 rpm,

3 min), the collecting vessel was changed and the phosphopeptides were eluted with 10 µL of ACN/0.1% FA aqueous solution (60:40) (centrifugation at 5000 rpm, 3 min). Four subsequent elutions were made on the same tip, then the eluates were combined into a final volume of 40 µL and dried in the SpeedVac apparatus; afterwards, phosphopeptides were recovered through re-dissolution into 50 µL of 0.4 % ammonium bicarbonate solution (pH 9.0 adjusted with 25% ammonia). In the last step of the procedure, this solution was incubated at 37°C for 1 hour after addition of 1 µL of alkaline phosphatase, in order to achieve the dephosphorylation of peptides. After the enzymatic reaction, the sample was cooled with ice, purified through an additional step with StageTips, as previously reported, and analyzed by LC-ESI-QTOF-MS/MS, as described below.

*6.3.5 LC-ESI-QTOF-MS/MS*

The HPLC-MS system adopted in this work consisted of an HPLC 1200 Series system (Agilent Technologies, Palo Alto, CA), equipped with a vacuum degasser (G1322A, Agilent), an autosampler (G1377A, Agilent), a quaternary pump, and a thermostatically controlled column compartment. The chromatographic separation was achieved with a Zorbax SB-C18 column (2.1 × 100 mm i.d., 1.8 µm packing; Agilent). The mobile phase was 0.1% formic acid aqueous solution (eluent A) and acetonitrile (eluent B) and the separation was conducted applying a gradient (0-5.0 min 5 % B, 5.0-35.0 min 5-45 % B), followed by a column washing and equilibration step, with a flow rate of 0.2 mL/min. The injection volume of the samples was 15 µL. The HPLC device was connected online to a MicroTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source (ESI). The time-of-flight (TOF) detector, used for accurate mass measurements, operated in positive mode (nebulizer gas, nitrogen, 1 bar; dry gas, nitrogen, 6.5 L/min, 200°C; endplate offset -500 V; capillary voltage -4.5 kV). External calibrations were made using a 100-L KD Scientific (Holliston, MA) syringe pump with a reference solution made up of 10 µL of formic acid (98%), 10 µL of aqueous sodium hydroxide (1.0 M), 490 µL of isopropanol and 490 µL of deionized water. The raw file data were collected as continuum mass spectrum at a regular time interval using DataAnalysis 4.0 software. Typically, 2 runs were performed for the HPLC-ESI-MS analysis of each sample. First, an MS full-scan acquisition (spectral rate of 1 spectrum/s with rolling averages of 3, m/z range of 400–1400 Da) was performed to obtain preliminary information on the predominant m/z ratios observed during the elution. Then, the mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition selecting the 3 most abundant precursor ions. The tandem MS data were

deconvoluted and deisotoped and exported in a generic Mascot format before database searching. Phosphopeptides were tentatively identified by spectra MS/MS searched against UniProtKB database (UniProt Consortium, 2015) by both search engines, Mascot (version 2.6, Matrix Science, Boston, MA) and Protein Prospector (version 5.20, UCSF, San Francisco, CA). Search parameters for Mascot were as follows: taxonomy, other mammals; enzyme, semiTrypsin; mass tolerance 0.2 Da for precursor ions and 0.5 Da for product ions. Since search with the parameter semiTrypsin may not reveal all peptides, subsequent search against the UniProtKB database consisting of $\alpha_{s1}$-(P02662), $\alpha_{s2}$-(P02663), ß- (P02666), and κ-casein (P02668), serum albumin (P02769), GlyCAM-1 (P80195) and osteopontin (P31096) was performed by Ms-Tag in Protein Prospector software, without enzyme restriction. Mass tolerance was set to 0.2 Da for precursor ion spectra and 0.5 Da for product ion spectra. Assignment of the peptides with the lowest scores (< 34 and 15 for Mascot and ProteinProspector, respectively) were verified by manual checking of product ion spectra.

Absolute quantification of all the peptides identified was not possible due to the unavailability of all the peptide standards; however, the ions of interest were manually integrated for the comparison of the same peptide in different samples. To take into account both the variability of matrix and method, reproducibility was evaluated by analyzing two phosphopeptide extracts for each replicate Kc experiment on different days (six HPLC-ESI-MS in six days) and expressed as percent relative standard deviation (% RSD).

### *6.3.6 Sensory evaluation*

Sensory evaluations were performed by 10 tasters familiar with kefir products, consisting of men and women between 25-45 years of age (students and staff of the SAFE Department, Foggia University, Italy). Samples were served at 10°C, in glasses coded with three digit random codes and placed in a random order, 24 h after kefir maturation step. The judges were asked to score the visual (appearance and consistency), olfactory (odor), gustatory (taste and acidity) characteristics, as well as the general acceptability of the product, on a scale of 0–5, according to increasing intensity.

## 6.4 Results and discussion

### *6.4.1 Microbiological characteristics and pH*

As described before, during the present study kefir was manufactured under different temperature (18 or 25°C) and/or pH (4.0 or 4.7) conditions and with a 1- or 2-step fermentation

(back slopping approach). The milk pH was measured before and 24 h after inoculation (at 25°C) with kefir grains and its average values found to be 6.59 (± 0.05) and 5.88 (± 0.03), respectively. Acidification was clearly a consequence of the growth of lactic acid bacteria (LAB) and, subsequently, of lactose breakdown and production of lactic acid (Guzel-Seydim et al., 2005).

As far as incubation times are concerned, Kc (25 °C, pH 4.7) and K25bs (25 °C, pH 4.7, back slopping approach) samples were incubated for 44 and 31 h respectively, whereas K18 (18°C, pH 4.7) and K18a (18°C, pH 4.0) samples for 75 h and 141 h, respectively. In accordance with literature, a faster lactic acid bacteria growth and faster lactic acid production occurred in kefir samples incubated at an higher temperature (Schoevers and Britz, 2003; Dimitreli and Antoniou, 2011). Similar results were reported for yoghurt samples incubated at different temperatures (Haque et al., 2001).

The microbial population was enumerated in activation milk, activated grains, kefir grains at the end of fermentation and in kefir products. Microbial composition was qualitatively similar in each sample and was mainly composed of LAB, acetic acid bacteria (AAB) and yeasts (Farnworth, 2005;Arslan, 2015). Levels of LAB, AAB and yeasts in milk kefir grains and kefir were similar to the values reported in the literature (Witthuhn et al., 2004; Guzel-Seydim et al. 2005; Magalhães et al., 2011; Kim et al., 2015).

Plate count agar (PCA), LAB, AAB and yeasts counts were not significantly different between grains after fermentation and kefir products (data not shown), whereas, as shown in Figure 1A, they were lower in milk (M) and grains (G) activated than in kefir and grains after fermentation. The incubation time (24 h) was therefore sufficient to allow the microorganisms activation and growth and not long enough to produce any change in the microbial population of the grains (Witthuhn et al., 2004). The predominant microorganisms after 24 h fermentation (Figure 1 A) in M and G were LAB and yeasts, followed by AAB. The level of AAB was significantly lower than that reported in previous studies (Irigoyen et al., 2005; Kim et al., 2015); however, the viable count of AAB is highly variable in kefir grains, as a function of the storage temperature, and of the duration of fermentation (Bottazzi, 2004). Significant differences in the microbial population were found according to the kefir production technology (Figure 1 B). In particular, microbial growth, at the investigated conditions, was affected only quantitatively by the fermentation temperature whereas both quantitatively and qualitatively by the final pH. In fact, LAB, AAB and yeasts in Kc and K18 samples were all at the same level, whereas the predominant microorganisms in K18a were yeasts, as they are more resistant under harsh conditions. Yeasts have an important role because they are responsible for $CO_2$ and

ethanol production during fermentation, thus providing unique, slightly effervescent and mildly alcoholic taste to kefir (Beshkova et al., 2002). As concerns kefir produced through back-slopping at 25°C, LAB and AAB counts were significantly higher compared to the other kefir products, whereas yeasts were similar to the ones observed in Kc, showing that LAB and AAB growth was favored by the double fermentation. Finally, owing to the afore discussed differences, the highest PCA was found in K18a and K25bs samples.

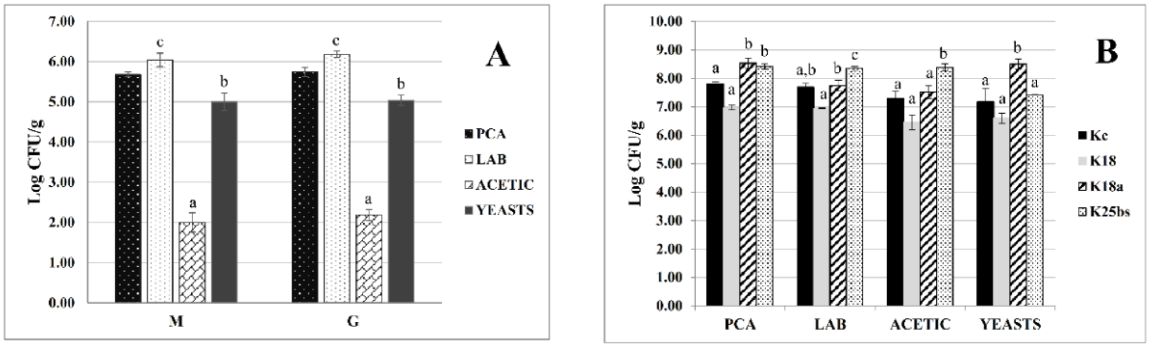


**Figure 1.** Plate count agar (PCA), lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts in [A] activation milk (M) and activated grains (G) and [B] in kefir manufactured at different condition (Kc at 25°C, pH 4.7; K18 at 18°C, pH 4.7; K18a at 18°C, pH 4.0; K25bs at 25°c, pH4.7) are expressed in colony forming units (CFU). Values are mean ± SD of CFU of three independent productions and each measured in duplicate.

### *6.4.2 Identification of kefir phosphopeptides by LC-ESI-QTOF-MS/MS*

Phosphopeptides relevant to Kc, K18, K18a and K25bs, obtained through a hydroxyapatite-mediated enrichment were identified in terms of aminoacidic sequence using LC-ESI-QTOF-MS/MS analysis and the results are showed in Table 1. For structure assignment, MS/MS spectra interpretation was performed using Mascot and Protein Prospector softwares searching the UniProt database (http://www.uniprot.org), and manually checked to achieve an unambiguous identification. Ninety-three phosphopeptides, deriving from the main phosphorylated proteins of milk, were identified in kefir products. Fifteen phosphopeptides were identified with Mascot software, all of them were recognized as unique peptides and with a score major or equal to 45 (34 value was indicated as the identification threshold). Seventy-eight phosphopeptides were identified with Protein Prospector (Ms-Tag), only two of them were considered putatively identified as the score was minor than 15, although several MS/MS fragments corresponded to the fragments of the proposed structure by a manually check.

A summary of phosphopeptide sequences identified in kefir extracts using MS/MS data-based searches has been reported in Table 1.

**Table 1** Chromatographic-mass spectrometric information, parent protein, aminoacid sequences and database search-related scores of phosphopeptides identified by LC-ESI-QTOF-MS/MS analysis after hydroxyapatite-based enrichment from kefir extracts, followed by enzymatic dephosphorylation. Legend for special characters or symbols: cleavage sites are indicated by dots; peak numbers of phosphopeptides containing motif (SSSEE) in their sequence are reported in bold characters; serine residues cited in the literature as possible phosphorylation sites are underlined (S); oxidized methionine residues in a sequence are shown sideways.

| Peak | RT | Ion Observed | Charge of precursor ion | $[M+H]^+$ observed | $[M+H]^+$ calculated | Parent Protein[a] | Sequence[b] | | Score[c] |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 4.5 | 598.287 | 2 | 1195.5597 | 1195.5728 | β-casein$_{15-25}$ | E.SLSSSEESITR.I | | 55* |
| **2** | 8.5 | 1039.474 | 1 | 1039.4746 | 1039.479 | β-casein$_{15-24}$ | E.SLSSSEESIT.R | | 18.8 |
| **3** | 11.5 | 716.327 | 2 | 1431.6465 | 1431.6598 | $\alpha_{s2}$-casein$_{6-18}$ | E.HVSSSEESIISQE.T | | 39.8 |
| **4** | 14.5 | 517.599 | 3 | 1550.7857 | 1550.7947 | β-casein$_{15-28}$ | E.SLSSSEESITRINK.K | | 51* |
| 5 | 14.5 | 511.596 | 3 | 1532.7737 | 1532.7075 | osteopontin$_{57-70}$ | N.TLPSKSNESPEQTD.D | | 15.4 |
| 6 | 14. | 632.325 | 2 | 1263.6466 | 1263.6539 | β-casein$_{18-28}$ | S.SSEESITRINK.K | | 24.3 |
| 7 | 14.5 | 588.807 | 2 | 1176.6072 | 1176.6219 | β-casein$_{19-28}$ | S.SEESITRINK.K | | 33 |
| **8** | 14.5 | 780.853 | 2 | 1560.6997 | 1560.7024 | $\alpha_{s2}$-casein$_{5-18}$ | M.EHVSSSEESIISQE.T | | 16 |
| 9 | 14.9 | 870.375 | 2 | 1739.7425 | 1739.764 | $\alpha_{s1}$-casein$_{39-54}$ | N.ELSKDIGSESTEDQAM.E | | 21.9 |
| **10** | 15.1 | 683.703 | 3 | 2049.0956 | 2049.1186 | β-casein$_{15-32}$ | E.SLSSSEESITRINKKIEK.F | | 29.6 |
| **11** | 15.2 | 854.366 | 2 | 1707.7242 | 1707.7378 | $\alpha_{s2}$-casein$_{4-18}$ | T.MEHVSSSEESIISQE.T | $M_4$(O) | 38.9 |
| **12** | 15.3 | 611.530 | 4 | 2443.0998 | 2443.1293 | $\alpha_{s2}$-casein$_{1-21}$ | KNTMEHVSSSEESIISQETYK.Q | $M_4$(O) | 105.8 |
| **13** | 15.9 | 1161.557 | 1 | 1161.5573 | 1161.5634 | $\alpha_{s1}$-casein$_{64-74}$ | E.SISSSEEIVPN.S | | 43.6 |
| **14** | 15.9 | 678.973 | 3 | 2034.9042 | 2034.9284 | $\alpha_{s2}$-casein$_{1-18}$ | A.KNTMEHVSSSEESIISQE.T | | 80.9 |
| **15** | 15.9 | 647.798 | 2 | 1294.5897 | 1294.6009 | $\alpha_{s2}$-casein$_{7-18}$ | H.VSSSEESIISQE.T | | 14.6 |
| **16** | 16.3 | 574.301 | 3 | 1720.8893 | 1720.9089 | β-casein$_{17-31}$ | L.SSSEESITRINKKIE.K | | 23.9 |
| **17** | 16.3 | 641.006 | 3 | 1921.0035 | 1921.0237 | β-casein$_{15-31}$ | E.SLSSSEESITRINKKIE.K | | 64.5 |
| **18** | 16.4 | 866.927 | 2 | 1732.8406 | 1732.8526 | $\alpha_{s1}$-casein$_{64-79}$ | E.SISSSEEIVPNSVEQK.H | | 73* |
| **19** | 16.6 | 846.367 | 2 | 1691.7267 | 1691.7429 | $\alpha_{s2}$-casein$_{4-18}$ | T.MEHVSSSEESIISQE.T | | 28.6 |
| **20** | 16.6 | 711.851 | 2 | 1422.695 | 1422.7071 | β-casein$_{15-27}$ | E.SLSSSEESITRIN.K | | 52.1 |
| **21** | 16.6 | 712.343 | 2 | 1423.6791 | 1423.6911 | β-casein$_{13-25}$ | I.VESLSSSEESITR.I | | 22.1 |
| **22** | 16.7 | 1056.022 | 2 | 2111.0363 | 2111.0615 | $\alpha_{s1}$-casein$_{64-82}$ | E.SISSSEEIVPNSVEQKHIQ.K | | 65 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **23** | 17.0 | 966.966 | 2 | 1932.9173 | 1932.9323 | $\alpha_{s1}$-casein$_{62-79}$ | E.AESISSSEEIVPNSVEQK.H | 90* |
| **24** | 17.2 | 702.8487 | 3 | 2106.5315 | 2105.9907 | $\alpha_{s1}$-casein$_{56-74}$ | E.DIKQMEAESISSSEEIVPN.S | 7.8 |
| **25** | 17.6 | 848.382 | 2 | 1695.7568 | 1695.7708 | $\alpha_{s2}$-casein$_{6-20}$ | E.HVSSSEESIISQETY.K | 50.5 |
| **26** | 17.7 | 946.492 | 2 | 1891.9701 | 1891.9898 | β-casein$_{12-28}$ | E.IVESLSSSEESITRINK.K | 85* |
| **27** | 17.9 | 802.878 | 2 | 1604.7488 | 1604.765 | $\alpha_{s1}$-casein$_{64-78}$ | E.SISSSEEIVPNSVEQ.K | 29.6 |
| **28** | 17.9 | 690.837 | 2 | 1380.6666 | 1380.674 | β-casein$_{12-24}$ | E.IVESLSSSEESIT.R | 18.9 |
| **29** | 18.2 | 732.724 | 3 | 2196.1583 | 2196.187 | β-casein$_{15-33}$ | E.SLSSSEESITRINKKIEKF.Q | 35.4 |
| **30** | 18.3 | 654.829 | 2 | 1308.65 | 1308.6642 | β-casein$_{15-26}$ | E.SLSSSEESITRI.N | 54.1 |
| **31** | 18.3 | 738.852 | 2 | 1476.6969 | 1476.7064 | $\alpha_{s1}$-casein$_{62-76}$ | A.ESISSSEEIVPNSV.E | 17.2 |
| **32** | 18.9 | 736.048 | 3 | 2206.1306 | 2206.1561 | β-casein$_{10-29}$ | P.GEIVESLSSSEESITRINKK.I | 102.8 |
| **33** | 19.1 | 882.449 | 2 | 1763.8911 | 1763.9021 | β-casein$_{12-27}$ | E.IVESLSSSEESITRIN.K | 84.5 |
| **34** | 19.2 | 861.915 | 2 | 1722.8163 | 1722.8319 | β-casein$_{10-25}$ | P.GEIVESLSSSEESITR.I | 91* |
| **35** | 19.3 | 674.346 | 3 | 2021.0158 | 2021.0324 | β-casein$_{11-28}$ | G.EIVESLSSSEESITRINK.K | 62* |
| **36** | 19.7 | 774.862 | 2 | 1548.716 | 1548.7275 | β-casein$_{8-22}$ | N.VPGEIVESLSSSEES.I | 19.4 |
| **37** | 19.9 | 1039.524 | 2 | 2079.0344 | 2079.0538 | β-casein$_{10-28}$ | P.GEIVESLSSSEESITRINK.K | 79* |
| **38** | 20.1 | 783.866 | 2 | 1566.725 | 1566.7381 | β-casein$_{10-24}$ | P.GEIVESLSSSEESIT.R | 21.8 |
| 39 | 20.3 | 817.759 | 3 | 2451.2641 | 2451.1998 | osteopontin$_{4-26}$ | V.KPTSSGSSEEKQLNNKYPDAVAT.W | 21.7 |
| **40** | 20.5 | 946.969 | 1 | 1892.9321 | 1892.9447 | β-casein$_{11-27}$ | G.EIVESLSSSEESITRIN.K | 69.3 |
| **41** | 20.8 | 801.419 | 3 | 2402.244 | 2402.2773 | β-casein$_{8-29}$ | N.VPGEIVESLSSSEESITRINKK.I | 123.9 |
| **42** | 21.1 | 650.655 | 3 | 1949.9497 | 1949.9662 | β-casein$_{10-27}$ | P.GEIVESLSSSEESITRIN.K | 110.7 |
| **43** | 21.2 | 693.872 | 4 | 2772.4675 | 2772.4989 | β-casein$_{8-32}$ | N.VPGEIVESLSSSEESITRINKKIEK.F | 88.1 |
| **44** | 21.3 | 959.975 | 2 | 1918.9352 | 1918.9531 | β-casein$_{8-25}$ | N.VPGEIVESLSSSEESITR.I | 60* |
| **45** | 21.5 | 681.609 | 4 | 2724.4153 | 2724.4462 | β-casein$_{10-33}$ | P.GEIVESLSSSEESITRINKKIEKF.Q | 86.2 |
| **46** | 21.8 | 1137.582 | 2 | 2274.1504 | 2274.175 | β-casein$_{8-18}$ | N.VPGEIVESLSSSEESITRINK.K | 90* |
| **47** | 22.1 | 882.128 | 3 | 2644.3707 | 2644.404 | β-casein$_{8-31}$ | N.VPGEIVESLSSSEESITRINKKIE.K | 118.5 |
| **48** | 22.2 | 831.882 | 2 | 1662.7572 | 1662.7705 | β-casein$_{7-22}$ | L.NVPGEIVESLSSSEES.I | 23 |
| **49** | 22.2 | 1762.844 | 1 | 1762.8449 | 1762.8593 | β-casein$_{8-24}$ | N.VPGEIVESLSSSEESIT.R | 44 |
| 50 | 22.8 | 836.9132 | 4 | 3344.631 | 3344.6717 | GlyCAM1$_{13-42}$ | E.AQPTDASAQFIRNLQISNEDLSKEPSISRE.D | 34.1 |
| **51** | 23.0 | 1073.538 | 2 | 2146.0688 | 2146.0874 | β-casein$_{8-27}$ | N.VPGEIVESLSSSEESITRIN.K | 95.5 |
| **52** | 23.0 | 717.031 | 3 | 2149.078 | 2149.1347 | β-casein$_{13-31}$ | I.VESLSSSEESITRINKKIE.K | 32.6 |
| **53** | 23.2 | 730.639 | 4 | 2919.5349 | 2919.5673 | β-casein$_{8-34}$ | N.VPGEIVESLSSSEESITRINKKIEKF.Q | 127.8 |
| **54** | 23.2 | 666.022 | 3 | 1996.0515 | 1996.0709 | β-casein$_{17-33}$ | L.SSSEESITRINKKIEKF.Q | 49 |

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|---|---|---|---|---|---|---|---|---|---|
| **55** | 23.3 | 629.828 | 4 | 2516.2904 | 2516.3202 | β-casein$_{7-29}$ | L.NVPGEIVESLSSSEESITRINKK.I | | 136.5 |
| **56** | 23.3 | 816.672 | 4 | 3263.6645 | 3263.7005 | β-casein$_{8-36}$ | N.VPGEIVESLSSSEESITRINKKIEKFQSE.E | | 59.9 |
| 57 | 23.4 | 723.133 | 4 | 2889.5101 | 2889.4523 | $\alpha_{s1}$-casein$_{109-133}$ | Q.LEIVPNSAEERLHSMKEGIHAQQKE.P | $M_{123}$(O) | 25.6 |
| 58 | 23.4 | 964.174 | 3 | 2890.5087 | 2890.2452 | $\alpha_{s1}$-casein$_{41-66}$ | L.SKDIGSESTEDQAMEDIKQMEAESIS.S | $M_{54}$ $M_{60}$ (O) | 20.4 |
| **59** | 23.7 | 848.932 | 4 | 3392.7072 | 3392.7431 | β-casein$_{8-37}$ | N.VPGEIVESLSSSEESITRINKKIEKFQSEE.Q | | 84.2 |
| **60** | 23.8 | 1016.995 | 2 | 2032.9752 | 2032.996 | β-casein$_{7-25}$ | L.NVPGEIVESLSSSEESITR.I | | 45* |
| **61** | 24.2 | 796.737 | 3 | 2388.1905 | 2388.218 | β-casein$_{7-28}$ | L.NVPGEIVESLSSSEESITRINK.K | | 53* |
| **62** | 24.2 | 1088.050 | 2 | 2175.0935 | 2175.1139 | β-casein$_{9-28}$ | V.PGEIVESLSSSEESITRINK.K | | 25.2 |
| **63** | 24.4 | 938.947 | 2 | 1876.8866 | 1876.9022 | β-casein$_{7-24}$ | L.NVPGEIVESLSSSEESIT.R | | 18.6 |
| 64 | 25.1 | 607.911 | 4 | 2428.6625 | 2428.3455 | $\alpha_{s2}$-casein$_{115-136}$ | R.NAVPITPTLNREQLSTSEENSK.K | | 21.1 |
| **65** | 25.1 | 888.424 | 2 | 1775.8416 | 1775.8545 | β-casein$_{6-22}$ | E.LNVPGEIVESLSSSEES.I | | 28.7 |
| **66** | 25.1 | 1011.864 | 3 | 3033.5772 | 3033.6103 | β-casein$_{7-33}$ | L.NVPGEIVESLSSSEESITRINKKIEKF.Q | | 67.5 |
| **67** | 25.3 | 754.042 | 3 | 2260.1127 | 2260.1303 | β-casein$_{7-27}$ | L.NVPGEIVESLSSSEESITRIN.K | | 117 |
| **68** | 25.4 | 658.0945 | 4 | 2629.3561 | 2629.4043 | β-casein$_{6-29}$ | E.LNVPGEIVESLSSSEESITRINKK.I | | 129.2 |
| **69** | 26.0 | 690.356 | 4 | 2758.4033 | 2758.4469 | β-casein$_{5-29}$ | E.ELNVPGEIVESLSSSEESITRINKK.I | | 90.9 |
| **70** | 26.1 | 815.173 | 4 | 3257.6691 | 3257.7111 | β-casein$_{4-32}$ | L.EELNVPGEIVESLSSSEESITRINKKIEK.F | | 87.8 |
| **71** | 26.2 | 722.619 | 4 | 2887.4544 | 2887.4895 | β-casein$_{4-29}$ | L.EELNVPGEIVESLSSSEESITRINKK.I | | 126.8 |
| **72** | 26.3 | 957.837 | 3 | 2871.4964 | 2871.531 | β-casein$_{6-31}$ | E.LNVPGEIVESLSSSEESITRINKKIE.K | | 80.8 |
| 73 | 26.3 | 719.383 | 4 | 2874.5091 | 2874.2503 | $\alpha_{s1}$-casein$_{41-66}$ | L.SKDIGSESTEDQAMEDIKQMEAESIS.S | $M_{60}$(O) | 18.1 |
| **74** | 26.4 | 834.433 | 3 | 2501.2777 | 2501.302 | β-casein$_{6-28}$ | E.LNVPGEIVESLSSSEESITRINK.K | | 57* |
| **75** | 26.8 | 882.708 | 4 | 3527.8091 | 3527.8439 | β-casein$_{1-31}$ | RELEELNVPGEIVESLSSSEESITRINKKIE.K | | 98 |
| **76** | 26.8 | 750.890 | 4 | 3000.5398 | 3000.5736 | β-casein$_{5-31}$ | E.ELNVPGEIVESLSSSEESITRINKKIE.K | | 113.3 |
| **77** | 26.9 | 790.153 | 4 | 3157.5891 | 3157.6223 | β-casein$_{1-28}$ | RELEELNVPGEIVESLSSSEESITRINK.K | | 99 |
| **78** | 26.9 | 1017.465 | 2 | 2033.9233 | 2033.9397 | β-casein$_{4-22}$ | L.EELNVPGEIVESLSSSEES.I | | 20.4 |
| **79** | 26.9 | 995.489 | 2 | 1989.9717 | 1989.9863 | β-casein$_{6-24}$ | E.LNVPGEIVESLSSSEESIT.R | | 25.3 |
| 80 | 26.9 | 878.447 | 2 | 1755.8877 | 1756.0367 | $\alpha_{s1}$-casein$_{101-115}$ | R.LKKYKVPQLEIVPNS.A | | 33.8 |
| **81** | 27.0 | 877.447 | 3 | 2630.32 | 2630.3446 | β-casein$_{5-28}$ | E.ELNVPGEIVESLSSSEESITRINK.K | | 59* |
| **82** | 27.0 | 783.151 | 4 | 3129.5835 | 3129.6161 | β-casein$_{4-31}$ | L.EELNVPGEIVESLSSSEESITRINKKIE.K | | 106.5 |
| **83** | 27.1 | 920.461 | 3 | 2759.3623 | 2759.3872 | β-casein$_{4-28}$ | L.EELNVPGEIVESLSSSEESITRINK.K | | 86* |
| **84** | 27.3 | 819.681 | 4 | 3275.7037 | 3275.7369 | β-casein$_{5-33}$ | E.ELNVPGEIVESLSSSEESITRINKKIEKF.Q | | 73.5 |
| **85** | 27.4 | 851.941 | 4 | 3404.7435 | 3404.7795 | β-casein$_{4-33}$ | L.EELNVPGEIVESLSSSEESITRINKKIEKF.Q | | 85.2 |
| **86** | 27.5 | 1060.008 | 2 | 2119.0095 | 2119.0289 | β-casein$_{5-24}$ | E.ELNVPGEIVESLSSSEESIT.R | | 39.1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **87** | 27.5 | 1187.101 | 2 | 2373.1949 | 2373.2144 | β-casein$_{6-27}$ | E.LNVPGEIVESLSSSEESITRIN.K | 116.3 |
| **88** | 27.8 | 1010.504 | 3 | 3029.499 | 3029.5273 | β-casein$_{1-27}$ | RELEELNVPGEIVESLSSSEESITRIN.K | 120.2 |
| **89** | 28.1 | 1251.623 | 2 | 2502.2379 | 2502.257 | β-casein$_{5-27}$ | E.ELNVPGEIVESLSSSEESITRIN.K | 119.8 |
| **90** | 28.2 | 877.7652 | 3 | 2631.2809 | 2631.2996 | β-casein$_{4-27}$ | L.EELNVPGEIVESLSSSEESITRIN.K | 111.4 |
| **91** | 31.9 | 1323.644 | 2 | 2646.2807 | 2646.2992 | β-casein$_{1-24}$ | RELEELNVPGEIVESLSSSEESIT.R | 94.8 |
| **92** | 31.9 | 630.083 | 4 | 2517.312 | 2517.2566 | β-casein$_{4-26}$ | L.EELNVPGEIVESLSSSEESITRI.N | 16.9 |
| **93** | 32.5 | 970.235 | 4 | 3877.9181 | 3877.9553 | β-casein$_{4-37}$ | L.EELNVPGEIVESLSSSEESITRINKKIEKFQSEE.Q | 28.1 |

[a] The amino acid sequence of the parent protein is based on UniProtKB database entries: $\alpha_{S1}$-casein (P02662), $\alpha_{S2}$-casein (P02663), β-casein (P02666); κ-casein (P02668), GlyCAM1 (P80195) and osteopontin (P31096).

[b] O= oxidation (modification post-translational)

[c] Protein Prospector Ion score obtained for phosphopeptide sequence by database search.

***** Mascot Ion score obtained for phosphopeptides sequence by database search. Ion score > 34 indicate identification ($p<0.05$).

Their molecular weights were found to be comprised between 1039 and 3878 Da, corresponding to peptides having a lengths ranging from 10 to 43 amino acids. Just three of the 93 detected peptides showed only one serine residue, suitable for phosphorylation, two of them bore two serine residues, whereas the remaining 88 peptides included from three to five serine residues in their sequences. Potential phosphorylation sites emerging from sequences reported in Table 1 were in agreement with those reported in the literature and also on information available in the UniProt database. A multiple phosphorylation could be clearly hypothesized for several peptides, thus confirming, hydroxyapatite-based enrichment to be a selective technique for multi-phosphorylated peptides analysis preconcentration (Mamone et al., 2010). This feature is reasonable, since this enrichment is based on the interaction between peptide phosphate groups and $Ca^{2+}$ ions embedded into C-sites of hydroxyapatite (Bernardi and Cook, 1960; Gorbunoff 1984a; Gorbunoff 1984b), thus the presence of several phosphate groups on the same peptide makes the binding to hydroxyapatite tighter (Mamone et al., 2010). Therefore, although phosphorylation level was not determined in the present study, it appears reasonable to hypothesize that mainly multi-phosphorylated peptides were detected.

Turning to proteins that were found to generate kefir phosphopeptides, the presence of caseins among them was not surprising, since milk proteins are constituted for about 80% of caseins, $\alpha_{s1}$-casein (34%) being the most abundant, followed by β-casein (25%), κ-casein (9%), $\alpha_{s2}$-casein (8%) and β-casein breakdown products, and all caseins include several phosphorylation sites: up to 8-9 for $\alpha_{s1}$-casein, 11 to 13 for $\alpha_{s2}$-casein, 5 for β-casein and 1-2 for κ-casein (Belitz et al., 2009). In particular, 74% of identified kefir phosphopeptides were found to arise from β-casein, 13% from $\alpha_{s1}$-casein and 10% from $\alpha_{s2}$-casein.

As for the whey accounting for about the 20% of whole milk proteome, with β-lactoglobulin (9%), α-lactalbumin (4%) prevailing, and the attention was focused on minor proteins, such as serum albumin, osteopontin and bovine glycosylation-dependent adhesion molecule (GlyCAM 1), since the latter proteins have known phosphorylation sites. Indeed, serum albumin and osteopontin may include 6 phosphoserines and 4 phosphotreonines, and 33 phosphoserines and 4 phosphothreonines, respectively, and GlyCAM 1 may include 5 phosphoserines (UniProt database). Actually, 2 and 1%, respectively, of the identified kefir phosphopeptides were related to osteopontin and GlyCAM 1, respectively (Table 1).

From a general point of view, the results reported in Table 1 indicate β-casein as the major precursor of kefir phosphopeptides. These results are in agreement with the previous studies on the whole peptide profile of kefir, indicating the β-casein as the preferential substrate for protein microbiological degradation during transformation of milk into kefir (Ebner et al., 2015; Dallas

et al., 2016). This finding is probably due to the higher accessibility of β-casein to microorganisms, compared to other milk proteins (Dallas et al., 2016). As for the enzymes potentially involved into the generation of kefir phosphopeptides, two general specificity classes of proteinases are reported for *Lactococcus* could be considered, namely PI-type ones, preferentially hydrolyzing β-casein to a lesser extent, κ-casein, and the PIII-type ones, capable of hydrolyzing $\alpha_{s1}$-, β- and κ-casein (Kunji, 1996).

Among specific peptides reported in Table 1, $\alpha_{s2}$-casein$_{1-21}$, β-casein$_{1-27}$ and β-casein$_{1-28}$, were previously found in milk (Baum et al., 2013). It could be hypothesized that these phosphopeptides, generated through the action of milk proteases and survived fermentation. Peptides $\alpha_{s1}$-casein$_{39-54}$, $\alpha_{s2}$-casein$_{115-136}$ and β-casein$_{15-28}$ were previously identified in yoghurt (Jin et al., 2016). Homologous series of β-casein peptides, e. g. (f4-28), (f5-28), (6-28), (f7-28), (f9-28), (f10-28), (f11-28), (f12-28), (f15-28), (f18-28), (f19-28) were also found in kefir. The presence of these peptides could suggest the further breakdown of the β-casein$_{1-28}$ peptide by the action of proteinases of LAB, such as *Lactococcus Lactis* or *Lactobacillus helveticus* species, by analogy with data reported by Gagnaire et al., (2011) for cheeses during ripening. Indeed, *Lactococcus Lactis* or *Lactobacillus helveticus* species were indeed identified also in kefir (Farnworth, 2005; Dallas et al., 2016).

Finally, 82 phosphopeptides derived from β-casein (67), $\alpha_{s2}$-casein (8) and $\alpha_{s1}$-casein (7) showed the typical acid motif "SSSEE" which is crucial for the ability of caseins to bind to minerals, in particular $Ca^{2+}$ (Baumy et al., 1989; FitzGerald 1998; Ferraretto et al., 2003; Perego et al., 2013), showing that they may efficiently bind to minerals and in particular $Ca^{2+}$. Calcium dissolution is facilitated by phosphopeptides thanks to the capability of phosphoserine residues to combine with calcium forming amorphous $Ca_3(PO_4)_2$ nanoclusters (Ferraretto et al., 2003). Mineral binding of CPP may exert anticariogenic activity and promote calcium absorption, and thus increase bone mineral density and strength, and consequently reduce the risk of osteoporosis (FitzGerald, 1998).

### *6.4.3 Influence of technology production on phosphopeptide profile of kefir*

To investigate the influence of temperature and pH and the repeated use of grains on the phosphopeptides profile in kefir, the phosphopeptide profiles of kefir samples obtained under different conditions were compared. The influence of temperature was evaluated at a temperature of 18°C, thus comparing K18 and K18a samples. As described in the Materials and Methods section a semi-quantitative comparison between samples, based on peak areas obtained from LC-ESI-QTOF-MS/MS Extracted Ion Current (EIC) chromatograms referred to

specific peptides, was performed. The mean relative standard deviation (n = 6) of peak areas for the investigated compounds in control sample, expressed as percentage, was considered as reproducibility of the method, and was multiplied for the t value at (n-1) freedom degrees to estimate the 95% p level. The histogram of the relevant peak areas, reporting the 95% p level for Kc sample, shows the distribution of peptides in the two samples (Figure 2). Kc and K18 phosphopeptide profiles were very similar, but K18 was richer in phosphopetides (peaks 15, 69, 75, 77, 82, 88 and 91) in Table 1 eluting in the second part of the chromatogram (Figure 2), regarding thus larger compounds with an increasing hydrophobicity. The low temperature slowed down the proteolytic activity in K18, yielding to more abundance of large peptides. Afterwards, the influence of pH was evaluated comparing K18 and K18a (Figure 3A, 3B). K18 can be considered as an intermediate, at 18°C, pH4.7, before becoming k18a which was at pH 4.0. As depicted in Fig 3A, phosphopeptide chromatographic profiles showed marked differences after 21 min of elution time, regarding thus compounds with an increasing hydrophobicity (compounds 42-93), which were predominant in K18 sample. Accordingly, the histogram (Figure 3B) of the relevant peak areas shows the distribution of peptides in the two samples, revealing as several compounds in K18 ranging from peaks 42 to 93 fall out the 95% p level (peaks 44, 69, 70, 71, 75, 77, 78, 80, 82, 83, 85, 88, 90, 91, 92, 93). Most of these compounds corresponded to the largest phosphopeptides detected in this study and all of them showed the sequence motif “SSSEE”, except phosphopeptide 80. K18 sample showed, therefore, a greater level of large peptides, whereas microbial population in K18a, at lower pH and for a longer fermentation time, had likely sufficient time to degrade more large peptides to survive at harsh pH conditions, though significant differences for small peptides, within the range explored, were not observed. Indeed, LAB and yeasts counts were major in K18a than in K18. Finally, the influence of the repeated use of grains was investigated; the phosphopeptide chromatographic profiles of Kc (a) and K25bs (b), displayed in Figure 4A, were significantly different, showing a major crowding of peaks in the range 19-24 min for Kc sample, whereas K25bs showed higher peaks in the range 24-28 min. Accordingly, the histogram shown in Figure 4B revealed that K25bs showed larger compounds with higher hydrophobicity compared to Kc. Results suggest an intense proteolytic activity though the reproducibility in the kefir phosphopeptide when grains are reused is poor. Previous studies revealed that the microflora of kefir grains is a symbiotic equilibrium, but the species and quantitative ratios of the various microbial groups change significantly along the pathway kefir grains followed kefir and then second batch kefir (Simova et al., 2002). This, indeed, is the main reason for using commercial starter cultures in the dairy industry for kefir making.

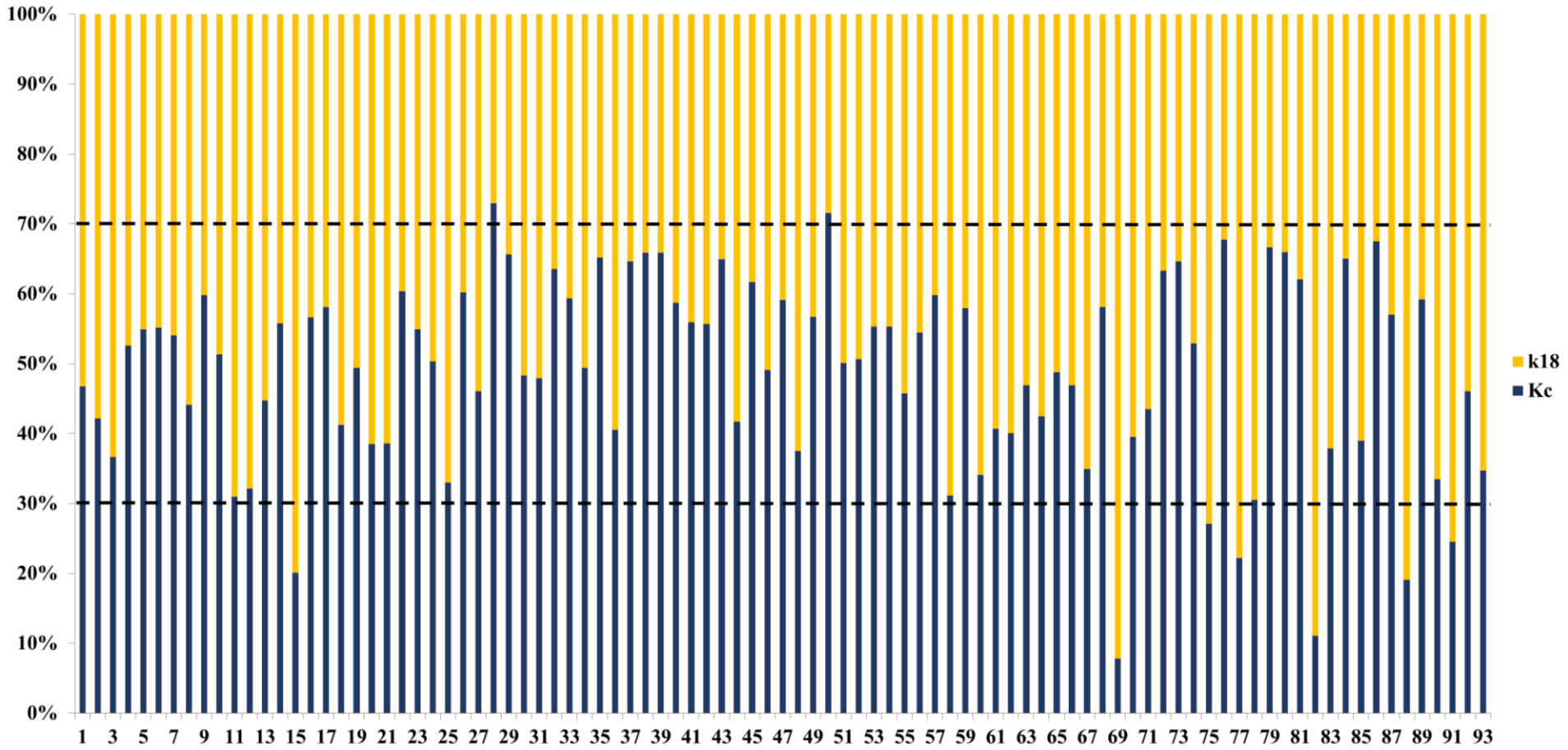



**Figure 2.** Comparison between phosphopeptides peak area of kefir control, at 25°C, pH 4.7 (Kc) and kefir sample at 18°C, pH 4.7 (K18). The peak areas reported for each phosphopeptide are mean values obtained from three replicated analyses.

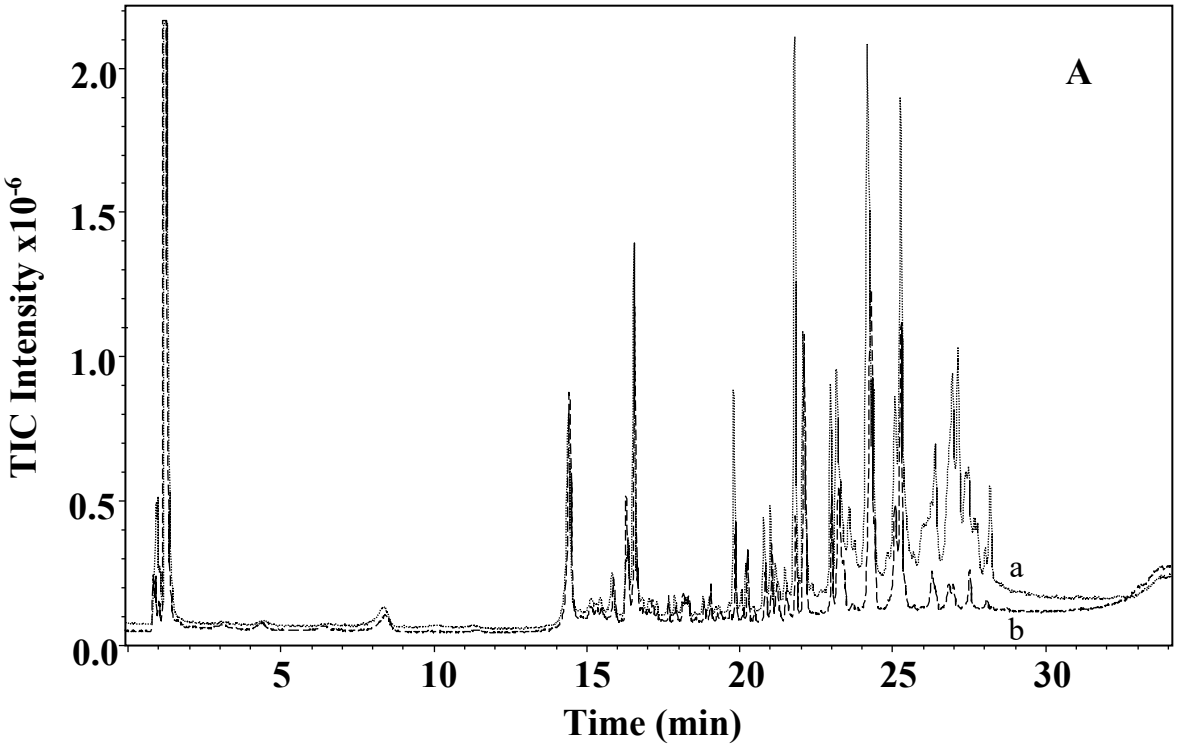
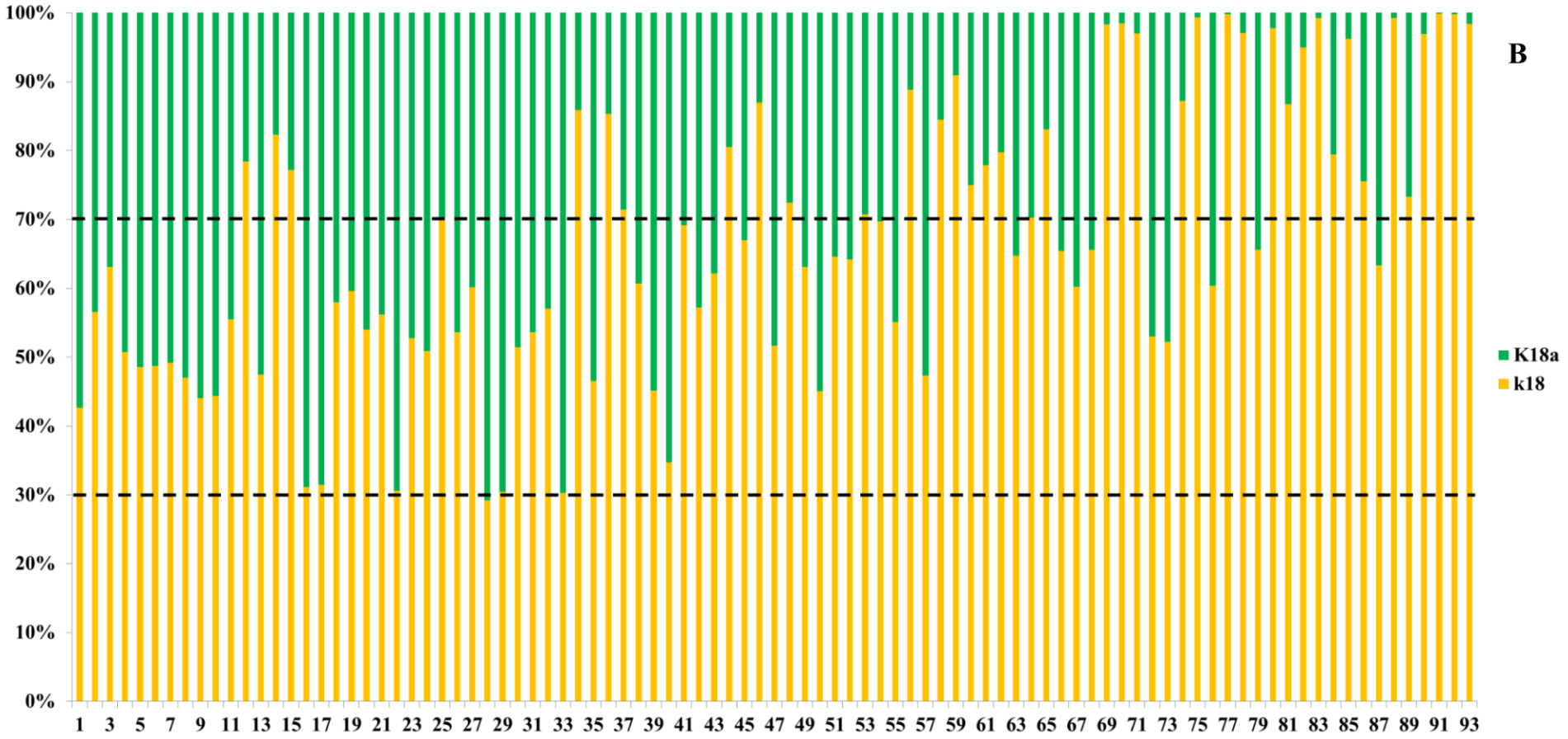



**Figure 3**. Evaluation of effect of the influence of pH on the phosphopeptides profile of two kefir treatment groups. A) Chromatographic traces referred to: a) kefir sample at 18°C, pH 4.7 (K18) and b) kefir sample at 18°C and pH 4.0 (K18a). B) Comparison between phosphopeptides peak area of two kefir samples reported above.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K18a
k18

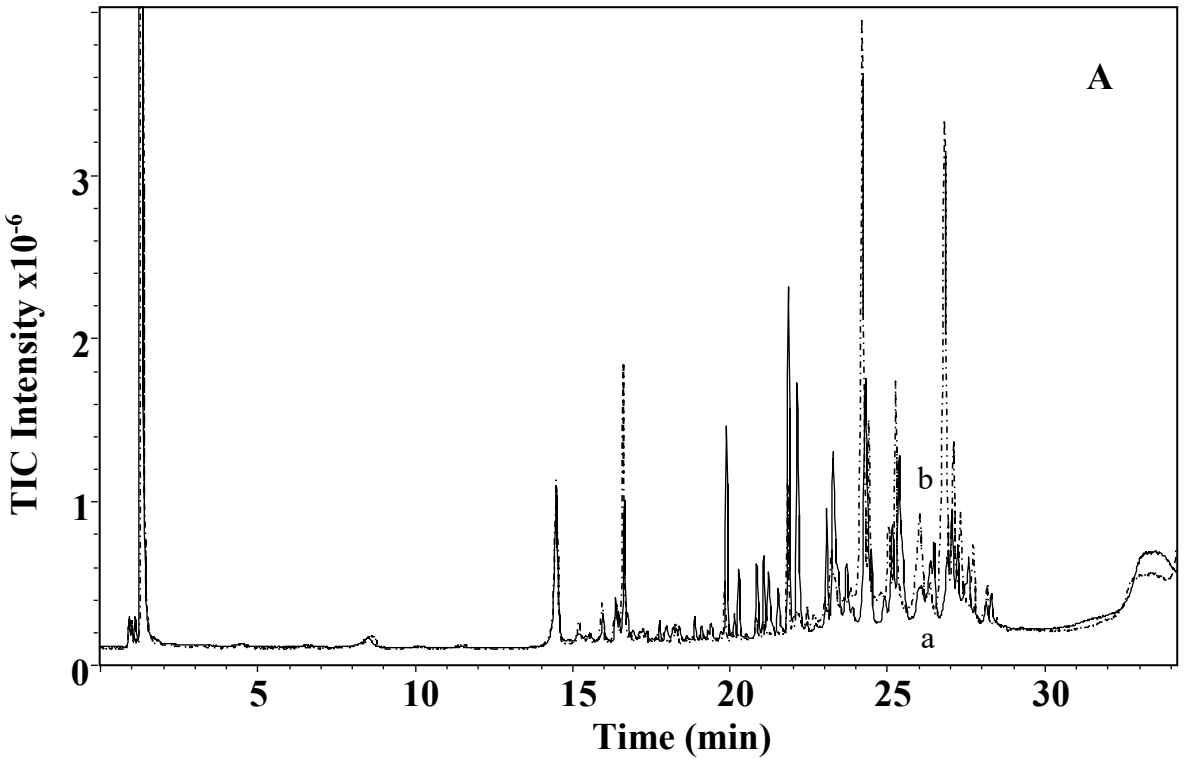
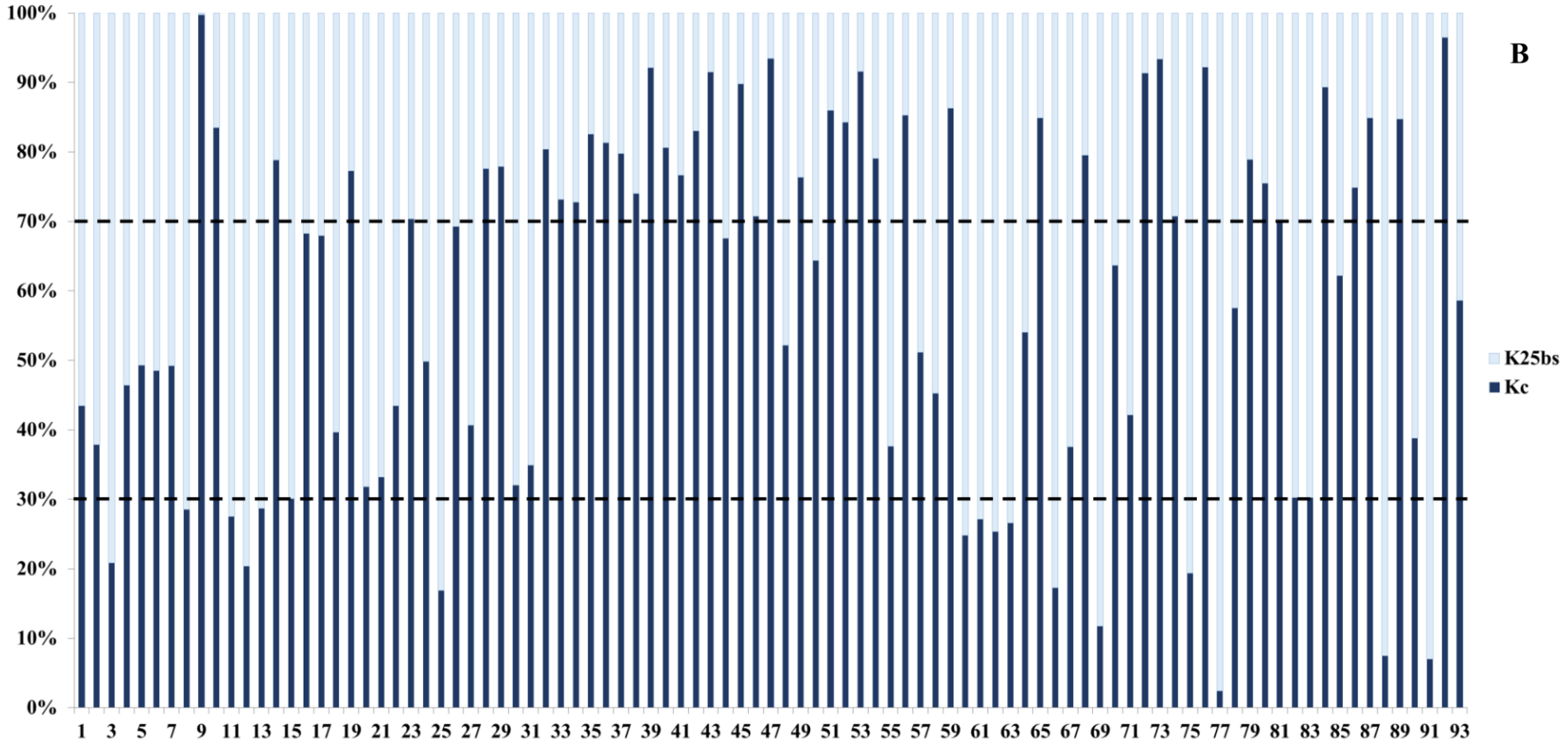



**Figure 4**. Evaluation of influence of the repeated use of grains on the phosphopeptides profile of two kefir treatment groups. A) Chromatographic traces referred to: a) kefir control, at 25°C, pH 4.7 (Kc) and b) kefir sample at 25°C, pH 4.7 and double fermentation (K25bs). B) Comparison between phosphopeptides peak area of two kefir samples reported above.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K25bs
Kc

*6.4.4 Sensory analysis*

The sensory evaluation was carried out on the kefir control (Kc), kefir fermented at 18°C until pH 4.7 (K18), kefir fermented 18°C until pH 4.0 (K18a) and kefir obtained using a back-slopping approach (K25sb). No statistically significant differences ($p > 0.05$) were observed in odor, taste and acidity between kefir products.

## 6.5 Conclusion

The present study revealed a kefir phosphopeptide profile that include 93 phosphopeptides of which 82 showed the typical acid motif "SSSEE", crucial for exhibiting the bioactive properties of mineral binding. Furthermore, kefir processing, in particular pH, largely influences the proteolysis of kefir grains microorganisms and the profile of microbial population. A reduced proteolysis rate was observed in kefir prepared with back-slopping approach, indicating a low reproducibility of proteolytic activity of kefir grains.

## Author contributions

This research work was carried out at "Sciences Agriculture, Food and Environment Department" of Foggia University and "Engineering Department" of Bari University.

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# CHAPTER 7

## MICROBIOLOGICAL EVALUATION AND LC-QTOF-MS/MS PEPTIDE PROFILING OF KEFIR OBTAINED BY SOY-DRINK

### 7.1 Abstract

Soy drink kefir is a product obtained by inoculation of kefir grains or selected cultures into soy drink and may be considered a good source of both probiotic microorganisms and bioactive peptides. To investigate the potential beneficial properties in soy drink kefir a comprehensive analysis of peptide profile was performed by LC-ESI-QTOF-MS/MS. Thus, 179 peptides were identified mainly derived from glycinin and lipoxygenase through microbial degradation by lactic acid bacteria present in kefir grains. Additionally, only lactic acid bacteria and yeasts, were found in soy drink kefir. The peptide identification was then used to look for the presence of bioactive peptides in the identified peptides. Results showed 62 bioactive sequences encrypted within the investigated peptides, which are known for ace-inhibitory, antihypertensive, antioxidative, hypocholesterolemic and antimicrobial activities.

### 7.2 Introduction

Soybean (*Glycine max* (L.) Merrill) is an ancient crop cultivated in China and other Asian countries, which is one of the most important sources of vegetable proteins in the human diet for East populations and recently also for people in the rest of world (Singh et al., 2014). Moreover, soy-based foods gained massive interest worldwide due to their beneficial effects on health (Singh et al., 2014; Sanjukta and Rai, 2016). Indeed, soybean and its derivatives contain important components for health benefits, such as proteins, essential fatty acids, oligosaccharides, isoflavones and fiber (Liu, 1997). Soy proteins possess a high nutritional value, similar to high-value animal proteins, and show excellent functional properties as source of bioactive peptides, which are known to exhibit immunomodulatory, antihypertensive, antioxidative, hypocholesterolemic, antiobesity and anticancer activities (Singh et al., 2014).

The major storage proteins are glycinin (11S globulin), which is composed of five major subunits (G1, G2, G3, G4 and G5), and β-conglycinin (7S globulin) composed of four subunits (α, α', β and γ); the sum of them constitutes 65-85% of the total soy proteins. Minor proteins include trypsin inhibitors, lectins, lipoxygenases, sucrose binding proteins, urease, which are recognized to be bioactive and allergenic, and other low abundance proteins (Herman et al., 2014). Moreover, lunasin is a peptide isolated from soybean cotyledon and has been reported to show anti-inflammatory, anticancer and antioxidant activities (Hernandez-Ledesma et al., 2009a; Hernandez-Ledesma et al., 2009b). Glycinin and β-conglycinin are the main precursors of the isolated bioactive peptides (Yang et al., 2000; Vasconcellos et al., 2014). Recently, Capriotti et al. (2015) characterized potential bioactive peptides in soybean and soy milk obtained by simulated in vitro gastrointestinal digestion.

Soybean can be utilized as unfermented, such as soybean, soy powder, soybean oil, soy aqueous extract (improperly called soy milk), and others, or as fermented products, such as miso, natto, soy cheese, soy yogurt, soy drink kefir and others. Fermentation is a good process to improve the digestibility of soybean proteins, by reducing proteinase and trypsin inhibitors, of indigestible oligosaccharides (raffinose and stachyose) and the beany flavor and taste (Egounlety and Aworh, 2003; LeBlanc et al., 2004). In addition, the microorganism proteolytic system during fermentation hydrolyzes the proteins into peptides and amino acids, increasing the bioactive peptides content in soy-fermented foods (Donkor et al., 2005; Tsai et al., 2006; Ibe et al., 2009; Singh and Vij, 2017).

Soy drink kefir is a product obtained by inoculation of kefir grains or selected cultures into soy drink (a soy aqueous extract) and represents an alternative to dairy fermented beverages or a substitute for vegans and people suffering from lactose intolerance or allergies. Grains are a symbiotic community of lactic acid and acetic bacteria (about 83-90%) and yeasts (about 10-17%) embedded with casein and complex sugars in a polysaccharide matrix (Prado et al., 2015; Baschali et al., 2017). Therefore, soy drink-kefir may be considered a good source of both probiotic microorganisms (Baú et al., 2014) and bioactive peptides (Kitawaki et al., 2009; Martinez-Villaluenga et al., 2012; Singh and Vij, 2017). The benefits derived from consuming of soy-drink kefir are numerous, including immunological, antitumoral, hycholesterolemic, antioxidant and antibacterial effects (Liu et al., 2005). Peptide profiling in food products can be efficiently achieved by liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS); however, to the best of our knowledge, a systematic LC-MS/MS study about peptides in soy-drink kefir and their potentially bioactivity has not been yet reported in the literature.

Therefore, the objective of this study was the characterization of microbiological profile and the structural elucidation of peptides in laboratory-made soy-drink kefir prepared with traditional kefir grains. The presence of potential bioactive peptides was also investigated.

## 7.3 Materials and Methods

### *7.3.1 Materials and reagents*

Formic acid (FA, LC-MS grade), sodium chloride (NaCl) and cycloheximide were obtained from Sigma-Aldrich (Milan, Italy). Acetonitrile (ACN, LC-MS grade) and water (LC-MS grade) were purchased from Merck (Vimodrone, Italy). Glucose was from Biolife (Milan, Italy) and calcium carbonate ($CaCO_3$) was obtained from Avantor (Arnhem, Netherlands). Plate Count Agar (PCA), Man, Rogosa and Sharpe broth (MRS), WL Nutrient Agar, Yeast extract and Agar were obtained from Oxoid (Milan, Italy).

Pasteurized UHT soy-drink, enriched with sugars, calcium and vitamins (B2, B12, D2, E), was acquired from a local supermarket. Kefir grains were purchased from Burumart Commerce S.L (Arrasate/Mondragón, Gipuzkoa, Spain) and consisted of *Lactobacillus Kefiranofaciens*, *Lactococcus lactis* ssp., and other lactic and acetic acid bacteria and yeasts as specified in the product.

### *7.3.2 Production of soy drink kefir*

Kefir grains were activated by inoculation of soy-drink with 18 g/L of grains and incubation at 25°C for 24 h, in accordance with the manufacturer information. Afterwards, grains were recovered from soy-drink with sterile sieve and added (8%, w/w) to 150 mL sample of soy-drink. Immediately after inoculation, sample was incubated at 25°C in a thermostatically controlled incubator. The desired final pH of soy-drink kefir was 4.0 (± 0.1) after approximately 24 h incubation. After fermentation, samples were left to mature at 4°C for 24 h before evaluation. During the incubation and maturation, kefir samples were kept open to allow the aerobic conditions typical for kefir production. The pH was periodically measured and sample manually agitated to favor the exchange of nutrients between grains and drink. Production of soy-drink kefir was performed in triplicate.

*7.3.3 Microbiological analyses*

Microbiological analyses were performed on activation soy-drink (S), activated kefir grains (G), kefir (K) and grains after fermentation (Gk). Initially, the samples of kefir and the grains used for fermentation were diluted 1:10 with a sterile saline solution (0.9% NaCl) and homogenized through a Sterilmixer (Pbi Intl., Milan, Italy), at 4000 rpm for 5 min prior to plating. Then, serial dilutions of homogenized samples were executed and plated onto selective media. Total count of viable mesophilic microorganisms was estimated on Plate Count Agar (PCA) by incubation at 30°C for 24 to 48 h. Lactic acid bacteria (LAB) counts were performed on MRS Agar, which was added with 0.17 g/L of cycloheximide to inhibit yeast growth, and incubated at 30°C under anaerobic conditions for 48 to 72 h. Acetic acid bacteria (AAB) count was carried out on GYC medium (Glucose 10%,Yeast extract 1%, $CaCO_3$ 1.5%, Agar 1.5%, w/v) in according to Gullo and Giudici (2008) with some modification, at 30°C for 24-48 h. Finally, yeasts count was performed on WL Nutrient Agar by incubation at 30°C for 24-48 h. All analyses were performed in triplicate.

*7.3.4 Sample preparation and peptide extraction*

Kefir sample was centrifuged at 3900 rpm, at 4 °C for 30 min, for the separation of proteins and other insoluble components. Then, supernatant was filtered through 0.22 µm sterile membrane filter (Roth, Karlsruhe, Germany) and the water soluble fraction was parceled into 10 mL aliquots. Afterwards, peptides extraction was performed, in duplicate for each sample, by solid-phase extraction (SPE) using Bond Elut C18 (500 mg, 3 mL) cartridges (Varian, Harbor City, USA) and a vacuum manifold (Varian), as reported by Ebner et al. (2015) with some modification. The cartridge was first conditioned with 2 mL of acetonitrile followed by 2 mL of 0.1% formic acid in ultrapure water. Then, 1 mL of the water-soluble kefir fraction was passed through the C18 cartridge. After a washing step with 2 mL of 0.1% formic acid, elution was carried out with 2 mL of acetonitrile/ 0.1% formic acid solution (60/40, v/v) and the extract was stored at -80°C until use.

Extracts were dried in a SpeedVac concentrator system (Thermo Scientific, Waltham, MA USA) and then were recovered with 1 mL of acetonitrile and 0.5% formic acid (60/40, v/v) solution. Finally, the samples were filtered using centrifugal filter units with molecular weight 10 kDa cutoff (Merck, Darmstadt, Germany) at 12000 rpm, 4°C for 90 min and stored at -80°C until use.

*7.3.5 LC-ESI-QTOF-MS/MS*

For peptide analysis by LC-ESI-QTOF-MS/MS consider the paragraph 6.4.2.

The amino acid sequence of the parent proteins is based on UniProtKB database entries: Glycinin G1 (P04775), G2 (P04405), G3 (P11828), G4 (P02858), glycinin (P04347), β-conglycinin with subunits β-(P25974), α'-(P11827) and α-(P13916), Trypsin inhibitor A (P01070), Lectin (P05046), seed Linoleate 13S-lipoxygenase-1 (P08170), 9S-lipoxygenase-2 (P09439), 9S-lipoxygenase-3 (P09186), 9S-lipoxygenase-4 (P38417), 9S-lipoxygenase (P24095), P24 oleosin isoform A-(P29530) and B-(P29531), Sucrose bonding protein (Q04672), Urease (P08298), Profilin 1-(O65809) and 2-(O65810). The MS/MS data were subjected to Mascot (version 2.6, Matrix Science, Boston, MA) and Protein Prospector Ms-Tag software (version 5.20, UCSF, San Francisco, CA) for peptide identification. Assignment of the peptides with the lowest scores (< 45 and 25 for Mascot and Protein Prospector, respectively) were verified by manual checking of product ion spectra. Peptides were also searched for their bioactivities in BIOPEP database (www.uwm.edu.pl/biochemia/index.php/pl/biopep; Minkiewicz et al., 2008) as well as available literature of soy peptides.

*7.3.6 Sensory evaluation*

Sensory evaluation was performed by 10 tasters familiar with kefir products, consisting of men and women between 25-45 years of age (students and staff of the SAFE Department, Foggia University, Italy). Samples were served at 10°C, in glasses coded with three digit random codes and placed in a random order, 24 h after kefir maturation step. The judges were asked to score the visual (appearance and consistency), olfactory (odor), gustatory (taste and acidity) characteristics, as well as the general acceptability of the product, on a scale of 0–5, according to increasing intensity.

*7.3.7 Statistical analysis*

Statistical analysis of microbiological and sensory data was conducted with Statistica software (version 7.0). Differences between the means were verified using one-way analysis of variance (ANOVA) followed by Tukey test for statistical comparison. The level of significance was preset at $p < 0.05$.

## 7.4. **Results and discussion**

### *7.4.1 Microbiological profile of soy-drink kefir*

The microbial population was evaluated in the activation soy-drink, activated grains, kefir grains at the end of fermentation and in soy-drink kefir. Plate count agar (PCA), lactic acid bacteria (LAB) and yeasts counts of kefir grains (G) and soy-drink (S), after 24 h activation time, are shown in Figure 1 A. The average pH of soy-drink was 6.9 (± 0.09) before inoculation with kefir grains, whereas the final pH after 24 h from inoculation, at 25°C, was 6.2 (± 0.05) as a result of the growth of grain microorganisms and their ability to produce lactic acid in soy-drink (Liu and Lin, 2000). Microbial composition was qualitatively and quantitatively similar in G and S. The predominant microorganisms after activation time were LAB, followed by yeasts; acetic acid bacteria (AAB) were not detected on plates in both G and S. The concentration of LAB and yeasts in S and G were about 6 Log CFU/g and about 5 Log CFU/g respectively (Figure 1 A); some authors reported higher values of lactic acid bacteria (Liu and Lin, 2000; Donkor et al., 2007; Farnworth et al., 2007; Baú et al., 2014), but our data were in agreement with the results of Silva Fernandez et al., (2017) and Singh and Vji (2018). Yeast counts in G and S, at time 0 h, were in agreement with Liu and Lin (2000).

Figure 1 B shows the microbial counts of soy-drink kefir (K) and kefir grains after fermentation (Gk). LAB and yeasts were at 7 Log CFU/g and 6 Log CFU/g, respectively. LAB levels were significantly lower than those reported in previous studies (Liu and Lin, 2000; Baú et al., 2014; Abdolmaleki et al., 2015; da Silva Fernandez et al., 2017; Singh and Vji, 2018), whereas yeasts counts were similar to the results reported by Liu and Lin (2000) and higher to the ones described in Abdolmaleki et al. (2015). Since the soy-drink utilized in this study was enriched with sugars and vitamins, this may have influenced the growth of kefir grain microorganisms. Liu and Lin (2000) have shown that the addition of carbohydrate (e. g. 1% glucose, lactose or sucrose) to soy-drink increased yeast count and not favored LAB, compared to kefir produced from soy-drink without additions. Indeed, some LAB possess the α-galactosidase enzyme to utilize low molecular weight oligosaccharides, such as sucrose, raffinose and stachyose for their growth, but its synthesis in *Lactobacillus fermentum* was inhibited by the supplied glucose (Garro et al., 1996). Additionally, the low pH environment and release of organic acids could have inhibited or slowed the growth of LAB during fermentation of soy-drink. At the end of fermentation, the pH drop, due to the accumulation of organic acids produced by LAB and yeasts, caused soy-drink kefir coagulation, which exhibited a yoghurt-like consistency.

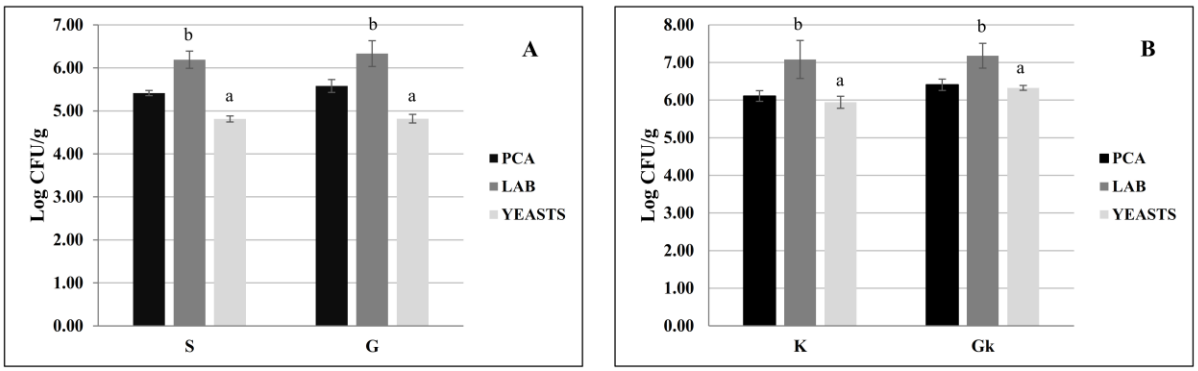


**Figure 1**. Plate count agar (PCA), lactic acid bacteria (LAB) and yeasts in [A] activation soy milk (S) and activated grains (G), and in [B] soy milk kefir (K) and kefir grains separated from kefir product (Gk). Values are means ± standard deviation of Log CFU/g of three independent productions and each measured in duplicate.

### *7.4.2 Peptide profile of soy-drink kefir*

The characterization of peptide profile of soy-drink kefir was performed by LC-ESI-QTOF-MS/MS analysis and the results are showed in Table 1. To elucidate their structure, MS/MS spectra analysis was conducted in combination with Mascot and Protein Prospector software search based on UniProt database. One hundred and seventy-nine peptides sequences were identified in kefir. In particular, six peptides were identified with Mascot software with a score greater than 45; the remaining peptide sequences were assigned with Protein Prospector (Ms-Tag) search. Therefore, Protein Prospector software demonstrated to be the most effective to identify peptide sequences randomly generated by kefir microflora; eleven of them were considered putatively identified as the score was minor than 25, although several MS/MS fragments corresponded to the fragments of the proposed structure by a manually check.

The sequences of the identified peptides (Table 1) ranged from 9 to 37 amino acid with a mass range between 1066 and 4093 Da, in agreement with a previous study (Singh and Vij, 2017). Singh and Vij (2017) revealed 35 bioactive peptides between 10 and 40 amino acids in soy-drink fermented with *Lactobacillus plantarum* strain C2. However, in the present study, only three peptide sequences reported by Singh and Vij (2017) were encrypted within peptides identified in soy-drink kefir (compounds 172, 178 and 179), likely due to the complex microbiological composition of the kefir grains compared to the single strain utilized in the authors work.

**Table 1.** Peptide sequences identified in soy-drink kefir by LC-ESI-QTOF-MS/MS followed by search in UniProtKB. Cleavage sites are indicated by dots and peptides masses are given in Da. Peaks related to potential bioactive peptides are in bold.

| Peaks | RT | Observed | Charge of precursor ion | Ms (expt) | Ms (calc) | Peptides[a] | Sequence[b] | | Score[c] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4.4 | 780.3754 | 2 | 1559.7434 | 1559.7548 | Glycinin $G1_{224-238}$ | N.LQGENEGEDKGAIVT.V | | 60.1 |
| 2 | 4.6 | 638.7994 | 2 | 1276.5916 | 1276.6029 | β-conglycinin-β $chain_{338-348}$<br>β-conglycinin-α'$chain_{539-549}$ | A.FGINAENNQRN.F | | 49.8 |
| **3** | 5.5 | 662.6681 | 3 | 1985.9898 | 1986.0138 | Glycinin $G1_{220-238}$ | Q.IAKNLQGENEGEDKGAIVT.V | | 53.1 |
| 4 | 5.9 | 596.2783 | 2 | 1191.5494 | 1191.5640 | $Glycinin_{165-175}$ | Y.LAGNPDIEHPE.T | | 39 |
| 5 | 6.9 | 579.3462 | 2 | 1157.6778 | 1157.6928 | Glycinin $G2_{231-242}$ | D.SGAIVTVKGGLR.V | | 77* |
| 6 | 7.2 | 646.8009 | 2 | 1292.5945 | 1292.6117 | $Glycinin_{165-176}$ | Y.LAGNPDIEHPET.M | | 40.4 |
| 7 | 13.5 | 679.3323 | 2 | 1357.6572 | 1357.6747 | Glycinin $G4_{248-258}$ | S.VISPKWQEQQD.E | | 30.3 |
| 8 | 14.1 | 778.8475 | 3 | 2334.528 | 2334.2915 | Linoleate 9S-lipoxygenase-$4_{46-68}$ | A.VDALTFAATKISIQLISATKADG.G | | 32.5 |
| **9** | 14.2 | 701.3927 | 4 | 2802.5491 | 2802.3938 | Glycinin $G2_{216-241}$ | N.MQIVRNLQGENEEEDSGAIVTVKGGL.R | $M_{216}$(O) | 42 |
| 10 | 14.2 | 881.1379 | 4 | 3521.5298 | 3521.8288 | $Lectin_{120-150}$ | Q.VVAVEFDTFRNSWDPPNPHIGINVNSIRSIK.T | | 41.7 |
| **11** | 14.2 | 705.3532 | 3 | 2114.0451 | 2113.9421 | Glycinin $G4_{195-231}$ | Q.HQQEEEEEGGSVLSGFSKH.F | | 21 |
| **12** | 14.6 | 679.8227 | 3 | 2037.4535 | 2036.9825 | β-conglycinin-β $chain_{93-110}$ | S.YNLHPGDAQRIPAGTTYY.L | | 26.6 |
| 13 | 14.6 | 622.3087 | 3 | 1864.9115 | 1865.003 | $Urease_{63-78}$ | L.VLWKPSFFGAKPEMVI.K | $M_{76}$(O) | 31.4 |
| 14 | 14.7 | 602.7906 | 2 | 1204.5738 | 1204.5957 | Glycinin $G4_{202-213}$<br>$Glycinin_{200-211}$ | E.EGGSVLSGFSKH.F | | 19.4 |
| 15 | 14.8 | 663.3249 | 3 | 1987.9602 | 1987.9931 | Glycinin $G1_{225-244}$ | L.QGENEGEDKGAIVTVKGGLS.V | | 80.2 |
| **16** | 15 | 600.8134 | 2 | 1200.6194 | 1200.6735 | Glycinin $G3_{447-456}$ | S.FLVPPKESQR.R | | 44 |
| 17 | 15 | 586.3022 | 3 | 1756.892 | 1756.8647 | $Urease_{9-24}$ | T.ADKMKSQRGPLQPGED.N | | 30.4 |
| 18 | 15.1 | 765.8977 | 2 | 1530.7808 | 1530.8049 | Glycinin $G2_{228-242}$ | E.EEDSGAIVTVKGGLR.V | | 103* |
| 19 | 15.2 | 504.9309 | 3 | 1512.7781 | 1512.8744 | Seed linoleate 9S-lipoxygenase-$2_{39-54}$ | D.LTKGNVGGLIGTGLNV.V | | 37.3 |
| 20 | 15.2 | 639.8276 | 2 | 1278.6479 | 1278.6688 | β-conglycinin-α'$chain_{516-526}$ | N.FLAGSKDNVISQ.I | | 27.7 |
| 21 | 15.3 | 636.3124 | 2 | 1271.6176 | 1271.6339 | Glycinin $G2_{410-422}$<br>Glycinin $G3_{403-415}$ | N.DRPSIGNLAGANS.L | | 29.6 |
| **22** | 15.4 | 596.5391 | 4 | 2383.1346 | 2383.2827 | $Lectin_{24-46}$ | D.AIVTSSGKLQLNKVDENGTPKPS.S | | 36.7 |
| **23** | 15.5 | 650.6536 | 3 | 1949.9464 | 1950.0596 | Glycinin $G1_{330-346}$ | D.FPALSWLRLSAEFGSLR.K | | 23.3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 24 | 15.5 | 1032.5144 | 3 | 3095.5286 | 3095.7762 | Profilin-1$_{71-110}$<br>Profilin-2$_{71-110}$ | K.YMVIQGEPGAVIRGKKGPGGVTVKKTGAALI.I | | 54.4 |
| 25 | 15.5 | 728.683 | 3 | 2184.0344 | 2184.1192 | Urease$_{91-110}$ | D.PNASIPTPEPVIMRPMFGTL.G | M$_{103}$(O) | 42.9 |
| **26** | 15.6 | 553.9454 | 3 | 1659.8145 | 1659.8475 | Glycinin G2$_{227-242}$ | N.EEEDSGAIVTVKGGLR.V | | 122* |
| 27 | 15.7 | 547.9446 | 3 | 1641.8192 | 1641.7768 | β-conglycinin-α' chain$_{535-548}$ | L.NFFAFGINAENNQR.N | | 23.9 |
| **28** | 15.9 | 653.9827 | 3 | 1959.9264 | 1959.9545 | Glycinin G2$_{224-242}$ | Q.GENEEEDSGAIVTVKGGLR.V | | 54 |
| **29** | 15.9 | 843.1046 | 3 | 2527.2993 | 2527.3362 | Glycinin G1$_{220-244}$ | Q.IAKNLQGENEGEDKGAIVTVKGGLS.V | | 117.2 |
| 30 | 16 | 667.3153 | 2 | 1333.6233 | 1333.6383 | Glycinin G4$_{201-213}$<br>Glycinin$_{199-211}$ | E.EEGGSVLSGFSKH.F | | 53.4 |
| 31 | 16.1 | 722.8495 | 2 | 1444.6917 | 1444.7642 | Seed linoleate 9S-lipoxygenase$_{38-53}$ | I.DTATGILGQGVSLVGG.V | | 31.9 |
| 32 | 16.2 | 1051.0293 | 2 | 2101.0514 | 2101.0772 | Glycinin G1$_{224-244}$ | N.LQGENEGEDKGAIVTVKGGLS.V | | 93 |
| **33** | 16.2 | 549.7953 | 2 | 1098.5833 | 1098.6041 | Glycinin G1$_{243-252}$ | G.LSVIKPPTDE.Q | | 24.6 |
| 34 | 16.3 | 587.9593 | 3 | 1761.8634 | 1761.8878 | Glycinin G2$_{406-422}$<br>Glycinin G3$_{399-415}$ | S.FKTNDRPSIGNLAGANS.L | | 53.5 |
| 35 | 16.4 | 693.8633 | 2 | 1386.7193 | 1386.7111 | β-conglycinin-α chain$_{445-457}$<br>β-conglycinin-β chain$_{278-290}$ | N.EGDANIELVGLKE.Q/N.EGDANIELVGIKE.Q | | 32.6 |
| **36** | 16.4 | 885.7899 | 3 | 2655.3550 | 2655.4149 | Urease$_{102-126}$ | V.IMRPMFGTLGKAGSSLSIAFVSKAW.L | | 48.8 |
| **37** | 16.5 | 723.3412 | 2 | 1445.6752 | 1445.7482 | Glycinin G2$_{142-154}$<br>Glycinin G1$_{144-156}$ | A.VSIIDTNSLENQL.D | | 27.7 |
| **38** | 16.5 | 793.0367 | 3 | 2377.0954 | 2377.2432 | Seed linoleate 9S-lipoxygenase$_{18-40}$ | L.MPKNVLDFNAITSIGKGGVIDTA.T | M$_{18}$(O) | 32.2 |
| 39 | 16.6 | 729.3903 | 3 | 2186.1562 | 2186.1816 | Seed linoleate 9S-lipoxygenase-2$_{472-493}$ | L.KPVAIELSLPHPAGDLSGAVSQ.V | | 28.1 |
| **40** | 16.8 | 704.3229 | 3 | 2110.9542 | 2111.207 | Seed linoleate 9S-lipoxygenase$_{33-55}$ | G.KGGVIDTATGILGQGVSLVGGVI.D | | 31.6 |
| 41 | 16.8 | 537.2785 | 2 | 1073.5497 | 1073.5626 | Glycinin G4$_{247-255}$<br>Glycinin$_{245-253}$ | L.SVISPKWQE.Q | | 27.8 |
| **42** | 16.9 | 531.2405 | 3 | 1591.707 | 1591.7235 | Glycinin G4$_{199-213}$ | E.EEEEGGSVLSGFSKH.F | | 73.8 |
| **43** | 16.9 | 895.1261 | 3 | 2683.3637 | 2683.4261 | Glycinin G1$_{216-240}$ | S.VDKQIAKNLQGENEGEDKGAIVTVK.G | | 41.9 |
| 44 | 17 | 614.3236 | 2 | 1227.6399 | 1227.6579 | β-conglycinin-α' chain$_{558-568}$<br>β-conglycinin-α chain$_{524-534}$ | N.VISQIPSQVQE.L | | 31.6 |
| 45 | 17 | 700.3612 | 3 | 2099.0692 | 2099.1707 | Lectin$_{19-38}$ | M.ILQGDAIVTSSGKLQLNKVD.E | | 31.4 |
| **46** | 17.1 | 671.5967 | 4 | 2683.3649 | 2683.4009 | Glycinin G2$_{218-242}$ | Q.IVRNLQGENEEEDSGAIVTVKGGLR.V | | 106.2 |
| 47 | 17.1 | 858.3882 | 2 | 1715.7692 | 1715.7615 | Urease$_{75-90}$ | P.EMVIKGGEVAYANMGD.P | M$_{76}$(O) M$_{88}$(O) | 36 |
| 48 | 17.4 | 609.323 | 2 | 1217.6387 | 1217.6525 | β-conglycinin-α chain$_{535-546}$ | E.LAFPGSAQAVEK.L | | 40.4 |
| 49 | 17.4 | 584.2814 | 3 | 1750.8297 | 1750.8507 | Glycinin G3$_{379-394}$ | I.VPQNFAVAARSQSDNF.E | | 23.7 |

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|---|---|---|---|---|---|---|---|---|---|
| 50 | 17.5 | 770.3539 | 3 | 2309.0471 | 2309.2475 | Seed linoleate 9S-lipoxygenase-2$_{352-371}$ | E.QVLKFPPPHVIQVSKSAWMT.D | $M_{370}$(O) | 28.3 |
| 51 | 17.6 | 780.7037 | 3 | 2340.0966 | 2340.2592 | Lectin$_{12-35}$ | F.VPKQPNMILQGDAIVTSSGKLQ.L | $M_{18}$(O) | 35.5 |
| 52 | 17.8 | 645.3188 | | 1289.6231 | 1289.6412 | β-conglycinin-β chain$_{368-379}$ | E.LAFPGSAQDVER.L | | 51* |
| 53 | 17.8 | 671.3566 | 3 | 2012.0552 | 2012.1063 | β-conglycinin-β chain$_{228-246}$ | N.HADADYLIVILNGTAILSL.V | | 27.7 |
| 54 | 17.9 | 794.3661 | 3 | 2381.0839 | 2381.2071 | Seed linoleate 9S-lipoxygenase$_{678-697}$ | E.AVEKGHGDLKEKPWWPKMQT.T | $M_{695}$(O) | 33.5 |
| **55** | 18 | 691.0186 | 3 | 2071.0411 | 2070.9801 | β-conglycinin-α' chain$_{291-315}$ | N.LRMIAGTTFYVVNPDNDE.N | $M_{293}$(O) | 29.9 |
| 56 | 18.2 | 659.8074 | 2 | 1318.6074 | 1318.6712 | Profilin-1$_{62-73}$ Profilin-2$_{62-73}$ | P.TGLYLGGTKYMV.I | $M_{72}$(O) | 29.4 |
| 57 | 18.4 | 574.2961 | 2 | 1147.5849 | 1147.5014 | Seed linoleate 9S-lipoxygenase$_{479-489}$ | K.PHPDGDNLGPE.S | | 29.3 |
| 58 | 18.8 | 635.8314 | 2 | 1270.6554 | 1270.7841 | Glycinin G2$_{232-244}$ | S.GAIVTVKGGLRVT.A | | 22.8 |
| **59** | 18.9 | 921.1295 | 3 | 2761.3739 | 2761.2351 | Glycinin G3$_{111-133}$ | Y.HFREGDLIAVPTGFAYWMYNNED.T | $M_{28}$(O) | 43.1 |
| 60 | 19 | 793.8454 | 3 | 2379.5218 | 2379.1173 | Glycinin G1$_{410-431}$ | N.FEYVSFKTNDTPMIGTLAGANS.L | $M_{152}$(O) | 38.6 |
| **61** | 19 | 863.9079 | 2 | 1726.8012 | 1726.8210 | Glycinin G4$_{214-238}$ | H.FLAQSFNTNEDIAEK.L | | 26.8 |
| 62 | 19.1 | 625.6458 | 3 | 1874.9227 | 1874.9719 | Glycinin G2$_{406-423}$ Glycinin G3$_{399-416}$ | S.FKTNDRPSIGNLAGANSL.L | | 44.6 |
| 63 | 19.1 | 938.4589 | 2 | 1875.9032 | 1875.9122 | Glycinin G4$_{147-162}$ | S.LLDTSNFNNQLDQTPR.V | | 106* |
| **64** | 19.2 | 852.4466 | 4 | 3406.7647 | 3406.7886 | Seed linoleate 9S-lipoxygenase$_{36-69}$ | G.VIDTATGILGQGVSLVGGVIDTATSFLGRNISMQ.L | $M_{68}$(O) | 53 |
| 65 | 19.3 | 864.9169 | 2 | 1728.8265 | 1728.9167 | Glycinin G4$_{240-255}$ Glycinin$_{238-253}$ | I.VTVEGGLSVISPKWQE.Q | | 28.8 |
| **66** | 19.4 | 1021.2237 | 3 | 3061.6566 | 3061.6892 | Glycinin G1$_{220-249}$ | Q.IAKNLQGENEGEDKGAIVTVKGGLSVIKPP.T | | 30.9 |
| **67** | 19.4 | 608.2884 | 4 | 2430.1319 | 2430.2769 | Linoleate 9S-lipoxygenase-4$_{547-568}$ | Y.RDTMNINSLARKALVNADGIIE.K | $M_{550}$(O) | 34.1 |
| 68 | 19.6 | 548.2639 | 3 | 1642.7771 | 1642.8759 | β-conglycinin-β chain$_{180-193}$ | E.QRQQEGVIVELSKE.Q | | 40.8 |
| **69** | 19.9 | 557.8311 | 2 | 1114.6475 | 1114.6580 | Glycinin G4$_{68-78}$ | R.MIIIAQGKGAL.G | | 35.4 |
| **70** | 20 | 847.4342 | 3 | 2540.2879 | 2540.3694 | Urease$_{104-127}$ | M.RPMFGTLGKAGSSLSIAFVSKAWL.N | $M_{106}$(O) | 39.9 |
| 71 | 20 | 735.949 | 2 | 1470.8908 | 1470.9043 | Trypsin inhibitor A$_{161-173}$ | R.LVVSKNKPLVVQF.Q | | 28.4 |
| 72 | 20.2 | 533.7635 | 2 | 1066.5197 | 1066.5164 | Glycinin G2$_{392-401}$ | A.VAAKSQSDNF.E | | 29.1 |
| 73 | 20.2 | 693.3462 | 2 | 1385.6851 | 1385.7635 | Seed linoleate 9S-lipoxygenase$_{44-58}$ | I.LGQGVSLVGGVIDTA.T | | 25.8 |
| 74 | 20.3 | 747.3976 | 3 | 2240.1784 | 2240.1921 | β-conglycinin-α chain$_{527-547}$ | S.QIPSQVQELAFPGSAQAVEKL.L | | 27.4 |
| 75 | 20.2 | 1030.5275 | 2 | 2060.0476 | 2060.0295 | β-conglycinin-α chain$_{516-534}$ | N.FLAGSQDNVISQIPSQVQE.L | | 55.7 |

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|---|---|---|---|---|---|---|---|---|---|
| 76 | 20.4 | 650.8414 | 2 | 1300.6756 | 1300.7219 | Seed linoleate 9S-lipoxygenase-2$_{40-52}$ | L.TKGNVGGLIGTGLN.V | | 26.9 |
| 77 | 20.5 | 526.2718 | 2 | 1051.5364 | 1051.5459 | Glycinin G2$_{203-211}$ | G.FAPEFLKEA.F | | 24.6 |
| 78 | 20.6 | 740.8471 | 3 | 2220.5267 | 2220.1482 | Seed linoleate 9S-lipoxygenase-3$_{541-559}$ | Y.KDWVFTDQALPADLIKRGM.A | M$_{559}$(O) | 38 |
| 79 | 20.6 | 852.4 | 2 | 1703.7928 | 1703.8195 | β-conglycinin-α chain$_{339-354}$ | L.NGTAILSLVNNDDRDS.Y | | 24.1 |
| 80 | 20.6 | 627.6881 | 3 | 1881.0498 | 1880.9245 | Urease$_{71-88}$ | F.GAKPEMVIKGGEVAYANM.G | M$_{88}$(O) | 52.1 |
| 81 | 20.6 | 834.973 | 2 | 1668.9388 | 1668.9643 | Seed linoleate 9S-lipoxygenase-2$_{39-56}$ | D.LTKGNVGGLIGTGLNVVG.S | | 37.5 |
| 82 | 20.6 | 941.0285 | 2 | 1881.0498 | 1881.0692 | Glycinin G3$_{228-246}$ | E.EEKGAIVTVKGGLSVISPP.T | | 33.2 |
| **83** | 20.7 | 707.0119 | 3 | 2119.0212 | 2119.1506 | Trypsin inhibitor A$_{46-64}$ | Q.SRNELDKGIGTIISSPYRI.R | | 47 |
| **84** | 20.7 | 671.3683 | 3 | 2012.0903 | 2012.1797 | Glycinin G2$_{231-250}$ | D.SGAIVTVKGGLRVTAPAMRK.P | | 46.5 |
| 85 | 20.8 | 987.9869 | 2 | 1974.9666 | 1974.9879 | Glycinin G4$_{147-163}$ | S.LLDTSNFNNQLDQTPRV.F | | 39.7 |
| 86 | 20.9 | 949.4604 | 2 | 1897.9063 | 1897.9217 | Glycinin G1$_{26-42}$ | R.IESEGGLIETWNPNNKP.F | | 31.1 |
| **87** | 21 | 706.9643 | 3 | 2118.8782 | 2119.1104 | Profilin-1$_{94-113}$ Profilin-2$_{94-113}$ | V.KKTGAALIIGIYDEPMTPGQ.C | M$_{109}$(O) | 35.2 |
| **88** | 21 | 580.2628 | 3 | 1738.7738 | 1738.7919 | Glycinin G4$_{199-214}$ | E.EEEEGGSVLSGFSKHF.L | | 60.1 |
| **89** | 21.1 | 1050.1835 | 3 | 3148.5358 | 3148.4853 | Profilin-1$_{44-72}$ Profilin-2$_{44-72}$ | P.EEITAIMNDFNEPGSLAPTGLYLGGTKYM.V | M$_{72}$(O) | 33.2 |
| 90 | 21.1 | 1029.985 | 2 | 2058.9626 | 2059.1618 | Linoleate 9S-lipoxygenase-4$_{61-81}$ | L.ISATKADGGKGKIGKSTNLRG.K | | 36.5 |
| 91 | 21.2 | 597.3044 | 2 | 1193.6015 | 1193.5983 | Glycinin G2$_{72-82}$ | Y.IQQGNGIFGMI.F | M$_{80}$(O) | 20.6 |
| **92** | 21.2 | 837.3882 | 4 | 3346.5309 | 3346.6226 | Glycinin G3$_{189-218}$ | Q.QEEENEGGSILSGFAPEFLEHAFVVDRQIV.R | | 43.5 |
| 93 | 21.3 | 670.8684 | 2 | 1340.7295 | 1340.7169 | Glycinin G2$_{412-425}$ Glycinin G3$_{405-418}$ | R.PSIGNLAGANSLLN.A | | 43.2 |
| 94 | 21.4 | 552.3074 | 2 | 1103.6002 | 1103.6135 | Seed linoleate 13S-lipoxygenase-1$_{830-839}$ 9S-lipoxygenase-3$_{848-857}$ 9S-lipoxygenase-4$_{844-853}$ | T.FRGIPNSISI | | 35 |
| 95 | 21.5 | 663.3491 | 3 | 1988.0328 | 1988.056 | Glycinin G2$_{406-424}$ Glycinin G3$_{399-417}$ | S.FKTNDRPSIGNLAGANSLL.N | | 37.5 |
| **96** | 21.6 | 805.4007 | 4 | 3218.5811 | 3218.6844 | Seed linoleate 9S-lipoxygenase-3$_{258-286}$ | Y.LPRDEAFGHLKSSDFLTYGLKSVSQNVLP.L | | 46.8 |
| 97 | 21.6 | 918.7023 | 3 | 2754.0924 | 2754.4785 | Seed linoleate 13S-lipoxygenase-1$_{63-93}$ | T.KADAHGKGKVGKDTFLEGINTSLPTLG.A | | 46.4 |
| **98** | 21.7 | 1223.2631 | 3 | 3667.7749 | 3667.8344 | Glycinin G2$_{221-254}$ | R.NLQGENEEEDSGAIVTVKGGLRVTAPAMRKPQQE.E | M$_{248}$(O) | 27.9 |
| 99 | 21.8 | 687.3426 | 3 | 2060.0132 | 2060.0771 | Glycinin G2$_{415-434}$ | I.GNLAGANSLLNALPEEVIQH.T | | 39.4 |

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|---|---|---|---|---|---|---|---|---|---|
| 100 | 21.8 | 800.4348 | 3 | 2399.2898 | 2399.3948 | P24 oleosin isoform A$_{92-116}$ | F.VLFSPVLVPATVAIGLAVAGFLTSG.A | | 42.4 |
| 101 | 21.9 | 609.2955 | 2 | 1217.5838 | 1217.5949 | β-conglycinin-α chain$_{174-183}$ | P.FLFGSNRFET.L | | 40.5 |
| **102** | 22 | 776.3641 | 2 | 1551.721 | 1551.9356 | Seed linoleate 9S-lipoxygenase-3$_{320-333}$ | T.DIISKISPLPVLKE.I | | 29.9 |
| 103 | 22 | 1134.0348 | 2 | 2267.0624 | 2267.1085 | Seed linoleate 9S-lipoxygenase$_{61-82}$ | S.FLGRNISMQLISATQTDGSGNG.K | | 37.5 |
| **104** | 22 | 756.359 | 3 | 2267.0624 | 2267.3373 | Seed linoleate 9S-lipoxygenase-3$_{318-337}$ | L.PTDIISKISPLPVLKEIFRT.D | | 34.2 |
| 105 | 22 | 965.9362 | 2 | 1930.8652 | 1931.1001 | Seed linoleate 9S-lipoxygenase$_{278-294}$ | F.LTYGIKSLSHDVIPLFK.S | | 28.1 |
| **106** | 22.1 | 673.3062 | 3 | 2017.9039 | 2018.009 | Profilin-1$_{14-32}$ Profilin-2$_{14-34}$ | D.IEGNHLTHAAIIGQDGSVW.A | | 43.9 |
| 107 | 22.2 | 687.8611 | 2 | 1374.715 | 1374.7297 | Glycinin G1$_{420-433}$ | D.TPMIGTLAGANSLL.N | M$_{422}$(O) | 34.9 |
| 108 | 22.2 | 725.3506 | 3 | 2174.0372 | 2174.007 | Seed linoleate 13S-lipoxygenase-1$_{715-732}$ | L.LPEKGTPEYEEMINNHEK.A | M$_{727}$(O) | 28.9 |
| 109 | 22.4 | 969.1274 | 3 | 2905.3676 | 2905.5279 | Glycinin$_{406-432}$ | C.QGNAVFDGELRRGQLLVVPQNPAVAEQ.G | | 32.8 |
| **110** | 22.5 | 1260.2856 | 3 | 3778.8423 | 3778.9868 | Glycinin G2$_{217-251}$ | M.QIVRNLQGENEEEDSGAIVTVKGGLRVTAPAMRKP.Q | M$_{248}$(O) | 23.8 |
| 111 | 22.5 | 709.876 | 2 | 1418.7447 | 1418.7573 | Glycinin G2$_{212-223}$ | A.FGVNMQIVRNLQ.G | | 27.9 |
| 112 | 22.6 | 924.451 | 2 | 1847.8948 | 1847.9069 | Glycinin G2$_{212-227}$ | A.FGVNMQIVRNLQGENE.E | | 34 |
| **113** | 22.6 | 859.9308 | 2 | 1718.8543 | 1718.9323 | Seed linoleate 9S-lipoxygenase$_{22-38}$ | N.VLDFNAITSIGKGGVID.T | | 41 |
| 114 | 22.6 | 749.3979 | 2 | 1497.7885 | 1497.802 | Glycinin G2$_{410-424}$ Glycinin G3$_{403-417}$ | N.DRPSIGNLAGANSLL.N | | 56.6 |
| 115 | 22.7 | 752.3669 | 3 | 2255.0861 | 2255.2605 | Seed linoleate 9S-lipoxygenase-2$_{36-59}$ | S.VADLTKGNVGGLIGTGLNVVGSTL.D | | 32.3 |
| **116** | 22.8 | 1030.8351 | 3 | 3090.4909 | 3090.5161 | Glycinin G2$_{212-240}$ | A.FGVNMQIVRNLQGENEEEDSGAIVTVKGG.L | | 60.1 |
| 117 | 22.9 | 737.3796 | 4 | 2946.4965 | 2946.5419 | Seed linoleate 9S-lipoxygenase-2$_{31-60}$ | V.LDFNSVADLTKGNVGGLIGTGLNVVGSTLD.N | | 55.8 |
| 118 | 23 | 1230.9335 | 3 | 3690.786 | 3690.8328 | Urease$_{86-121}$ | Y.ANMGDPNASIPTPEPVIMRPMFGTLGKAGSSLSIAF.V | M$_{106}$(O) | 40 |
| 119 | 23.2 | 1361.672 | 2 | 2722.3368 | 2722.4675 | Glycinin G4$_{376-399}$ Glycinin$_{342-365}$ | A.DFYNPKAGRISTLNSLTLPALRQF.Q | | 27.9 |
| **120** | 23.2 | 1104.0131 | 2 | 2207.0188 | 2207.0404 | Glycinin G2$_{24-42}$ Glycinin G3$_{24-42}$ | R.IESEGGFIETWNPNNKPFQ.C | | 47.2 |
| **121** | 23.3 | 1058.9367 | 2 | 2116.8661 | 2117.0907 | Glycinin G2$_{228-248}$ | E.EEDSGAIVTVKGGLRVTAPAM.R | M$_{248}$(O) | 36.8 |
| **122** | 23.3 | 1262.5703 | 3 | 3785.6964 | 3785.7274 | Glycinin G2$_{174-207}$ | K.YQQQQQGGSQSQKGKQQEEENEGSNILSGFAPEF.L | | 51.7 |
| 123 | 23.4 | 1087.067 | 2 | 2173.1267 | 2173.1135 | β-conglycinin-α chain$_{516-535}$ | N.FLAGSQDNVISQIPSQVQEL.A | | 40 |
| 124 | 23.5 | 881.4429 | 3 | 2642.314 | 2642.5312 | Linoleate 9S-lipoxygenase-4$_{59-84}$ | I.QLISATKADGGKGKIGKSTNLRGKIT.L | | 45.5 |

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|---|---|---|---|---|---|---|---|---|---|
| 125 | 23.5 | 832.0926 | 3 | 2494.2632 | 2494.2896 | Glycinin $G2_{407-430}$<br>Glycinin $G3_{400-423}$ | F.KTNDRPSIGNLAGANSLLNALPEE.V | | 53.1 |
| 126 | 23.6 | 616.9537 | 3 | 1848.8465 | 1848.9199 | Glycinin $G2_{405-422}$<br>Glycinin $G3_{397-315}$ | V.SFKTNDRPSIGNLAGANS.L | | 37.4 |
| 127 | 23.7 | 717.3585 | 2 | 1433.7098 | 1433.7423 | Glycinin $G1_{209-220}$ | E.FLEHAFSVDKQI.A | | 29.6 |
| 128 | 23.8 | 659.3711 | 2 | 1317.7276 | 1317.7452 | Seed linoleate<br>13S-lipoxygenase-$1_{828-839}$<br>9S-lipoxygenase-$3_{846-857}$<br>9Slipoxygenase-$4_{842-853}$ | G.LTFRGIPNSISI | | 32.2 |
| 129 | 23.9 | 640.6235 | 3 | 1919.8559 | 1919.9168 | Urease$_{78-96}$ | V.IKGGEVAYANMGDPNASIP.T | $M_{88}$(O) | 35.9 |
| 130 | 23.9 | 786.7279 | 3 | 2358.1691 | 2358.2452 | Sucrose-binding protein$_{226-246}$ | F.SWNVLQAALQTPKGKLENVFD.Q | | 36.8 |
| **131** | 23.9 | 994.3987 | 2 | 1987.7901 | 1987.9984 | P24 oleosin isoform $A_{14-32}$ | T.TTTHRYEAGVVPPGARFE.T | | 44.9 |
| 132 | 24 | 881.1168 | 3 | 2641.3357 | 2641.358 | Glycinin $G2_{406-430}$<br>Glycinin $G3_{399-423}$ | S.FKTNDRPSIGNLAGANSLLNALPEE.V | | 72.3 |
| **133** | 24.1 | 1903.8949 | 2 | 3806.7824 | 3806.9348 | β-conglycinin-α' chain$_{241-274}$ | L.LPHHADADYLIVILNGTAILTLVNNDDRDSYNLQ.S | | 33.2 |
| 134 | 24.1 | 838.1009 | 3 | 2512.2881 | 2512.3154 | Glycinin $G2_{406-429}$<br>Glycinin $G3_{399-422}$ | S.FKTNDRPSIGNLAGANSLLNALPE.E | | 66.5 |
| 135 | 24.3 | 943.1495 | 3 | 2827.4339 | 2827.4585 | Glycinin $G2_{404-430}$<br>Glycinin $G3_{397-423}$ | Y.VSFKTNDRPSIGNLAGANSLLNALPEE.V | | 47 |
| 136 | 24.3 | 994.3966 | 2 | 1987.7858 | 1988.0016 | P24 oleosin isoform $B_{177-194}$ | Q.SKAQDTREAAARDARDAR.E | | 24.9 |
| 137 | 24.5 | 991.161 | 3 | 2971.4684 | 2971.5596 | Glycinin $G2_{407-434}$ | F.KTNDRPSIGNLAGANSLLNALPEEVIQH.T | | 36.2 |
| 138 | 24.6 | 683.0200 | 3 | 2047.0453 | 2047.0818 | Linoleate<br>9S-lipoxygenase-$4_{397-415}$ | I.AKEHLEPNLGGLTVEQAIQ.N | | 36.1 |
| 139 | 24.6 | 640.0057 | 3 | 1918.0027 | 1917.9925 | Urease$_{97-113}$ | P.TPEPVIMRPMFGTLGKAG.S | $M_{107}$(O) | 30.8 |
| 140 | 24.7 | 729.7194 | 3 | 2187.1438 | 2187.1768 | β-conglycinin-α' chain$_{567-585}$ | V.QELAFPRSAKDIENLIKSQ.S | | 50.3 |
| **141** | 24.8 | 791.085 | 3 | 2371.2406 | 2371.2504 | Linoleate<br>9S-lipoxygenase-$4_{356-376}$ | T.DEEFARETIAGLNPNVIKIIE.E | | 36.2 |
| 142 | 25 | 1076.0471 | 2 | 2151.0869 | 2151.104 | Glycinin $G2_{410-430}$<br>Glycinin $G3_{403-423}$ | N.DRPSIGNLAGANSLLNALPEE.V | | 66.2 |
| 143 | 25.1 | 848.443 | 2 | 1695.8788 | 1695.8912 | Glycinin $G2_{414-430}$<br>Glycinin $G3_{407-423}$ | S.IGNLAGANSLLNALPEE.V | | 40.5 |
| 144 | 25.1 | 725.0371 | 3 | 2173.0969 | 2173.1499 | β-conglycinin-α' chain$_{550-569}$ | N.FLAGSKDNVISQIPSQVQEL.A | | 49 |
| 145 | 25.2 | 1050.9389 | 2 | 2100.8704 | 2100.9754 | Seed linoleate<br>9S-lipoxygenase$_{400-418}$ | L.YGDQTSTITKEQLEINMGG.V | $M_{416}$(O) | 38.1 |
| 146 | 25.3 | 1011.5276 | 2 | 2022.0479 | 2022.0614 | Glycinin $G2_{410-429}$<br>Glycinin $G3_{403-422}$ | N.DRPSIGNLAGANSLLNALPE.E | | 48 |
| 147 | 25.4 | 976.4887 | 3 | 2927.4516 | 2927.4745 | β-conglycinin-α chain$_{519-546}$ | GSQDNVISQIPSQVQELAFPGSAQAVEK | | 69.3 |
| **148** | 25.4 | 791.364 | 3 | 2372.0773 | 2372.2205 | β-conglycinin-β chain$_{169-188}$ | E.INRVLFGEEEEQRQQEGVIV.E | | 31 |

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|---|---|---|---|---|---|---|---|---|---|
| **149** | 25.5 | 1101.5555 | 3 | 3302.6519 | 3302.7842 | Seed linoleate 9S-lipoxygenase$_{25-59}$ | D.FNAITSIGKGGVIDTATGILGQGVSLVGGVIDTAT.S | | 48.5 |
| 150 | 25.5 | 878.4362 | 3 | 2633.294 | 2633.3127 | Glycinin G1$_{415-439}$ | S.FKTNDTPMIGTLAGANSLLNALPEE.V | M$_{422}$(O) | 37.4 |
| 151 | 25.6 | 673.3071 | 2 | 1345.6068 | 1345.678 | Trypsin inhibitor A$_{102-114}$ | E.GPAVKIGENKDAM.D | M$_{114}$(O) | 32.9 |
| **152** | 25.7 | 1172.4999 | 2 | 2343.9925 | 2344.2217 | Seed linoleate 9S-lipoxygenase-2$_{574-594}$ | S.LINADGIIEKSFLPSKHSVEM.S | M$_{594}$(O) | 50.9 |
| **153** | 25.7 | 1101.5585 | 3 | 3302.6611 | 3302.7665 | Seed linoleate 9S-lipoxygenase$_{18-50}$ | L.MPKNVLDFNAITSIGKGGVIDTATGILGQGVSL.V | M$_{18}$(O) | 42.3 |
| 154 | 25.7 | 914.1448 | 3 | 2740.4199 | 2740.4264 | Glycinin G2$_{406-431}$ Glycinin G3$_{399-424}$ | S.FKTNDRPSIGNLAGANSLLNALPEEV.I | | 43.1 |
| 155 | 25.8 | 748.3789 | 3 | 2243.1221 | 2243.2183 | Sucrose-binding protein$_{225-244}$ | A.FSWNVLQAALQTPKGKLENV.F | | 30.6 |
| 156 | 25.9 | 1063.5426 | 3 | 3188.6132 | 3188.7017 | Linoleate 9S-lipoxygenase-4$_{9-38}$ | G.QKIKGTMVVMQKNVLDINSITSVGGIVDQG.L | M$_{18}$(O) | 56.4 |
| 157 | 26 | 1026.5285 | 3 | 3077.571 | 3077.4884 | Glycinin G1$_{400-427}$ | F.VVAARSQSDNFEYVSFKTNDTPMIGTLA.G | M$_{422}$(O) | 45.8 |
| **158** | 26.2 | 1148.5254 | 2 | 2296.0435 | 2296.1357 | Profilin-1$_{18-38}$ Profilin-2$_{18-38}$ | N.HLTHAAIIGQDGSVWLQSTDF.P | | 39 |
| 159 | 26.2 | 805.6666 | 4 | 3219.6445 | 3219.6757 | Glycinin G2$_{406-435}$ | S.FKTNDRPSIGNLAGANSLLNALPEEVIQHT.F | | 63.1 |
| 160 | 26.4 | 995.525 | 2 | 1990.0428 | 1990.0604 | Glycinin G2$_{417-435}$ Glycinin G1$_{426-444}$ | N.LAGANSLLNALPEEVIQHT.F | | 50.2 |
| **161** | 26.6 | 1107.5555 | 3 | 3320.6519 | 3320.8253 | β-conglycinin-α' chain$_{463-492}$ | L.FLPHFNSKAIVVLVINEGEANIELVGIKEQ.Q | | 37.5 |
| 162 | 26.6 | 1086.8813 | 3 | 3258.6223 | 3258.6568 | β-conglycinin-α chain$_{516-546}$ | N.FLAGSQDNVISQIPSQVQELAFPGSAQAVEK.L | | 61* |
| **163** | 26.7 | 1279.9062 | 3 | 3837.7042 | 3838.0538 | Glycinin G2$_{305-339}$ | D.IYNPQAGSITTATSLDFPALWLLKLSAQYGSLRKN.A | | 38.2 |
| **164** | 26.8 | 1013.8736 | 3 | 3039.6064 | 3039.5647 | β-conglycinin-α chain$_{207-231}$ | Q.NLRDYRILEFNSKPNTLLLPNHADAD.Y | | 32 |
| 165 | 26.9 | 1310.6527 | 3 | 2620.2982 | 2620.4531 | Seed linoleate 9S-lipoxygenase$_{4-26}$ | G.IFDKGQKIKGTVVLMPKNVLDFN.A | M$_{18}$(O) | 43.1 |
| **166** | 27 | 1139.588 | 3 | 3416.7493 | 3416.6753 | Profilin-1$_{46-78}$ Profilin-2$_{46-78}$ | E.ITAIMNDFNEPGSLAPTGLYLGGTKYMVIQGE.P | M$_{72}$(O) | 42.5 |
| 167 | 27.1 | 1280.2353 | 3 | 3838.6914 | 3838.9727 | P24 oleosin isoform B$_{275-309}$ | D.IQSKAQDTREAAARDARDAREAAARDARDAKVEAR.D | | 43.1 |
| 168 | 27.5 | 671.3459 | 3 | 2012.0231 | 2012.0369 | Glycinin G1$_{420-439}$ | D.TPMIGTLAGANSLLNALPEE.V | | 71.7 |
| **169** | 27.6 | 918.8018 | 3 | 2754.3908 | 2754.4097 | Glycinin G4$_{142-165}$ | P.VVAISLLDTSNFNNQLDQTPRVFY.L | | 55.1 |
| **170** | 27.7 | 1024 | 4 | 4092.9782 | 4093.091 | Seed linoleate 9S-lipoxygenase$_{383-418}$ | V.IRRLQEFPPKSTLDPTLYGDQTSTITKEQLEINMGG.V | M$_{416}$(O) | 38.8 |
| 171 | 27.8 | 1032.1934 | 3 | 3094.5656 | 3094.5878 | Glycinin G1$_{415-443}$ | S.FKTNDTPMIGTLAGANSLLNALPEEVIQH.T | | 66 |
| **172** | 28.3 | 945.8293 | 3 | 2835.4733 | 2835.4999 | Glycinin G2$_{417-442}$ Glycinin G1$_{426-451}$ | N.LAGANSLLNALPEEVIQHTFNLKSQQ.A | | 102 |
| 173 | 28.4 | 1162.5447 | 3 | 3485.6194 | 3485.9148 | Seed linoleate 9S-lipoxygenase-2$_{23-56}$ | T.VVLMRKNVLDFNSVADLTKGNVGGLIGTGLNVVG.S | M$_{26}$(O) | 42.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 174 | 28.6 | 1222.5502 | 2 | 2444.0932 | 2444.1688 | Glycinin $G3_{288-311}$ | R.HNIGQTSSPDIFNPQAGSITTATS.L | | 37.2 |
| **175** | 28.7 | 814.3648 | 3 | 2441.08 | 2441.2632 | Seed linoleate 9S-lipoxygenase$_{572-593}$ | S.LINADGIIEKSFLPGKYSIEMS.S | $M_{592}$(O) | 46.5 |
| 176 | 28.9 | 1065.8725 | 3 | 3195.6031 | 3195.6354 | Glycinin $G1_{415-444}$ | S.FKTNDTPMIGTLAGANSLLNALPEEVIQHT.F | | 91.1 |
| **177** | 30 | 1107.5159 | 3 | 3320.5332 | 3320.6719 | Seed linoleate 13S-lipoxygenase-1$_{544-573}$ | R.QSLINANGIIETTFLPSKYSVEMSSAVYKN.W | $M_{566}$(O) | 44.5 |
| **178** | 30.3 | 1011.013 | 4 | 4041.0302 | 4041.075 | Glycinin $G1_{415-451}$ | S.FKTNDTPMIGTLAGANSLLNALPEEVIQHTFNLKSQQ.A | | 56.9 |
| **179** | 30.6 | 1152.9124 | 3 | 3456.7226 | 3456.7468 | Glycinin $G1_{415-446}$ | S.FKTNDTPMIGTLAGANSLLNALPEEVIQHTFN.L | | 85.2 |

[a] The amino acid sequence of the parent protein is based on UniProtKB database entries: Glycinin G1 (P04775), G2 (P04405), G3 (P11828), G4 (P02858), glycinin (P04347), β-conglycinin with subunits β-(P25974), α'-(P11827) and α-(P13916), Trypsin inhibitor A (P01070), Lectin (P05046), seed Linoleate 13S-lipoxygenase-1 (P08170), 9S-lipoxygenase-2 (P09439), 9S-lipoxygenase-3 (P09186), 9S-lipoxygenase-4 (P38417), 9S-lipoxygenase (P24095), P24 oleosin isoform A-(P29530) and B-(P29531), Sucrose bonding protein (Q04672), Urease (P08298), Profilin 1-(O65809) and 2-(O65810).

[b] O= oxidation (modification post-translational)

[c] Protein Prospector ion score obtained for phosphopeptide sequence by database search.

***** Mascot ion score obtained for phosphopeptides sequence by database search. Ion score > 45 indicates identification ($p<0.05$).

The 179 released peptides originated from 9 soybean proteins (Table 1 and Figure 2), including 84 peptides from glycinin, followed by 41 from lipoxygenases and 24 from β-conglycinin. The remaining 30 peptides derived from whey proteins, such as urease (10), profilin (7), lectin (4), oleosins (4), Kunitz-type trypsin inhibitor A (3) and sucrose binding protein (2). It is noteworthy that many peptides show a homology of amino acid sequences among parent proteins, in particular for glycinins, and thus the parent protein could not be established (Figure 3 and Table 1).

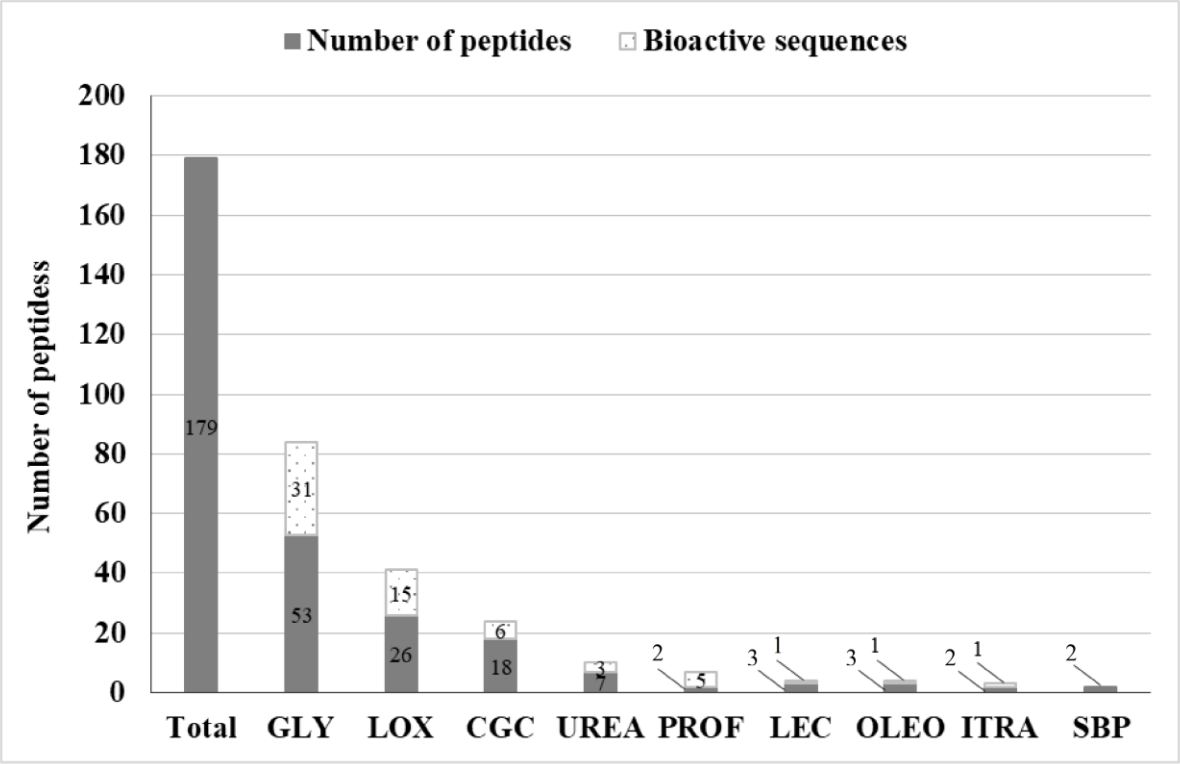


**Figure 2.** Distribution of peptides and bioactive sequences identified in soy-drink kefir from parent proteins: GLY = Glycinin (five subunit), CGC = β-Conglycinin (three subunit), LOX = Lipoxygenase (five isoenzymes), UREA= Urease, PROF = Profilin (two isoforms), LEC = Lecitin, ITRA = Trypsin inhibitor A, OLEO= P24 oleosin (isoforms A and B) and SBP = sucrose binding protein.

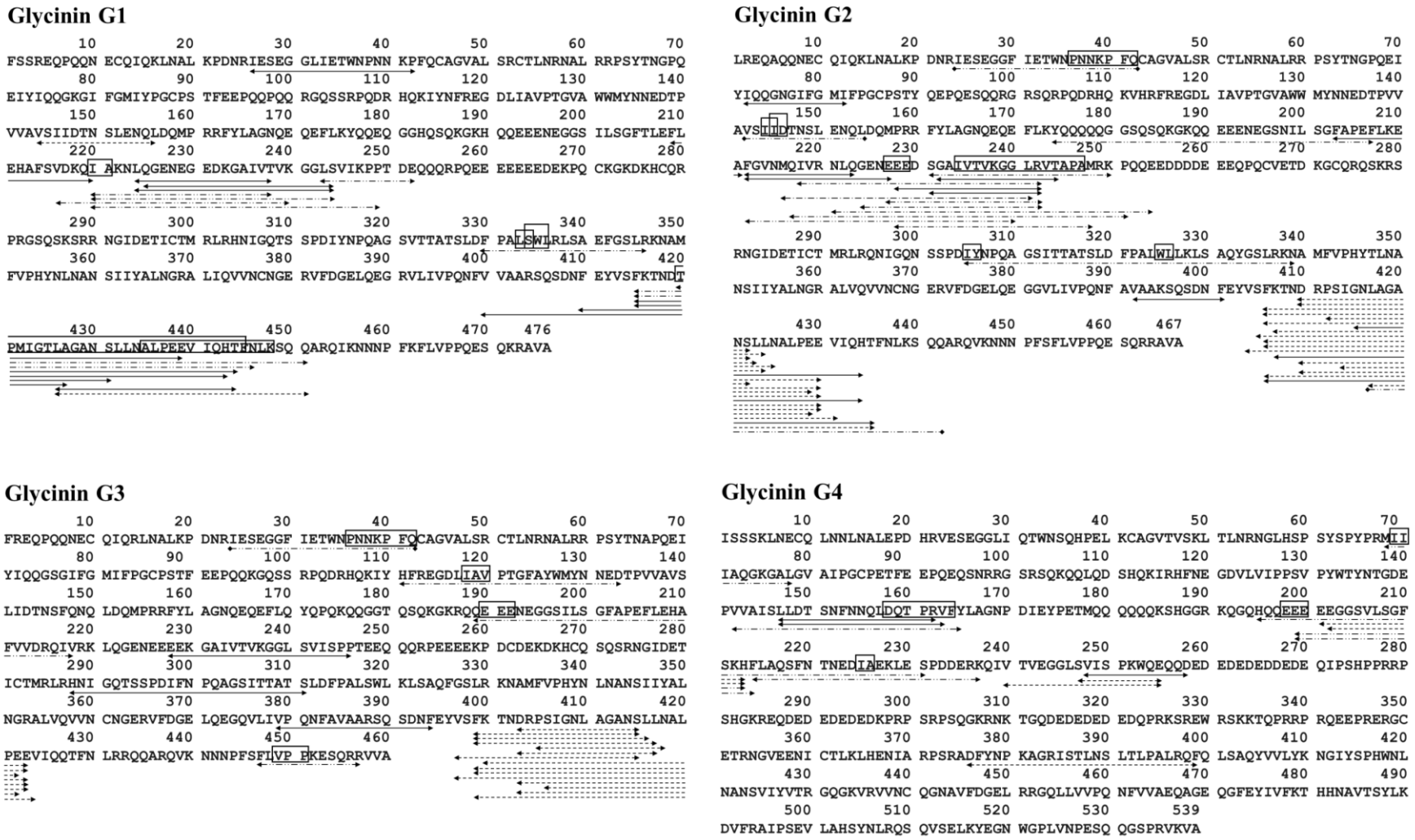


**Figure 3.** Amino acid sequence of soybean glycinin proteins in single letter code with the relevant identified peptides (◄—►) peptides homology (◄- - -►) and bioactive (◄- - -►) and homology (◆- - -◆) peptides by LC-ESI-MS/MS. Bioactive amino acid residues are marked with a square.

**Glycinin**

```
        10         20         30         40         50         60         70
ITSSKFNECQ LNNLNALEPD HRVESEGGLI ETWNSQHPEL QCAGVTVSKR TLNRNGSHLP SYLPYPQMII
        80         90        100        110        120        130        140
VVQGKGAIGF AFPGCPETFE KPQQQSSRRG SRSQQQLQDS HQKIRHFNEG DVLVIPLGVP YWTYNTGDEP
       150        160        170        180        190        200        210
VVAISPLDTS NFNNQLDQNP RVFYLAGNPD IEHPETMQQQ QQQKSHGGRK QGQHRQQEEE GGSVLSGFSK
       220        230        240        250        260        270        280
HFLAQSFNTN EDTAEKLRSP DDERKQIVTV EGGLSVISPK WQEQEDEDED EDEEYGRTPS YPPRRPSHGK
       290        300        310        320        330        340        350
HEDDEDEDEE EDQPRPDHPP QRPSRPEQQE PRGRGCQTRN GVEENICTMK LHENIARPSR ADFYNPKAGR
       360        370        380        390        400        410        420
ISTLNSLTLP ALRQFGLSAQ YVVLYRNGIY SPDWNLNANS VTMTRGKGRV RVVNCQGNAV FDGELRRGQL
       430        440        450        460        470        480        490
LVVPQNPAVA EQGGEQGLEY VVFKTHHNAV SSYIKDVFRV IPSEVLSNSY NLGQSQVRQL KYQGNSGPLV
492
NP
```

It is interesting to note that glycinin alone and glycinin and β-conglycinin together were precursors of about 47% and 58% of the peptides released by microbial fermentation. Glycinin (11S legumin type) and β-conglycinin (7S vicilin type) are the most abundant soybean proteins (65-80%). Glycinins is a hexamer composed of five major subunits (G1 to G5) linked together by disulfide bonds and β-conglycinin has three subunits (α, α' and β) which share a large degree of amino acids homologies between the respective subunits. This partly explains the most abundant peptides derived from the main proteins, in agreement with previous studies (Gibbs et al. 2004; Singh et al., 2017). In particular, Gibbs et al. (2004) have shown that glycinin was the precursor of 95% of the peptides formed in soybean protein hydrolysis in fermented soy foods (natto and themph), whereas β-conglycinin was found to be resistant to proteolytic attack even by multi-enzyme systems (Gibbs et al., 2004). The second source of peptides (about 23% of peptides) in soy-drink kefir are lipoxygenases, which are whey proteins and belong to the 7S fraction (Wang and Gonzalez de Mejia, 2005). Soybean contains at least four enzymes (L1, L2, L3 and L4) and 9S-lipoxygenase, which are involved in the oxidation of polyunsaturated fatty acids (converting linoleic acid in 13-and 9-hydroperoxide compounds) and are responsible of 'beany', 'green' and 'grassy' off-flavors produced during processing and storage of soy-based foods (Ma et al., 2015). Most of the peptides were identified from two lipoxygenases proteins, the 9S-lipoxygenase and the 9S-lipoxygenases-2 isoenzymes (Table 1). The remaining peptides (about 10%) derived from minor soybean proteins, which are recognized as antinutritional factors or allergenic and bioactive (Herman and Burks, 2011). Furthermore, no allergenic peptide has been reported with length of 2 to 40 amino acids and mass range of 200 to 5000 Da (Goodman et al., 2005), thus peptides found in this study could be consider no allergenic.

Finally, most of the released peptides from parent proteins (glycinins and lipoxygenases) are not distributed equally among the protein sequences as showed in Figure 3 (only glicinins are shown) and Table 1. Indeed, there were distinct parts in the proteins where more peptides are released than in other parts. In particular, a large number of peptides derived from C-terminal of glycinin G1, G2 and G3, and from the 210-250 amino acid residues of glycinin-G1 and -G2. Differently from the results reported by Singh and Vij (2017), and likely due to the different involved microflora, a few amino acid fragments were released by glycinin G4. Peptides released from breakdown of lipoxygenases were derived for about 50% from N-terminal of proteins (Table 1). The lipoxygenases residues are organized in two domains: N-terminal domain (domain I) with a β-sheet structure and C-terminal domain with a α-helicum (domain II) structure (Prigge et al., 1997). It is possible to suppose that lactic acid bacteria proteolytic system is able to produce peptides from N-terminal domain than peptides released from glycinin as more accessible than other residues. However, the specificity of these proteinases in soy-based fermented foods has not been equally investigated as the proteolytic behavior on milk proteins.

Finally, as an example, Figure 4 showed the MS/MS spectrum of peptide 29 with the relevant sequence scheme showing the amino acid bond cleavage yielding to the experimentally detected y-ions and b-ions fragments. This peptide is a breakdown product of glycinin G1 f(220-244) which contains a small bioactive sequence (IA) identified as the angiotensin I-converting enzyme inhibitory peptide isolated in the peptic digest of soybean proteins (Chen et al., 2003).

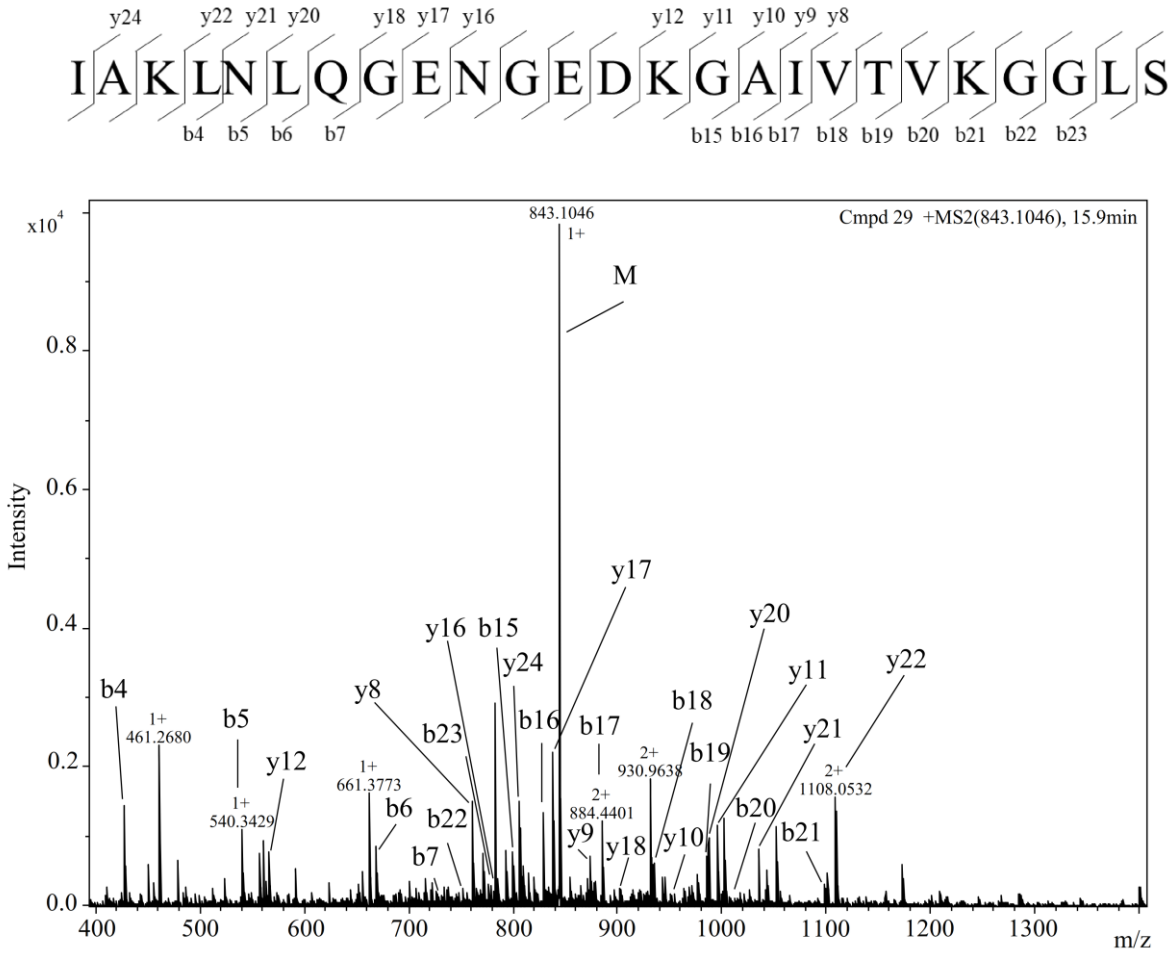


**Figure 4.** MS/MS spectrum of the peptide showing the precursor ion at 843.1046 m/z (triple charged). The b and y-ion series are from single to triple charged ions.

*7.4.3 Peptides from soy-drink kefir with encrypted bioactive sequence*

All peptides identified in the present study were compared for sequence matches both with an in-house database of known bioactive peptides derived a detailed literature study, and in BIOPEP database. Table 2 shows a list of 62 potential bioactive peptides, encrypted within the peptides identified in the present study, with the amino acid sequences could be recognized as bioactive. As expected, most of the peptides derived from glycinin (31), followed by lipoxygenases (15), β-conglycinin (6), urease (3), profilin (5), and only one from lectin, oleosins and Kunitz-type trypsin inhibitor A (Figure 2). Moreover, within the potential bioactive peptides, 32 revealed ACE inhibitory activity, 11 were stimulating vasoactive substance release activity, followed by 7 with antioxidant activity, 3 with hypocholesterolemic functionality and 2 antimicrobial (Table 2). The remaining 7 peptides showed from two to four activities of potential bioactivity. The angiotensin 1-converting enzyme (ACE inhibitory) peptides inhibit

conversing angiotensin I to angiotensin II, which have a strong vasoconstrictor action, thereby decreasing the blood pressure and risk of cardiovascular disease (Hartmann and Meisel, 2007). Prevalence of hydrophobic (A, Y, W, F, I, V, L and M) or positively charge (R and K) amino acids and/or P at C-terminal position of the ACE inhibitory peptides, as in some cases of the identified peptides, have been shown to have better affinity with ACE (Nakamura et al., 1995; Kuba et al., 2003; Shimakage et al., 2012 ect.). In particular, tripeptides as V-P-P are more active than peptides with 6 or 5 amino acids (T-T-M-P-L-W or F-F-V-A-P) (Pessione and Cirrincione, 2016). Bioactive ACE inhibitory identified peptides are found in several soy-based foods or soy proteins, such as soy-drink (Tomatsu et al., 2013), soybean protein or hydrolyzed (Gu and Wu, 2013; Kuba et al., 2005), processed soya-drink (Tomatsu et al., 2013), protease-treated hikiwari-natto (Shimakage et al., 2012), after gastrointestinal digestion of soy drink (Capriotti et al., 2015), after peptic digestion of soybean (Chen et al., 2003). In this study, most of ACE inhibitory peptides, encrypted in peptides, derived from lipoxygenases (12), followed by glycinin (10). Furthermore, 10 and one identified peptides from glycinin and β-conglycinin-β chain, respectively, were found stimulating vasoactive substance release (Table 2). The peptide sequence E-E-E was recognized to modulate vasoactive substance from human aortic endothelial cells (Ringseis et al., 2005). Antioxidant peptides can control both enzymatic and non-enzymatic peroxidation of fatty acids, act as radical scavengers or eliminate radical precursors (Hartmann and Meisel, 2007). In particular, Y, M, H, K, and W, are generally accepted to be antioxidants and the tripepetide P-H-H, encripted in β-conglycinin-α' chain$_{241-274}$ was demonstrated to possess the highest antioxidant activity (Chen et al., 1996). A small dipeptide with D-E amino acid sequence was found in three soy drink kefir peptides with hypocholesterolemic effect (Fatma et al. 2013). The authors have recognized that the peptide has potential in lowering total cholesterol, triglycerides, LDL, and in raising levels of HDL in Sprague dawley male rats (Fatma et al., 2013). Finally, two peptides encripted in glycinin G2 (compounds 84 and 121) fragments showed a potential antimicrobial activity predicted by Capriotti et al., (2015), which is appreciable in food as natural preservative of undesiderable contamination.

**Table 2.** Bioactive peptides sequences (bold) encrypted within the peptides identified by LC-ESI-QTOF-MS/MS. Bioactivity was established by search in BIOPEP database and literature of bioactive peptides.

| Peaks | Peptide containing bioactive sequence | Sequence | Bioactivity | References |
|---|---|---|---|---|
| 3 | Q.**IA**KNLQGENEGEDKGAIVT.V | Glycinin $G1_{220-238}$ | ACE inhibitory | Chen et al., 2003 |
| 16 | S.FL**VPP**KESQR.R | Glycinin $G3_{447-456}$ | ACE inhibitory | Nakamura et al., 1995 |
| 23 | D.FPA**LSWL**RLSAEFGSLR.K | Glycinin $G1_{330-346}$ | ACE inhibitory | Gu and Wu, 2013; Kuba et al., 2003 |
| 29 | Q.**IA**KNLQGENEGEDKGAIVTVKGGLS.V | Glycinin $G1_{220-244}$ | ACE inhibitory | Chen et al., 2003 |
| 36 | V.IMRPMFGTLGKAGSSLS**IA**FVSKAW.L | Urease$_{102-126}$ | ACE inhibitory | Chen et al., 2003 |
| 37 | A.VS**IID**TNSLENQL.D | Glycinin $G2_{142-154}$ Glycinin $G1_{144-156}$ | ACE inhibitory | Shimakage et al., 2012 |
| 38 | L.MPKNVLDFNAITSIGKGGV**ID**TA.T | Seed linoleate 9S-lipoxygenase$_{18-40}$ | ACE inhibitory | Shimakage et al., 2012 |
| 40 | G.KGGV**ID**TATGILGQGVSLVGGVI.D | Seed linoleate 9S-lipoxygenase$_{33-55}$ | ACE inhibitory | Shimakage et al.., 2012 |
| 43 | S.VDKQ**IA**KNLQGENEGEDKGAIVTVK.G | Glycinin $G1_{216-240}$ | ACE inhibitory | Chen et al., 2003 |
| 59 | Y.HFREGDL**IAV**PTGFAYWMYNNED.T | Glycinin $G3_{111-133}$ | ACE inhibitory | Tomatsu et al., 2013 |
| 61 | H.FLAQSFNTNED**IA**EK.L | Glycinin $G4_{214-238}$ | ACE inhibitory | Chen et al., 2003 |
| 64 | G.V**ID**TATGILGQGVSLVGGV**ID**TATSFLGRNISMQ.L | Seed linoleate 9S-lipoxygenase$_{36-69}$ | ACE inhibitory | Shimakage et al., 2012 |
| 66 | Q**.IA**KNLQGENEGEDKGAIVTVKGGLSVIKPP.T | Glycinin $G1_{220-249}$ | ACE inhibitory | Chen et al., 2003 |
| 67 | Y.RDTMNINSLARKALVNADG**II**E.K | Linoleate 9S-lipoxygenase-$4_{547-568}$ | ACE inhibitory | Shimakage et al., 2012 |
| 69 | R.MI**II**AQGKGAL.G | Glycinin $G4_{68-78}$ | ACE inhibitory | Shimakage et al., 2012 |
| 70 | M.RPMFGTLGKAGSSLS**IA**FVSKA**WL**.N | Urease$_{104-127}$ | ACE inhibitory | Chen et al., 2003; Kuba et al., 2003 |
| 83 | Q.SRNELDKGIGT**II**SSPYRI.R | Trypsin inhibitor $A_{46-64}$ | ACE inhibitory | Shimakage et al., 2012 |
| 102 | T.D**II**SKISPLPVLKE.I | Seed linoleate 9S-lipoxygenase-$3_{320-333}$ | ACE inhibitory | Shimakage et al., 2012 |
| 104 | L.PTD**II**SKISPLPVLKEIFRT.D | Seed linoleate 9S-lipoxygenase-$3_{318-337}$ | ACE inhibitory | Shimakage et al., 2012 |
| 106 | D.IEGNHLTHAA**II**GQDGSVW.A | Profilin-$1_{14-32}$ Profilin-$2_{14-34}$ | ACE inhibitory | Shimakage et al., 2012 |
| 113 | N.VLDFNAITSIGKGGV**ID**.T | Seed linoleate 9S-lipoxygenase$2_{2-38}$ | ACE inhibitory | Shimakage et al., 2012 |
| 120 | R.IESEGGFIETWN**PNNKPFQ**.C | Glycinin $G2_{24-42}$ Glycinin $G3_{24-42}$ | ACE inhibitory | Capriotti et al., 2015 |
| 131 | T.TTTHRYEAGV**VPP**GARFE.T | P24 oleosin isoform $A_{14-32}$ | ACE inhibitory | Nakamura et al., 1995 |
| 149 | D.FNAITSIGKGGVIDTATGILGQGVSLVGGV**ID**TAT.S | Seed linoleate 9S-lipoxygenase$_{25-59}$ | ACE inhibitory | Shimakage et al., 2012 |
| 152 | S.LINADG**II**EKSFLPSKHSVEM.S | Seed linoleate 9S-lipoxygenase-$2_{574-594}$ | ACE inhibitory | Shimakage et al., 2012 |
| 153 | L.MPKNVLDFNAITSIGKGGV**ID**TATGILGQGVSL.V | Seed linoleate 9S-lipoxygenase$_{18-50}$ | ACE inhibitory | Shimakage et al., 2012 |

| | | | | |
|---|---|---|---|---|
| 158 | N.HLTHAA**II**GQDGSV**WL**QSTDF.P | Profilin-1$_{18-38}$ Profilin-2$_{18-38}$ | ACE inhibitory | Shimakage et al., 2012; Kuba et al., 2003 |
| 161 | L.F**LPHF**NSKAIVVLVINEGEANIELVGIKEQ.Q | β-conglycinin-α' chain$_{463-492}$ | ACE inhibitory | Kuba et al., 2005 |
| 164 | Q.NLRDYRI**LEF**NSKPNTLLLPNHADAD.Y | β-conglycinin-α chain$_{207-231}$ | ACE inhibitory | Gu and Wu, 2013 |
| 169 | P.VVAISLLDTSNFNNQL**DQTPRVF**Y.L | Glycinin G4$_{142-165}$ | ACE inhibitory | Capriotti et al., 2015 |
| 175 | S.LINADG**II**EKSFLPGKYSIEMS.S | Seed linoleate 9S-lipoxygenase$_{572-593}$ | ACE inhibitory | Shimakage et al., 2012 |
| 177 | R.QSLINANG**II**ETTFLPSKYSVEMSSAVYKN.W | Seed linoleate 13S-lipoxygenase-1$_{544-573}$ | ACE inhibitory | Shimakage et al., 2012 |
| 84 | D.SGA**IVTVKGGLRVTAPA**MRK.P | Glycinin G2$_{231-250}$ | Antimicrobial | Capriotti et al., 2015 |
| 121 | E.EEDSGA**IVTVKGGLRVTAPA**M.R | Glycinin G2$_{228-248}$ | Antimicrobial | Capriotti et al., 2015 |
| 89 | P.EEITAIMNDFNEPGSLAPTG**LY**LGGTKYM.V | Profilin-1$_{44-72}$ Profilin-2$_{44-72}$ | Antioxidative | Matsufuji et al., 1994 |
| 133 | L.L**PHH**ADADYLIVILNGTAILTLVNNDDRDSYNLQ.S | β-conglycinin-α' chain$_{241-274}$ | Antioxidative | Chen et al., 1996 |
| 166 | E.ITAIMNDFNEPGSLAPTGL**YL**GGTKYMVIQGE.P | Profilin-1$_{46-78}$ Profilin-2$_{46-78}$ | Antioxidative | Matsufuji et al., 1994 |
| 170 | V.IRRLQEFPPKSTLDPT**LY**GDQTSTITKEQLEINMGG.V | Seed linoleate 9S-lipoxygenase3$_{83-418}$ | Antioxidative | Matsufuji et al., 1994 |
| 172 | N.LAGANSLLN**ALPEEVIQHTFNLK**SQQ.A | Glycinin G2$_{417-442}$ Glycinin G1$_{426-451}$ | Antioxidative | Singh B. P. and Vij, S. 2017 |
| 178 | S.FKTNDTPMIGTLAGANSLLN**ALPEEVIQHTFNLK**SQQ.A | Glycinin G1$_{415-451}$ | Antioxidative | Singh B. P. and Vij, S. 2017 |
| 179 | S.FKTND**TPMIGTLAGANSLLNALPEEVIQHTF**N.L | Glycinin G1$_{415-446}$ | Antioxidative | Singh B. P. and Vij, S. 2017 |
| 22 | D.AIVTSSGKLQLNKV**DE**NGTPKPS.S | Lectin$_{24-46}$ | Hypocholesterolemic | Fatma et al., 2013 |
| 33 | G.LSVIKPPT**DE**.Q | Glycinin G1$_{243-252}$ | Hypocholesterolemic | Fatma et al., 2013 |
| 96 | Y.LPR**DE**AFGHLKSSDFLTYGLKSVSQNVLP.L | Seed linoleate 9S-lipoxygenase-3$_{258-286}$ | Hypocholesterolemic | Fatma et al., 2013 |
| 9 | N.MQIVRNLQGEN**EEE**DSGAIVTVKGGL.R | Glycinin G2$_{216-241}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 11 | Q.HQQ**EEE**EEGGSVLSGFSKH.F | Glycinin G4$_{195-231}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 26 | N.**EEE**DSGAIVTVKGGLR.V | Glycinin G2$_{227-242}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 28 | Q.GEN**EEE**DSGAIVTVKGGLR.V | Glycinin G2$_{224-242}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 42 | E.E**EEE**GGSVLSGFSKH.F | Glycinin G4$_{199-213}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 46 | Q.IVRNLQGEN**EEE**DSGAIVTVKGGLR.V | Glycinin G2$_{218-242}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 88 | E.E**EEE**GGSVLSGFSKHF.L | Glycinin G4$_{199-214}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 92 | Q.Q**EEE**NEGGSILSGFAPEFLEHAFVVDRQIV.R | Glycinin G3$_{189-218}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 116 | A.FGVNMQIVRNLQGEN**EEE**DSGAIVTVKGG.L | Glycinin G2$_{212-240}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 122 | K.YQQQQQGGSQSQKGKQQ**EEE**NEGSNILSGFAPEF.L | Glycinin G2$_{174-207}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 148 | E.INRVLFG**EEE**EQRQQEGVIV.E | β-conglycinin-β chain$_{169-188}$ | Stimulating vasoactive substance release | Ringseis et al., 2005 |
| 12 | S.YN**LHPGDAQR**IPAG**TTYY**.L | β-conglycinin-β chain$_{93-110}$ | ACE inhibitory, antioxidant | Tomatsu et al., 2013; Beermann et al., 2009 |

| | | | | |
|---|---|---|---|---|
| 55 | N.LRM**IA**GTTFYV**VNP**DN**DE**.N | β-conglycinin-α' chain$_{291-315}$ | ACE inhibitory, hypocholesterolemic | Chen et al., 2003; Tomatsu et al., 2013; Fatma et al., 2013 |
| 87 | V.KKTGAAL**II**G**IYDE**PMTPGQ.C | Profilin-1$_{94-113}$ Profilin-2$_{94-113}$ | ACE inhibitory, antioxidant, hypocholesterolemic | Shimakage et al., 2012; Matsufuji et al., 1994; Fatma et al., 2013 |
| 141 | T.**DEE**FARET**IA**GLNPNVIK**II**E.E | Linoleate 9S-lipoxygenase-4$_{356-376}$ | Antithrombotic, ACE inhibitory | Lee and Kim, 2005; Chen et al., 2003; Shimakage et al., 2012 |
| 163 | D.**IY**NPQAGSITTATSLDFPAL**WL**LKLSAQYGSLRKN.A | Glycinin G2$_{305-339}$ | Antioxidant, ACE inhibitory | Matsufuji et al., 1994; Kuba et al., 2003; |
| 98 | R.NLQGEN**EEE**DSGA**IVTVKGGLRVTAPA**MRKPQQE.E | Glycinin G2$_{221-254}$ | Stimulating vasoactive substance release, antimicrobial | Ringseis et al., 2005; Capriotti et al., 2015 |
| 110 | M.QIVRNLQGEN**EEE**DSGA**IVTVKGGLRVTAPA**MRKP.Q | Glycinin G2$_{217-251}$ | Stimulating vasoactive substance release, antimicrobial | Ringseis et al., 2005; Capriotti et al., 2015 |

*7.4.4 Sensory analysis*

Odor, appearance, taste, acidity and texture attributes, as well as general acceptability of soy-drink kefir were evaluated by sensory analysis. All panelists classified soy-drink kefir as "good" for the general acceptability, with the highest value (score 4.5 ± 0.5) for the texture, followed by odor attribute (score 4.1 ± 0.7) and the lowest value for acidity (score 3.1 ± 0.8).

## 7.5 Conclusion

In conclusion, the present study is the first report to analyze the peptides released during soy drink kefir production; therefore, the results could get insight on the proteolytic process and serve as a basis for the discovery of bioactive components. Results showed a peptide profile of soy drink kefir that includes 179 sequences, mainly released from glycinin and lipoxygenase proteins during kefir fermentation. Bioactive sequences were found only encrypted in 62 peptides; further studies are required to explore the presence of bioactive peptides at m/z range lower than the one used in this work.

## Author contributions

This research work was carried out at "Sciences Agriculture, Food and Environment Department" of Foggia University and "Engineering Department" of Bari University.

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# CHAPTER 8

## CONCLUSIONS

The present research work concerned the study of influence of food-fermented processing on the composition and quality of foods in relation to the nutritional content and potential health-promoting effects, particularly focusing on two foods, wine and kefir. In the first part of the work, the study interested the investigation of several factors affecting quality of white and red wines, whereas, in the second part, the influence of some technological parameters on phosphopeptides production in kefir and finally, a microbiological and peptide characterization of alternative non-dairy kefir such as soy drink kefir.

As concerning wine, the behaviour of the main oenological parameters, the phenolic, and the volatile profiles of a white wine without added sulfites and produced by reductive winemaking, along 15 months storage at different light and temperature conditions, was investigated and compared with a control stored with sulfur dioxide. The study of phenolic compounds in wines stored without sulfites addition indicated that at 15 months, sample stored at high temperature (30°C) was the most browned, followed by samples stored at uncontrolled temperature and light conditions and at constant temperature of 12°C; otherwise, samples stored with sulfites were not significantly different at 15 storage months. Particularly samples stored at high temperature and uncontrolled conditions of temperature and light showed significant changes in volatile profiles. Finally, although the applied technology involved a reduced contact between oxygen and must in all the steps of winemaking in order to preserve quality attributes, the inadequate storage, primarily, and the absence of sulfites, secondarily, caused reactions of oxidation. Results demonstrated that the presence of sulfites helped the typical aroma of young wines; however, even more important seems to be the storage in the dark, at low temperature, as improper storage conditions favored aroma degradation regardless sulfite addition.

Afterwards, ochratoxin A (OTA) concentration was determined in thermovinified wines as affected by processing by using a new, simple, fast, accurate and validated method for the determination of OTA in wine requiring a microextraction by packed sorbent (MEPS) combined with HPLC-FLD detection. MicroExtraction by Packed Sorbent (MEPS) based on a C18 phase proved to be a successful approach to the extraction of Ochratoxin A from wine matrices, preliminary to its determination based on HPLC-FLD analysis. In particular, the

remarkable removal of wine interferents achievable using MEPS enabled an accurate determination of the analyte in real samples even using a solvent-matched calibration. This feature, along with the easiness, rapidity and possibility of automation make the proposed MEPS procedure a very promising, reliable alternative to consolidated analytical approaches like SPE or IAC, especially when a significant number of samples has to be analyzed in a relatively short time. The proposed method could then be successfully used for OTA monitoring and for risk-assessment purposes in the context of wine production. Finally, thermovinification processing did not affect OTA concentration in wines.

As concerning kefir, its increasingly production and consumption in different countries in the world is linked to health promoting effects. The caseino-phosphopeptides (CPPs), known to exert several physiological properties, were investigated in kefir prepared by traditional kefir grains in relation to milk kefir processing. The phosphopeptide sequences of dephosphopeptides were identified in kefir by UHPLC-ESI-MS/MS analysis. As a result, the influences of temperature, pH and the back-slopping approach of on phosphopeptide profile in kefir prepared using kefir grains, were assessed. The lower fermentation temperature (18°C vs 25°C) slowed down the proteolytic activity in kefir samples yielding to more abundance of large peptides, whereas the lower pH (4.0 vs 4.7) showed increase of degradation of more large peptides although significant differences for small peptides, within the range explored, were not observed. The back-slopping approach suggested an intense proteolytic activity though the reproducibility in the kefir phosphopeptide when grains are reused is reduced. Indeed, the symbiotic equilibrium between various microbial groups changes significantly along the first and second fermentation. As concerning the bioactive sequences, the polar acidic motif pSpSpSEE (pS = phosphoserine) of casein-phosphopeptides is known to have bioactivity such as mineral binding. Nine peptide sequences found in milk kefir showed the mineral binding motif. Besides the presence of the typical cluster sequence, other factors are important for mineral binding, as the total negative charge, the total number of amino acids, the phosphorylation degree and the amino acid composition around the phosphorylated region. Therefore, further studies are required to determine the potential bioactivity of other phosphopetide sequences. Finally, peptide profile of an alternative non-dairy kefir drink, a soy drink kefir, which is suitable for vegan and dairy-product allergic consumers, was investigated, as soy proteins possess a high nutritional value, similar to high-value animal proteins, and show excellent functional properties as source of bioactive peptides. To the best of our knowledge, the present study is the first report on the peptides released during soy drink kefir production;

therefore, the results could get insight on the proteolytic process and serve as a basis for the discovery of bioactive components.

Nevertheless, many other technological aspects, for instance, the enhancement of organoleptic quality, the development of new products and the study of fermentation kinetics remain of high scientific interest, especially in relation to kefir nutraceutical properties. Therefore, a further advancement of knowledge in this field can help industry operators by encouraging product innovation and differentiation.