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Antifungal and functional properties of polyphenols in food products

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"The secret of getting ahead is getting started."

Mark Twain

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ABSTRACT

Nowadays, the consumer's demand for food products free of synthetic chemicals impels the search for alternatives able to maintain product's properties and microbiological safety. The most common way to ensure microbiological stability and prevent spoilage during food products shelf-life is by adding preservatives such as potassium sorbate (E202). However, consumers' negative perception about industrially synthesized antimicrobials due to a possible association with potential toxicological problems, has created interest in food industries for natural compounds with antimicrobial effect. Plant polyphenols are well known for their antibacterial, antifungal and antiviral activities, as well as for their health benefits due to their antioxidant activity. Due to these bioactive properties, many studies have pointed out the potential of plant extracts rich in polyphenols as natural preservatives in food products, as well as functional ingredients. The aim of this study was to evaluate the ability of several plant extracts rich in polyphenols as potassium sorbate substitutes and as functional ingredients in cereal and forest fruit preparations and respective dosed yogurts.

Several plant extracts such as acai, cranberry, grape seed, green coffee, green tea, hibiscus and olive extract and the chemically synthesized molecule vanillin were tested for their antifungal activity against isolated fungi. These microorganisms were isolated from the raw material's preparations and from the environmental air of the Tortosendo unit. Vanillin and grape seed and green tea extracts were the ones with greater antifungal activity and were selected for further tests. These substances, when added to yogurt did not interfere with its natural microflora. Additionally, the total phenolic content and the antioxidant activity of these yogurts was higher than plain yogurt and stable over 28 days of refrigerated storage. When added to forest fruit preparations, grape seed and green tea extract were not able to ensure the absence of spoilage fungi during 4-week storage, whereas vanillin showed very similar results to potassium sorbate. The ability of grape seed extract to prevent a purposive contamination of *Aspergillus* sp. and *Penicillium* sp. was also tested. This extract did not have the ability to prevent the fungal load in the cereal and forest fruit preparations and corresponding yogurts.

Keywords: natural antimicrobials, food preservatives, plant extracts, polyphenols, antifungal activity, functional properties.

SUMÁRIO

Atualmente, a procura por alimentos sem ingredientes sintéticos é cada vez maior, o que leva à procura de alternativas que consigam assegurar as propriedades e segurança microbiológica destes produtos. A adição de conservantes, como o sorbato de potássio (E202), tem como função prevenir contaminações fúngicas durante o tempo de prateleira dos produtos alimentares. No entanto, os consumidores têm uma perceção negativa acerca de compostos sintéticos com função antimicrobiana, devido a uma possível associação com potenciais problemas toxicológicos. Assim sendo, há um interesse cada vez maior em compostos antimicrobianos naturais. Os polifenóis, provenientes de plantas, são bem conhecidos pela sua atividade antibacteriana, antifúngica e antiviral, assim como pelos seus benefícios para a saúde devido à sua capacidade antioxidante. Devido às suas propriedades bioativas, muitos estudos salientam a potencialidade do uso de extratos de plantas ricos em polifenóis como conservantes alimentares e como ingredientes funcionais. Neste sentido, o presente trabalho teve como objetivo avaliar a capacidade de vários extratos de plantas ricos em polifenóis como alternativas naturais ao sorbato de potássio, bem como na qualidade de ingredientes funcionais, num preparado de cereal e de frutos do bosque e respetivos iogurtes dosificados.

A atividade antifúngica dos extratos de açaí, arando, grainha de uva, café verde, chá verde, hibiscos e azeitona, e da molécula quimicamente sintetizada vanilina foi testada contra fungos previamente isolados das matérias-primas dos preparados e do ambiente fabril da unidade de Tortosendo. A vanilina e os extratos de chá verde e de grainha de uva obtiveram os melhores resultados em termos de atividade antifúngica, sendo selecionados para testes posteriores. Quando adicionados a iogurte, estes compostos não interferiram com as culturas *starter* do mesmo. Além disso, iogurtes com extratos mostraram um maior e estável conteúdo de fenólicos totais e maior capacidade antioxidante em comparação com iogurte simples, durante 28 dias de armazenamento sob refrigeração. Quando adicionados ao preparado de frutos do bosque, o extrato de chá verde e grainha de uva não conseguiram assegurar a ausência de contaminações durante 4 semanas de armazenamento. Por outro lado, a vanilina mostrou resultados bastante semelhantes aos do sorbato de potássio. Foi testada a capacidade do extrato de grainha de uva para prevenir contaminações provocadas por *Aspergillus* sp. e por *Penicillium* sp. Este extrato não mostrou qualquer capacidade de prevenir ou diminuir a contaminação fúngica ao longo do tempo nos preparados testados e respetivos iogurtes.

Palavras-chave: antimicrobianos naturais, conservantes alimentares, extratos de plantas, polifenóis, atividade antifúngica, propriedades funcionais.

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GLOSSARY

Abbreviations

ABTS$\cdot^+$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
a_w	Water activity
BA	Blackberry
BHA	Butylated hydroxyanisol
BHT	Butylated hydroxytoluene
BPW	Buffered peptone water
BR	Barley
BU	Blueberry
CFU	Colony forming units
CIP	Cleaning in place
CP	Cereal preparation
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGCG	Epigallocatechin gallate
FDA	Food and Drug Administration
FP	Forest fruit preparation
GCCA	Green coffee chlorogenic acids
GRAS	Generally regarded as safe
GS	Grape seed extract
GT	Green tea extract
HACCP	Hazard analysis critical control points
ISO	International Organization for Standardization
IQF	Individually quick-frozen
MGI	Mycelial growth inhibition
MIC	Minimum inhibitory concentration
MRD	Maximum recovery diluent
NA	No activity
NP	No preference
ORAC	Oxygen radical absorbance capacity
OT	Oats
PCA	Plate count agar

PDA	Potato dextrose agar
PS	Potassium sorbate
RA	Raspberry
RBC	Rose-bengal chloramphenicol agar
RG	Retarded growth
ROS	Reactive oxygen species
RSA	Radical scavenging activity
RT-PCR	Real time polymerase chain reaction
SIP	Sterilization in place
TPC	Total phenolic content
UV	Ultraviolet
VN	Vanillin
WT	Wheat

Chapter 1.

Work Outline

1.1 Thesis presentation

The present work was developed in association with FRULACT, an agro-food company founded in 1987 in Maia, where it has its headquarters. FRULACT mainly produces fruit based preparations for other food industries such as dairy, ice-creams, beverages, pastry and others. Approximately 90% of its clients are dairy industries, which makes this area their main focus of attention. FRULACT has several industrial units: in Portugal (Maia, Ferro and Tortosendo), France, Morocco and South Africa. Food Industry is a high competitive economic sector where differentiation, innovation and the development of new products are essential key strategic factors. In this framework, FRULACT has been investing efforts in research and development in order to answer its clients' needs and to reduce new products time-to-market. Thus, Frutech was created as a development centre to allow the company to follow the advances in food processing and the market new tendencies.

Nowadays, products with "clean label" are being increasingly demanded by consumers (FoodDIVE 2015). Basically, a product with "clean label" means that it is free of chemically-synthesized substances and others, that even though might be natural, have names that are not perceived as good ingredients for health by the consumer. One of the most used chemically-synthesized substances by FRULACT is the preservative potassium sorbate (E202). This preservative main function is to prevent the growth of fungi during the product shelf-life as well as to prevent contaminations by environmental sources when it is used by the client. However, consumers' negative perception about industrially synthesized antimicrobials due to a possible association with potential toxicological problems, has created interest in food industries for natural compounds with antimicrobial effect (Tomadoni *et al.* 2016). It is well known that plant phytochemicals have antimicrobial properties and several studies have pointed out their potential to be used in the food industry as natural preservatives (Juneja *et al.* 2012; Tomadoni *et al.* 2016; Aneja *et al.* 2014). This way, the main purpose of this study was the evaluation of the potential of natural phytochemicals, namely plant extracts rich in polyphenols, as

potassium sorbate substitutes. Plant polyphenols are of great interest in the food industry due to their numerous bioactive properties. Because of their antimicrobial and antioxidant activity they can prevent food spoilage and browning in products with fruits and vegetables, making it possible to reduce synthetic preservatives such as potassium sorbate and synthetic phenolic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA) (Pace *et al.* 2014). Polyphenols have also been reported as substances active in the prevention of several diseases and in the maintenance of a healthy lifestyle, making them potential substances to be part of a functional food product (Pace *et al.* 2014; Yao *et al.* 2004; Vladimir-Knežević *et al.* 2012).

1.2 Research objectives

The main goal of this work was the evaluation of the potential of a polyphenol-rich plant extract as a substitute of the commonly used preservative potassium sorbate (E202) and, therefore, if applicable, one could have a more natural final product free of artificial preservatives and with a functional activity. This hypothesis was tested in two formulations that were dosed in yogurt: one cereal preparation and one forest fruit preparation. This work comprised the following specific objectives:

- **To identify possible food spoilage fungi**: the cereals and fruits used in the preparations were analyzed with standard microbiological methods to quantify possible spoilage fungi. The factory environment in the Tortosendo unit was also screened in order to assess the most recurring fungi that eventually could contaminate the final product.

- **To test the antifungal activity of selected polyphenol-rich plant extracts**: the antifungal activity of selected phytochemicals was tested against the most recurring fungi identified in the previous bullet point.

- **To evaluate the effect of selected plant extracts in the final product:** the ability of selected extracts to prevent the growth of spoilage fungi in the preparations and in dosed yogurt was tested. Changes in organoleptic properties were also examined since the addition of phenolic compounds might provide an astringent flavor, often pointed by the consumer as a not appealing feature. As yogurt is a functional food itself, the extracts should not interfere with its natural lactic acid bacteria. This way, the effect of the extracts on the viability of the yogurt natural microflora was also assessed. The presence of phenolic

compounds in the yogurt and its antioxidant capacity was monitored over the yogurt shelf-life in order to evaluate if the food product maintains its functional properties.

The overall challenge consisted in the selection of a plant extract that while dosed in a certain percentage would act as a preservative, giving the final product functional properties, respecting its natural flavor and not interfering with its natural microflora.

1.3 Thesis organization

This work is divided into six chapters. Chapter 1 introduces and describes the main goals, background and motivations.

Chapter 2 provides a literature review about the potential of polyphenols in the food industry with a special emphasis in the use of these natural antimicrobials as a trend for a "clean label". The main chemical characteristics of polyphenols are described, as well as an extensive review about polyphenol-rich plant extracts antifungal properties. Their potential as a functional ingredient is also described in terms of health-promoting benefits. Industrial challenges related to the incorporation of polyphenols in food products are also analyzed, with a special emphasis in the ones related with polyphenols thermal stability.

Chapter 3, 4 and 5 have a brief introduction to the addressed subject, followed by the description of the materials and methods. The results and discussion are presented, followed by the overall conclusions. Chapter 3 focus on the characterization of the preparations' raw materials in terms of microorganisms at 30 ºC, yeasts and filamentous fungi, and on the fungal load in the air of the Tortosendo unit. Based on FRULACT experience, the most recurring yeasts and filamentous fungi were identified by real time polymerase chain reaction (RT-PCR).

Chapter 4 describes *in vitro* studies for the antifungal activity of selected extracts against the fungi identified in chapter 3. Chapter 5 describes the effect of the incorporation of the extracts in the final products in terms of microbiology, organoleptic and chemical properties.

Finally, chapter 6 provides an overview and achievements of the developed work, as well as suggestions for future work.

Chapter 2.

Literature review

2.1 Clean label products

The market of natural products with "clean label" has been growing worldwide (Penton 2015). Even though the term "clean label" is not yet well defined, and although the perception about what a product with "clean label" is, varies from person to person, it is clearly becoming a standard by the industry (Penton 2015; FoodDIVE 2015; FoodBusinessNews 2015). There are certain ingredients that clearly classify some food products as "unclean", which include high-fructose corn syrup, genetically modified ingredients and other substances such as artificial colours, flavours and preservatives, additives and the substances denoted by E numbers or other with names that consumers don't know or relate as artificial (Penton 2015). Nowadays, consumers are more conscious about nutrition, which has led to an increasing search for healthy foods with fewer synthetic ingredients (Penton 2015; FoodDIVE 2015). In the last two decades there has been an increasing demand for minimal processed foods with low levels of chemically-synthesized food additives (Juneja *et al.* 2012). Consumers are demanding products with fewer and recognisable ingredients that they relate as healthy (FoodBusinessNews 2015). Food producing companies are giving response by incorporating as many natural ingredients as possible that do not need to be declared as additives or that can label the products as "free from artificial ingredients" (FoodDIVE 2015).

2.2 Natural preservatives from plants

Control of food spoilage is mainly achieved by chemical control, generally using preservatives such as potassium sorbate (E202) or sodium sorbate (E201) (Raju & Bawa 2006). Consumers relate synthetic preservatives as artificial products, resulting in the rejection of foods containing these substances (Tribst *et al.* 2009). This negative perspective about industrially synthesized food preservatives has led to many studies regarding the search for natural antimicrobial compounds, so foods preserved with natural additives have become very popular (Daglia 2012; Tomadoni *et al.* 2016). Natural

antimicrobials have a purpose as a food preservative, extending the shelf-life, inhibiting the growth or killing spoilage microorganisms (Juneja *et al.* 2012).

Natural compounds with potential to act as food antimicrobials can be of microbial, animal or plant origin (Juneja *et al.* 2012). Plant-derived secondary metabolites such as phenolics and essential oils are of big interest, as many plant extracts have been shown to have antimicrobial activity against microorganisms related to food spoilage and safety (Rupasinghe *et al.* 2006). Essential oils contain antimicrobial compounds active against bacteria, yeast and filamentous fungi (Alvarez *et al.* 2014). Despite the good results obtained with essential oils in several studies and despite being classified as generally regarded as safe (GRAS) substances, their use in the food industry is very limited (Alvarez *et al.* 2014). This happens because the dose needed to exert antimicrobial effect surpasses what is accepted in terms of organoleptic properties (Juneja *et al.* 2012).

Polyphenols, besides essential oils, are another group of important bioactive phytochemicals, which are the most abundant secondary metabolites found in plants (Ignat *et al.* 2011). Polyphenols are known for their health-promoting effects and antimicrobial activity. Several studies have reported the potential of several plant extracts rich in polyphenols to inhibit the growth of spoilage fungi and bacteria, and have suggested their potential to be used in the food industry as natural preservatives (Tehranifar *et al.* 2011; Tomadoni *et al.* 2016; Daglia 2012). However, plant extracts can have an effect on the color, flavor, bitterness, astringency, odor and oxidative stability in food, which should be taken into account when applying the extract into a certain product (Pandey & Rizvi 2009). This thesis concerns just about antimicrobials of plant origin, namely plant extracts rich in polyphenols. Focusing in plant extracts rich in polyphenols rather than pure polyphenols is a marketing important aspect because to most consumers, polyphenols names sound like less appealing chemical stuff. For example, having a label with "pomegranate extract" is much more "consumer-friendly" than a label containing the terms "ellagic acid" and "punicalagin", which are the pomegranate extract's main ingredients.

2.3 Polyphenols

The term "polyphenols" includes more than 8,000 compounds with several different structures (Harborne & Williams 2000). Polyphenols can be divided into various subclasses depending on the basis of their origin, biological function or chemical

structure. Chemically, polyphenols are compounds with one or more aromatic rings with one or more hydroxyl groups (Pandey & Rizvi 2009). Polyphenols can be divided into different classes depending on their chemical structure: according to the number of phenolic rings, to the structural elements that bind the rings and to the substituents linked to the rings (Pandey & Rizvi 2009). Generally, polyphenols can be divided into flavonoids and non-flavonoids.

2.3.1 Flavonoids

More than 4000 flavonoids have been identified and the number is still growing (Ignat *et al.* 2011). Flavonoids are the most widely occurring phytochemicals in plants, and are the main pigment that colors flowers and seeds (Winkel-Shirley 2001). Flavonoids play diverse functional roles in plants, such as (1) protection against ultraviolet (UV) light; (2) protection against parasites and oxidative cell injury; (3) transport of auxins; (4) male fertility, among other functions (Falcone Ferreyra *et al.* 2012). Biological activities of flavonoids include antioxidant, antibacterial, anti-inflammatory, anticancer and antiviral activity (Kumar & Pandey 2013). In food, flavonoids are generally responsible for color, taste, protection of vitamins and enzymes and prevention of lipid oxidation (Yao *et al.* 2004). Flavonoids are compounds with 15 carbons and have the pattern structure C_6-C_3-C_6, in which two benzene rings are linked together by a group of three carbons and generally occur as glycosylated derivatives (Rauha *et al.* 2000). The arrangement of these three carbons determines how flavonoids are further divided and classified (Vermerris & Nicholson 2008). The major classes of flavonoids found in edible plants are flavones, flavanones, flavanols (also known as catechins), chalcones, anthocyanidins, isoflavones and flavonols (Ververidis *et al.* 2007). Flavanols and flavones are the most widely occurring and structurally diverse (Ignat *et al.* 2011). Moreover, flavanols can also be found as polymers, referred to as condensed tannins or proanthocyanidins. Flavones can be predominantly found in citrus fruits, isoflavonoids in legumes and flavones in herbs and anthocyanins and catechins in teas, fruits and vegetables (Yao *et al.* 2004).

2.3.2 Non-flavonoids

Concerning non-flavonoids, its main groups are phenolic acids. Phenolic acids can be further divided into: derivatives of benzoic acid (e.g., gallic acid, protocatechuic acid) and derivatives of cinnamic acid (e.g., coumaric acid, caffeic acid, ferulic acid), based on C_1-C_6 and C_3-C_6 skeletons, respectively (Cheynier 2005). Phenolic acids represent

approximately one third of the dietary phenols that can be present in edible plants in free and bound forms (Robbins 2003). In food, phenolic acids have been associated with color and sensory qualities as well as antioxidant properties (Quideau *et al.* 2011).

Other nonflavonoids classes include stilbenes (e.g., resveratrol) and complex molecules derived from them such as stilbenes oligomers, gallotannins, ellagitannins and lignans (Cheynier 2005). The structures of the main classes of polyphenols are elucidated in Figure 1.
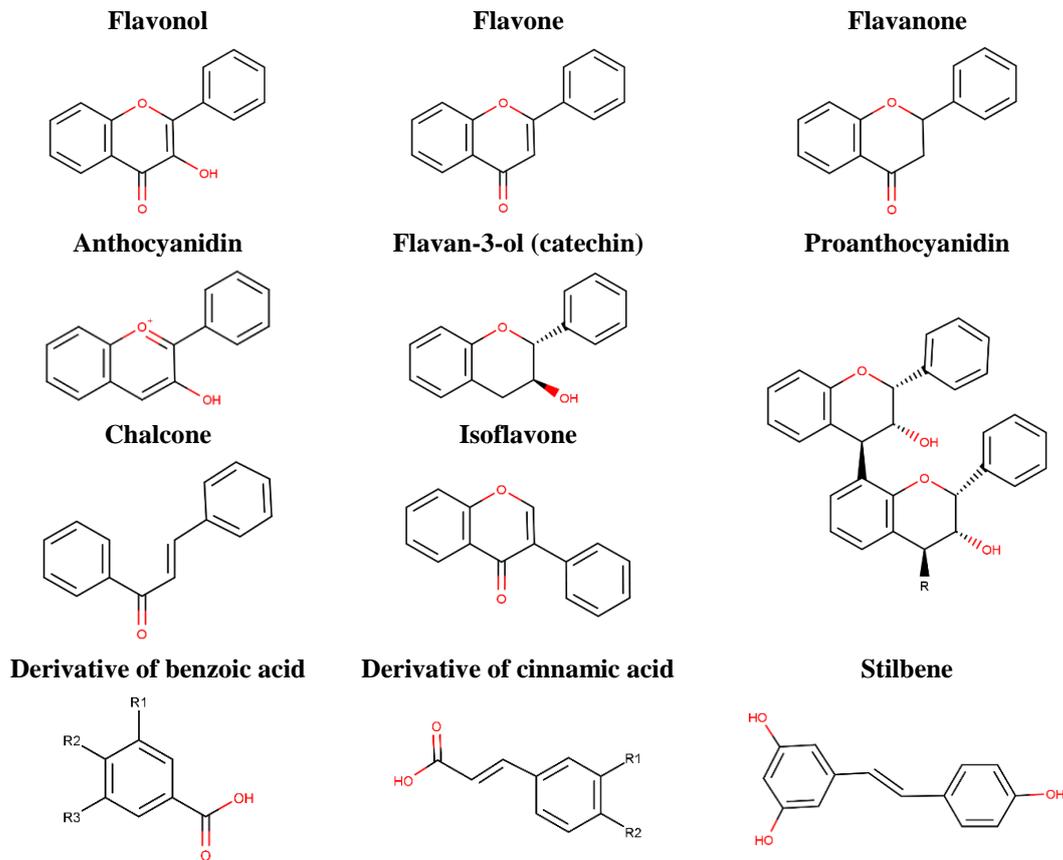
Figure 1. Chemical structures of the main classes of polyphenols.

2.4 Plant polyphenols as natural antimicrobial compounds

Plant polyphenols have been shown to exert antibacterial, antifungal and antiviral activity. Several studies have been done regarding the antimicrobial activity of polyphenols against food-borne bacteria such as *Salmonella typhimurium*, *Listeria monocytogenes* (Cetin-Karaca 2011) and *Escherichia coli* O157:H7 (Tomadoni *et al.* 2016), and their antifungal properties against human pathogenic fungi such as *Candida albicans* (Hirasawa & Takada 2004). However, studies regarding the effect of

polyphenols on the growth of spoilage fungi are scarce. This thesis focus on the antifungal activity of polyphenols against food spoilage fungi.

Flavonoids have shown to exert potent antifungal activity against a variety of fungi such as *Aspergillus* spp. and *Penicillium* spp.(Juneja *et al.* 2012). It was shown that flavonoids from chickpea and soybeans can have a potent antifungal activity against *Aspergillus ochraceus*, *Penicillium digitatum* and *Fusarium culmorum*, which are fungi that contaminate food (Krämer *et al.* 1984). Weidenbörner *et al.* (1990), also tested flavonoids antifungal activity against several species of *Aspergillus.* They found that an unsubstituted flavone and flavanone in concentrations ranging between 0.05 and 0.8 mM, where highly active against *A repens, A. amstelodami, A. chevalieri, A. flavus* and *A. petrakii.*

Pizzolitto *et al.* (2015) studied the effect of natural phenolic compounds on *A. parasiticus* growth. *A. parasiticus* is of a great importance in foods and feeds because it produces toxic metabolites, more specifically aflatoxins (Hocking 2001). They found that among the tested compounds, isoeugenol, carvacrol and thymol showed antifungal activity with MIC values of 1.26 mM, 1.47mM and 1.50 mM, respectively. Additionally, they also reported that creosol, p-cresol, o-cresol, m-cresol, vanillin and phenol had no antifungal activity within the range tested for the minimum inhibitory concentration (MIC) (6.19 to 11.68 mM).

Some plant extracts rich in polyphenols have been demonstrated to exert an antifungal activity. Tea is rich in polyphenols, mainly in catechins, which are the tea main active ingredient (Perumalla & Hettiarachchy 2011). Tea polyphenols have shown to have a good antifungal activity against fungi such as *Botrytis cinerea*, *Monilinia fructicola* and *Rizophus stolonifer*, which are post-harvested fruit pathogens (Yang *et al.* 2013; Chen *et al.* 2013; Yang & Jiang 2015). The minimum inhibitory concentration of tea polyphenols against *R. stolonifer* was shown to be 4 mg/mL and the minimum fungicidal concentration was 32 mg/mL (Yang & Jiang 2015)

Grapefruit extract, which is rich in naringin, nobiletin (which are flavonoids) and gallic acid (Xi *et al.* 2015), have shown antimicrobial activity against several bacteria and fungi. For example, a self-made grapefruit ethanolic extract, containing 3.92% of polyphenols, showed antifungal activity against *Saccharomyces cerevisiae* at concentrations up to 8.25% (Cvetnic & Vladimir-Knezevic 2004).

Pomegranate extracts, rich in polyphenols, namely flavonoids, condensed tannins and hydrolysable tannins have been tested for their antimicrobial and antifungal activity (Tanveer *et al.* 2014). For example, Mostafa *et al.* (2011) studied the antifungal and antiaflatoxigenic activities of methanolic plant extracts against a toxigenic *A. flavus* isolate. They found that pomegranate extract completely arrested the production of aflatoxin B1 at 0.5% and inhibited its growth at 1%. The pomegranate extract was analyzed by gas chromatography mass spectrometry, showing that it is rich in phenolic compounds, mainly composed by ellagic acid (37%), pedunculagin (6.4%), punicalagin (5.6%) and lumicolchicine (4.68%). Following pomegranate extract, ginger extract, which is rich in gingerol (47%), cedrene (8.4%), zingiberene (7.4%) and alfa-curcumene (7.3%), also showed significant antifungal and antiaflatoxigenic activity, completely inhibiting both the production and growth of aflatoxin B1 at 1.5% and 2%, respectively. The authors suggest that the phenolic compounds, as the main extract constituents, play a major role in growth and aflatoxin inhibition. Dahham *et al.* (2010), also studied the effects of pomegranate rind, seeds and whole fruit extracts, against different species of fungi. They found that methanolic extracts of pomegranate had high antifungal activity against *A. niger, P. citrinum, R. oryzae* and *Trichoderma reesei*, with rind extract having the highest inhibitory zone for all tested fungi. Pomegranate extract was also shown to have antifungal activity against *P. italicum, R. stolonifer* and *B. cinerea.* The seed and peel extract are the richest in polyphenols, and are the ones showing highest antifungal activity (Tehranifar *et al.* 2011). Pomegranate extract have already been tested as natural antimicrobial on strawberry juice (Tomadoni *et al.* 2016). Although it was effective in reducing mesophilic and psychrophilic bacteria, it showed no effect on yeast and mould population, at the concentrations tested (180 and 360 µg/mL). On the other hand, in this same study, vanillin showed efficacy as a natural antimicrobial, extending the microbiological shelf-life of the product, at the tested concentrations (2.5 and 5 mg/mL) (Tomadoni *et al.* 2016).

Vanillin is widely used as a flavoring compound and is also known to have antifungal and bacteriostatic effects (Rupasinghe *et al.* 2006). In a study carried out by Fitzgerald *et al.* (2004), vanillin has shown to be effective against *S. cerevisiae* and *C. parapsilosis* with a minimum inhibitory concentration of 17 and 9 mM, respectively, in laboratory media. The efficacy of vanillin as a preservative was also studied in soft drinks and fruit juices, and it was shown to vary depending on the storage temperature. Vanillin showed

to be effective at concentrations of 20 and 10 mM against *S. cerevisiae* and *C. parapsilosis,* respectively. This compound was able to completely inhibit, over 8-week storage at 25 ºC, both yeast strains that were inoculated in an apple juice and a soft drink at a level of approximately 10^4 colony forming units (CFU)/mL. In another study, Cerrutti and Alzamora (1996) showed that vanillin inhibited the growth of *S. cerevisae, Zygosaccharomyces rouxii, Debaryomyces hansenii* and *Z. baili* in culture media and apple purée containing 2000 ppm of vanillin for 40 days storage at 27 ºC. Vanillin (3 -7 mM) was also shown to inhibit the growth of *A. flavus, A. niger, A. ochraceus* and *A. parasiticus* for two months when incorporated into fruit-based agar (López-Malo *et al.* 1995).

Grape seed extract, which is a valuable by-product from wine processing, is rich in polyphenolic compounds such as catechin, epicatechin and epicatechin-3-O-gallate (Perumalla & Hettiarachchy 2011). The antimicrobial activity against several food-borne pathogens demonstrated by the grape seed extract is attributed to its high content in polyphenols (Perumalla & Hettiarachchy 2011). Despite its efficacy against bacteria, studies regarding its effectiveness against fungi are scarce.

Resveratrol, which is a stilbene, has been known for its broad spectrum antifungal activity (Jeandet *et al.* 2002). Extracts rich in resveratrol can be obtained from grape skins, berries and medicinal plants (Alvarez *et al.* 2014). Resveratrol has been demonstrated to be effective against *B. cinerea*, *S. cerevisiae, A. niger* and *P. expansum* (Filip *et al.* 2003).

There are a wide variety of natural plant extracts rich in polyphenols with antifungal and antibacterial activity. The selection of extracts with potential to be incorporated into food products as natural preservatives is not a straightforward task. Besides the selection of compounds with antifungal activity, one should not forget about the changes caused in the final product when the plant extract is used in the food formulation.

2.5   Polyphenols as a functional ingredient

2.5.1   Functional foods

Polyphenols have many bioactive properties. If in one hand they act as natural antimicrobial compounds, on the other hand their intake have an effect on human health, thus acting as antioxidants, anti-allergic, anti-inflammatory, anticancer, and antihypertensive, just to mention a few (Daglia 2012; Cheynier 2005; Pandey & Rizvi 2009). These properties make them suitable to be used in functional foods. According to

the International Food Information Council (1999), functional foods are "foods or food components that may provide benefits beyond basic nutrition". The consumption of functional foods are related with prevention of cancer, diabetes, atherosclerosis, between others (Hasler 2002).

The global market of functional foods had an annual growth rate of 6% between 2011 and 2015 and it is expected to grow 25% by 2017 (Statista 2015; Leatherhead food research 2013). In 2010 the total sales of functional foods worldwide had a value of 190 billion U.S. dollars (Statista 2010). Among the most popular categories are probiotic functional foods, that had a global retail sales value of approximately 31 billion U.S. Dollars in 2014 (Statista 2014). Thus, there has been an extensive search for the development of novel functional foods.

The main goal of this project was the evaluation of the enrichment effects of fruit and cereal preparations by adding polyphenols. Since these formulations are normally incorporated into yogurts, the sensory features of the final product were also evaluated. Yogurts are between the most common dairy products consumed worldwide and are a functional food itself (El-Said *et al.* 2014). Yogurt is obtained by the fermentation of milk by thermophilic lactic acid bacteria like *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. It has good nutritional properties due to its high levels of potassium, calcium, protein and vitamins and it is also an excellent vehicle to deliver probiotics, which improve the intestinal environment and enhance the body immunity (Pereira *et al.* 2013).

The recommended daily intake of polyphenols is approximately 1 g (Georgé *et al.* 2005).Consuming functional foods with polyphenols is a convenient way to increase polyphenols daily intake. Due to their antioxidant activity, they are becoming popular in the development of yogurts with antioxidant properties (Gad & El-Salam 2010; Sun-waterhouse *et al.* 2013). It has been suggested that fruit juices, extracts and powders rich in polyphenols are prone to be incorporated into dairy products, thus resulting in a final product with bioactive compounds (Sun-waterhouse *et al.* 2013; Coïsson *et al.* 2005; Najgebauer-lejko 2014).

2.5.2 Polyphenols health promoting benefits

Several epidemiological studies have shown that the consumption of a polyphenol-rich diet is associated with a lower risk of chronic human diseases, especially those associated with oxidative stress (Pandey & Rizvi 2009). Oxidative stress can be

defined as an imbalance between the production of free radicals, also called reactive oxygen species (ROS), and their elimination by antioxidants (Vladimir-Knežević *et al.* 2012). The excess of free radicals can cause oxidative damage to lipids, proteins, carbohydrates and DNA in cells and tissues, which can lead to many chronic diseases such as atherosclerosis, cancer, diabetes, autoimmune and neurodegenerative disorders (Uttara *et al.* 2009). Antioxidants are responsible for the removal of free radicals by (1) scavenging ROS or their precursors; (2) inhibiting ROS formation; (3) blocking the action of some enzymes; (4) and chelating metal ions involved in the free radical production (Uttara *et al.* 2009; Vladimir-Knežević *et al.* 2012). In case of overproduction of ROS, external supply of antioxidants is needed to countervail the consequences of oxidative stress. Polyphenols have been proposed to act as antioxidants, scavenging free radicals or suppressing its formation (Vladimir-Knežević *et al.* 2012). Polyphenols antioxidant activity makes them fundamental compounds to prevent the incidence of oxidative stress related diseases, such as cardiovascular diseases, cancer, diabetes, aging-related diseases, neurodegenerative diseases and hypertension (Pandey & Rizvi 2009).

2.5.3 Yogurts and polyphenols

There are several products in the market that combine yogurts with polyphenols. For a functional yogurt it is important to assure a maximum retention of polyphenols and their stability during storage. It is also important to assure the count number and metabolic activities of the starter cultures (Sun-waterhouse & Zhou 2012; Kailasapathy *et al.* 2008). Figure 2 shows some examples of products in the market that combine yogurts and polyphenols: "Iogurtes Nutrégi AntiOxidantes" that contain vitamin E and grape polyphenols; "Danone Silhouette 0+" , "Provamel apple green tea and lime lemon balm yogurts" and "Chobani greek yogurt "that contain natural green tea extract.

| (1) | (2) | (3) | (4) |

Figure 2. Some examples of yogurts with polyphenols from: (1) Nutrégi; (2) Danone; (3) Provamel and (4) Chobani.

2.6   Industrial challenges

The search for natural antimicrobials able to substitute the most used chemically-synthesized preservatives, like sodium benzoate (E201) and potassium sorbate (E202), is not a straightforward task. Many aspects should be taken into account as discussed below.

Addition of natural preservatives such as plant extracts usually increases the cost of a finished product (Alvarez *et al.* 2014). Plant extracts are subjected to extraction and refinement procedures that usually increase their cost, when comparing to synthetic chemicals. However, if the extracts are recovered from industry wastes such as the peel or seeds, one can have another scenario. Also, these antimicrobials should be added at concentrations able to produce the desired effects, while not affecting the natural flavor of the product. For example, polyphenols have been reported to confer an astringent flavor, so the dose applied should take this into account (Manach 2004). One should also not forget, that many times, substances that have shown antimicrobial activity *in vitro* have little or no effect in food matrices due to their interaction with food components such as carbohydrates, lipids and proteins (Alvarez *et al.* 2014). For example, polyphenols are known to interact with proteins mainly by hydrogen and hydrophobic bonds. These interactions can reduce polyphenols bioavailability and bioactivity (Ozdal *et al.* 2013).

During food processing many phytochemical compounds are susceptible to degradation due to factors such as temperature, light, oxygen, just to mention a few (Parisi *et al.* 2014). Therefore, special attention should be paid to the food processing technologies in order to preserve the quality of the bioactive compounds. Regarding polyphenols, its thermal stability is an important issue in food industry, since one wants to ensure that these compounds maintain the desired properties, activity and structure during different stages of processing such as pasteurization. One important aspect to bear in mind is the proper moment in the process to add polyphenols to the fruit and cereal formulations. It is important to know if polyphenols are still stable during the pasteurization to determine if they should be added before or after this operation. There are some studies on the thermal stability of polyphenols in terms of antioxidant activity, but there are fewer, when it comes to antimicrobial activity. According to some examples found in the literature it can be feasible to add polyphenols before pasteurization, but accordingly it depends on the binomial time-temperature. For example, apple polyphenols

have been reported to have a good stability, as shown by Chen *et al.* (2014) while studying the effect of heat treatment in apple polyphenol samples. With the rise of temperature from 70 to 95 ℃, the concentration in apple polyphenols decreased, but just slightly when compared with a sample control not subjected to heat treatment. Thus, apple polyphenols extracted from apple pomace could be used in several food products as a functional food substance and as natural preservative in foods subjected to pasteurization. In another study, Fischer *et al.* (2013) showed that the pomegranate juices' antioxidant capacity and total phenolic amounts were not markedly lowered upon heating at 90 ℃. Moreover, the bioactive compounds made responsible for the health-promoting effects of the pomegranate juices were not significantly affected after heating (Fischer *et al.* 2013). When it comes to green tea extracts, epigallocatechin gallate (EGCG) from green tea is probable to be affected by pasteurization, since it is vulnerable to heat treatment (Seto *et al.* 1997). The decrease in EGCG concentration depends on the used time/temperature pasteurization ratio. For example, the (30 seconds/90 ℃) pasteurization of tea beverage enriched with EGCG just slightly affects EGCG stability (Bazinet *et al.* 2010). Anthocyanins present a very high thermal sensitivity (Patras *et al.* 2010). However, in blueberry juices, the short pasteurization time used, typically 60-90 seconds at 90 ℃, generally results in minor losses of anthocyanins (Brownmiller *et al.* 2008; Skrede *et al.* 2000; Lee *et al.* 2002; Srivastava *et al.* 2007). Moreover, Volf *et al.* (2013) showed that polyphenols stability decrease with the increase of temperature, for example catechin from grape seed was shown to have a degradation rate of approximately 13% at 60 ℃ and 25% at 100 ℃, while gallic acid showed a degradation rate of approximately 12% at 60 ℃ and 20% at 100 ℃. In another study, the polyphenolic content, color and antioxidant activity of red grape pomace peels were not significantly affected at a drying temperature of 60 ℃. However, significant reduction in total extractable polyphenols and condensed tannins was observed, as well as a reduction of the antioxidant activity when the extract was subjected to higher temperatures, 100 and 140 ℃ (Larrauri & Rupe 1997).

Since the polyphenol properties should be preserved during the product life-cycle, the stability during storage is an important key factor. Bazinet *et al.* (2010) studied the effect of long-term storage of commercially green tea beverages on catechins stability. They found that the stability of catechins during long-term storage was best at a low temperature, 4 ℃, and acidic pH, 4.0, being relevant within the present study since these storage conditions met yogurts storage temperature and pH.

Chapter 3.

Raw materials and factory environment microbiology

3.1 Introduction

Due to their versatile environment requirements, yeasts and filamentous fungi have a great ability to attack many types of food, causing them premature spoilage. In food and drink matrices, yeasts usually grow in planktonic form, while filamentous fungi tend to grow on the surface of foods, creating a visible mycelium (Pitt & Hocking 2009). These microorganisms can cause different degrees of deterioration, and can invade and grow on virtually any type of food at any time. These spoilage fungi can be present in the raw materials, in our case, in the fruits and cereals, and consequently perpetuate into the food chain. Cross-contamination between several raw materials is a very common situation, especially when these products are stored and manipulated in the same space. Spoilage fungi can also be introduced during food processing or subsequent storage and via airborne spores. (Dijksterhuis *et al.* 2013). In the food processing chain and plant, some fungal spoilage organisms can develop a significant amount of biomass and act as a recurrent source of contamination when a proper cleaning strategy is not established (Dijksterhuis *et al.* 2013). In this field, well projected and implemented Hazard Analysis Critical Control Points (HACCP) systems should be considered not only as a legal duty, but as part of the organization quality strategy and policy.

Several foodborne moulds and yeasts can be hazardous to human or animal health. Some filamentous fungi can produce mycotoxins, so careful should be taken in the food industry, because, even though the microorganisms may not survive to food preparation and pasteurization, their mycotoxins may still be present. The presence of moulds and yeasts is mainly problematic in industry plants that produce foodstuffs with high sugar content, and/or low water activity, and/or low pH. This includes factories that produce fruit products, as is the case of FRULACT, factories of fermented dairy products, which includes the great majority of FRULACT's clients, among other industries such as confectionary and baking industries. Yeasts are very likely to cause spoilage in foods such as fruits and soft drinks because they contain fermentable sugars and have a low

pH (3 to 5) together with a high concentration of soluble carbohydrates (Deak & Beuchat, Larry 2000).

There is a relationship between certain types of food and the type of contamination that can occur. For example, normal fungal microflora of fruits can include moulds such as genus *Aspergillus, Penicillium, Rhizopus* and *Wallemia* and yeasts like genus *Saccharomyces, Zygosaccharomyces* and *Pichia* that should remain in the skin of fruits, but can entry into the soft tissue with the occurrence of cuts or bruises during postharvest processes (Kalia & Gupta 2006). In this work, the tested raw materials were fruits: blueberry, raspberry and blackberry, and cereals: oats, wheat and barley. As found in the literature, fungi mainly associated with blackberries, raspberries and blueberries include *Alternaria* spp*., Botrytis cinerea, Rhizopus stolonifer* and *Mucor mucedo* (ICMSF 2005; Dennis & Mountford 1975). Some thermoresistant fungi may also be associated with berries as is the case of *Neosartorya fischeri*. It is a ubiquitous fungus that commonly grows in soil, being frequently found in fruits at the ground level (Girardin *et al.* 1995). This fungus is thermoresistant and produces high numbers of spores, particularly ascospores. These characteristics make this kind of fungi very relevant, as it may survive pasteurization and so, it is very common in the spoilage of heat-processed fruit products in food industries (Girardin *et al.* 1995). Regarding dry cereals, the most common fungi found in cereals during cultivation, harvest, storage and transport include yeasts such as *Candida, Cryptococcus, Pichia, Sporobolomyces, Rhodotorula* and *Trichosporon*; and filamentous fungi like *Alternaria, Aureobasidium, Cladosporium* and *Claviceps* (Pitt & Hocking 1997). During processing, cereals are likely to be contaminated by xerophilic *A. glaucus* and *Penicillium* spp. (Oliveira *et al.* 2014)

In yogurts, particularly in the ones containing fruit, yeasts are very common and can sometimes cause spoilage with the production of gas and off-odors (Pitt & Hocking 2009; Fleet 1990). For instance, the shelf-life of yogurts can be quickly shortened mainly due to the growth of species like *Candida famata* and *Kluyveromyces marxianus* (Fleet 1990). Contamination by yeasts, usually has to do with the used fruits and/or poor hygienic practices during packaging operations (Deak 2007). What makes yeasts prone to grow in yogurts are their ability to grow at low refrigeration temperatures (< 10 ºC), and to metabolize the proteins, organic acids, and carbohydrates present in the yogurt (Fleet 1990).

This chapter describes the work developed as a requisite imposed by FRULACT. It had two main objectives: the first was the quantitative and qualitative analysis of the raw materials' microbial load in terms of microorganisms at 30 ℃, yeasts and moulds. The tested raw materials were the fruits and cereals used in the preparations' formulations; the second main objective was the analysis of the air fungal load of several departments in the FRULACT industrial unit located in Tortosendo. The most recurring moulds and yeasts obtained in these steps were isolated and identified by RT-PCR and used to determine the efficacy of selected compounds in chapter 4.

3.2   Materials and methods

3.2.1   Raw materials tested

The raw materials of a fruit preparation and the raw materials of a cereal preparation were examined for the quantification of the microbial load. For each raw material, several lots were assessed accordingly to their availability at FRULACT. The raw materials examined are described in Table 1.

Table 1. Raw materials tested for assessment of the microbial load

Preparation	Raw material
Forest fruit preparation	Blackberry (BA) (frozen, whole)
	Raspberry (RA) (frozen, pieces)
	Blueberry (BU) (IQF, wild)
Cereal preparation	Wheat (WT) (bran)
	Oatsl (OT) (flour)
	Barley (BR) (flour)

3.2.2   Preparation of samples

The microbial load of the raw materials was quantified in terms of microorganisms at 30 ℃, yeasts and moulds according to ISO 4833-1:2013 and ISO 21527:2008, respectively. The fruits and oats were stored at -20 ± 1 ℃ and were thawed before handling, and wheat and barley were stored at 4 ± 1 ℃. Briefly, the raw materials (10 g) were mixed with sterile Buffered Peptone Water (BPW) (Merck, Germany) (90 mL). This mixture was processed and blended in a BagMixer® (400P interscience) for 90 seconds, using a "stomacher" bag. After processing, the samples were left to rest for about 30 minutes to 1 hour. This procedure was done in duplicate for each lot of raw material

analyzed. Serial dilutions of each sample were made in Maximum Recovery Diluent (MRD) (Liofilchem, Italy) for counting purposes.

3.2.3 Culture of microorganisms from samples

The culture media for the quantification of microorganisms at 30 ℃ was Plate Count Agar (PCA) (VWR Chemicals, Belgium). The microorganisms were cultivated using the pour-plate method: 1 mL of sample of each dilution was placed into a sterile Petri dish and then, 15 to 20 mL of sterile molten PCA was added to the plate and mixed well with the sample. The plates were incubated at 30 ± 1 ℃ for approximately 72 hours. This procedure was done in duplicate.

The culture media used for the enumeration of yeasts and filamentous fungi was Rose-Bengal Chloramphenicol Agar (RBC) (Liofilchem, Italy). The microorganisms were cultivated using the spread-plate method: 0.1 mL of sample of each dilution was pipetted onto the surface of a plate containing sterile solid RBC, and then the sample was spread over the media surface using a sterilized glass spreader. This procedure was done in duplicate. The dishes were then incubated at 25 ± 1 ℃, for at least 5 days and for a maximum of 7 days.

3.2.4 Colony count

The colony count was done after the incubation period, whenever possible. When it was not possible, the plates were stored at 4 ± 1℃ for a maximum of 2 days. For microorganisms at 30 ℃, the number of Colony Forming Units (CFU) per gram of raw material was calculated according to ISO 4833-1:2013. Yeasts and filamentous fungi were enumerated according to ISO 21527:2008.

3.2.5 Collection of samples from the Tortosendo unit

In the Tortosendo unit, environmental air samples were collected in the several departments: (1) production; (2) fruit preparation room; (3) weighing of dry raw materials room; (4) washing room (section of Sterilization In Place - SIP, section of Cleaning In Place - CIP, section of washing); (5) refrigerated storage room; (6) freezing storage room; (7) and dry raw products storage room. For collection of the environmental samples, 3 places inside each department were chosen and Petri dishes with sterile Potato Dextrose Agar (PDA) (VWR Chemicals, Belgium) were left open for 10 minutes. In each zone, three different heights were sampled (1, 2, 3), in which height 1 is at the floor level, height 2 is approximately 1.20 m and height 3 is approximately 2.10 m. Although a volumetric

air sample would be much more reliable, the adopted method gives a direct indication of the types of fungi that are likely to come into contact with the food product.

3.2.6 Isolation of pure cultures of yeasts and filamentous fungi

The most often recurring colonies of yeasts and moulds were isolated for further tests. The selected cultures were subcultured in PDA. Yeasts were isolated scratching a sterile loop containing yeast biomass in a Petri dish containing PDA. For the isolation of filamentous fungi, a cube of approximately 5×5×5 mm of RBC (in case of raw materials' isolates) or PDA (in case of factory environmental isolates) containing the chosen colony was cut using a sterilized loop and placed in the middle of a PDA plate. The isolated cultures were incubated at 25 ± 1 ℃ for 7 days. After the incubation period, the cultures were stored at 4 ± 1℃ until reused.

3.2.7 RT-PCR for yeasts and filamentous fungi identification

RT-PCR was performed for selected isolated cultures of yeasts and filamentous fungi. The identification of these microorganisms was carried out by the company BIOPREMIER (Lisbon).

3.3 Results and Discussion:

3.3.1 Raw materials' microbial load

The microbial load of the raw materials are following presented in the form of figures and tables. Figure 3 shows the microbial load in terms of microorganisms at 30 ℃, yeasts and moulds for the fruits and cereals tested. Several lots of the same raw material were analysed. The most recurring and significant filamentous fungi and yeasts found were identified by RT-PCR and are discriminated in Table 2.

Raspberry and blackberry had the highest microbial load among the tested fruits. It is known that the type, composition, shape and surface of the fruit affects its microflora. If in one hand blueberries have a smooth and hard skin, which makes them impermeable to most microorganisms, on the other hand, raspberries and blackberries have rough surfaces with numerous indentations and protuberances, having a higher surface area for the attachment of microorganisms. Additionally, due to their significantly thinner skin, these fruits are more susceptible to breakage, and therefore making easier the contamination of the inner tissues. These results are in accordance with Tournas and

Katsoudas (2005) research. These authors studied the mould and yeast flora in berries and concluded that blueberries were the less contaminated fruit.

■ Microorganisms at 30 ℃ ▨ Yeasts ▨ Filamentous fungi   ▽ Log(CFU/g) < 1
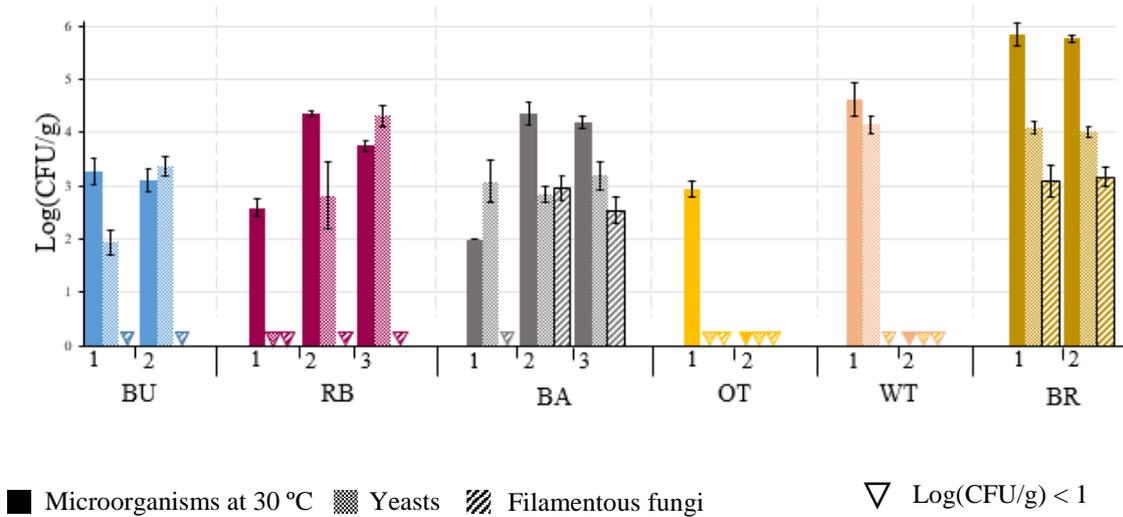
Figure 3. Microbial load of the raw materials in terms of microorganisms at 30 ℃, yeasts and moulds. BU refers to blueberry, RB to raspberry, BA to blackberry, OT to oats, WT to wheat and BR to barley. Numbers represent different lots of the same raw material. Bars represent average and error bars the standard deviation.

The most abundant fungi found in the tested fruits were *Cladosporium* sp., *Penicillium* sp. and *Mucor piriformis*. *Penicillium* and *Aspergillus* species are very common in berries as reported in several studies (Tournas & Katsoudas 2005; Kalia & Gupta 2006; Barth *et al.* 2009). Conidia of *Cladosporium* species are very prone to aerial dispersal, which facilitates environmental contamination (Pitt & Hocking 2009a). The presence of *Penicillium* is not surprising, since *Penicillium* species are ubiquitous and are undemanding nutritionally (Pitt & Hocking 2009a). *Mucor piriformis* have been found in soil, surface plant residues, air and moist environments (Pitt & Hocking 2009a). It causes rot of cold pears, apples and tomatoes and is a destructive pathogen of fresh strawberries (Pitt & Hocking 2009a; Sommer *et al.* 2002). Its presence on blackberry, probably has to do with contamination via air or via contact with surfaces that had been in contact with this fungus. As FRULACT deals with many types of fruits, mainly with strawberries, it is probable that sometimes some cross-contamination can occur. *Peyronellaea glomerata*, isolated from blackberry and that causes plant diseases, is an ubiquitous species that have been found on inorganic materials (Aveskamp *et al.* 2008). Thus, its presence in the tested blackberries was likely to be due to contact with a contaminated surface. The same explanation can be used to justify the presence of *Chaetomoium* sp. in raspberries, since this is a fungus commonly found in soil, air and decaying plant material. The mentioned spoilage microorganisms can be introduced during crop growth, harvesting, handling,

storage, distribution and processing. Due to their ground proximity during growth, most observed microorganisms on berries are soil inhabitants and fungal spores that can be further present in any food contact surface during the whole process (Barth *et al.* 2009).

Table 2. Identified occurring fungi in the raw materials. More information about the isolated fungi can be found in the appendix, Table A.1.

Raw material	Occurring fungi
Blackberry	*Cladosporium* sp.*, Cryptococcus* sp.*, Mucor piriformis, Penicillium* sp., *Peyronellaea glomerata*
Blueberry	*Cryptococcus* sp.
Raspberry	*Chaetomium* sp., *Cladosporium* sp., *Cryptococcus* sp.
Oats	*Penicillium* sp.
Barley	*Cladosporium* sp., *Cryptococcus* sp. *Mucor piriformis*
Wheat	*Aspergillus* sp., *Cryptococcus* sp.*, Mucor piriformis*, *Penicillium* sp.

Among the tested cereals, oats presented the lowest microbial load, while barley had the highest one. Oats were stored in the freezing chamber (-20 ℃), while barley and wheat were store in refrigerated conditions (4 ℃). Freezing conditions inhibit the proliferation of microorganisms, which explains oats lowest microbial load. As barley and wheat were stored at the same conditions, the differences in the microbial load might be related with different values of water activity (a_w). Moulds cannot grow when the water activity is below 13% (Beverly 2014). So, the highest level of contamination of barley can be related with its higher water activity. It is important to refer in this analysis that this property can be intrinsic, or can be eventually caused by differences in temperature and moisture due to improper handling and storage.

The most recurring fungi found in cereals were *Cladosporium* sp.*, Penicillium* sp. and *Mucor piriformis*. These same fungi were also identified in wheat by Al-defiery (2015) and are reported to be very common in cereal grains and flours (Pitt & Hocking 2009a; ICMSF 2005a; Tinatin 2010). It is important to mention that barley and wheat were stored at 4 ℃, and they come in flour and bran formulation, respectively, with lower water activity. Nevertheless, food storage at this temperatures is often accompanied by the growth of cold-tolerant fungi that can grow at low a_w such as the genus *Penicillium*, *Cladosporium*, *Alternaria* and *Fusarium,* among others (Dijksterhuis *et al.* 2013). Additionally, fungal spores introduced during any step of the distribution and processing chain, may still be present, although dormant, during storage, and germinate when there are propitious conditions.

When it comes to yeasts, these are very common and are associated with all raw materials except oats. It is important to notice that the counting of yeasts was done long before the identification of each species. Some *Pseudomonas* species are resistant to chloramphenicol (Fernández *et al.* 2012; Li *et al.* 1994) being able to grow on RBC media. As it forms colonies very similar to yeast's, it is likely that yeast counting is overestimated. In fact, this bacteria was isolated from RBC plates and was found in all raw materials, except oats. *Pseudomonas* species are ubiquitous in nature, soil and water, and constitute the normal microflora of fruits (Kalia & Gupta 2006). *Pseudomonas* spp. have a complex enzymatic system and simple nutritional requirements, which allows it to adapt to a wide variety of habitats. Many species of *Pseudomonas* are psychrotrophic, which means that they have a great ability to spoil chill foods (Franzetti & Scarpeluni 2007). Wheat and barley may have been contaminated with *Pseudomonas* species during crop or harvest and their storage conditions, 4 °C, do not prevent its growth.

Cryptococcus sp. was present in all raw materials, except oats. Other species of yeasts were also present but were not sequenced. According to available literature, yeasts mainly associated with the surface of fruits include species of *Rhodotorula*, *Cryptococcus* and *Sporobolomyces* (Fleet 2003). In cereals, *Cryptococcus* species have already been identified and other yeast species belonging to the genus *Candida, Rhodotorula, Saccharomyces* and *Trichosporon*, among others (Tudor & Board 1993).

From the results obtained it is also patent that in some cases, the microbial load varies from one lot to another. This situation is very noticeable between the different lots of wheat. This may be related with several factors: (1) the expiration date of the lot; (2) the storage time; (3) if the lot had already been opened and managed by several people; (4) or if the lot is brand new. If the lot had already been opened several times, it is more prone to be contaminated by spore producing moulds such as *Penicillium* and *Cladosporium.* Another possible reason for the differences between lots might be related to harvesting and selection operations.

3.3.2   Analysis of the air microbiology of the Tortosendo unit

The environmental air of the Tortosendo unit was analysed in order to identify the most recurring fungi. For a better understanding of the dynamics and connection between the several departments, a blueprint of the factory is presented in Figure 4.
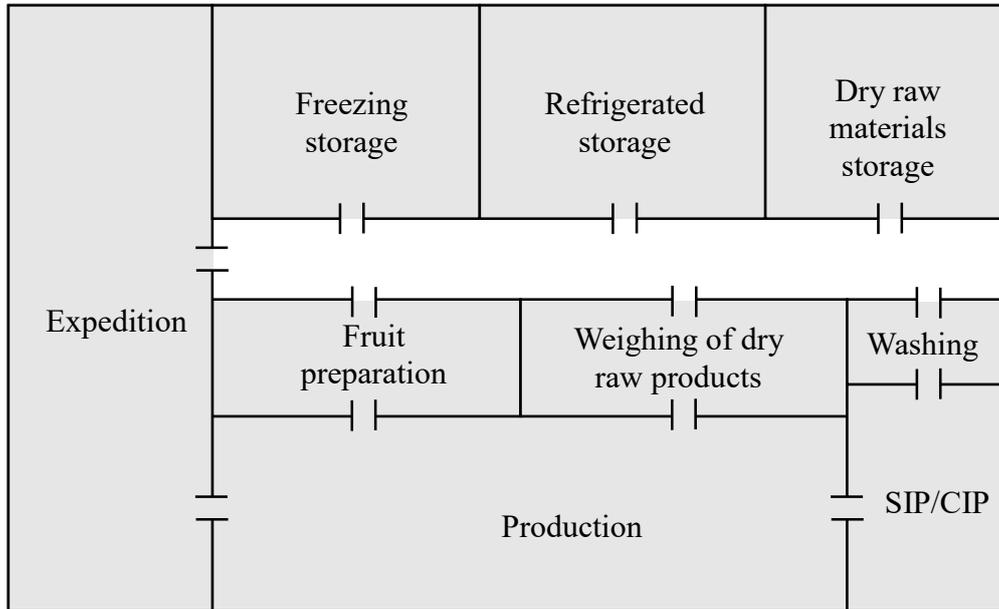
Figure 4. Tortosendo factory simplified blueprint showing the main areas. The figure is not to scale.

In the production room the fruit is processed and the final product is handled and packaged in metallic containers with different weights. This is the biggest room which has a direct connection with the fruit preparation room. In this latter, the fruits are cut and milled according to detailed process specifications. This area has proper ventilation in order to reduce the contamination risk. The production room has also direct access to the weighing of dry raw products room, where dry raw products such as flours and sugars are weighted. In the washing room there are three sections: the washing zone, where leftovers of fruits from trolleys are washed; the CIP, where the final product containers are washed; and the SIP, where the containers are sterilized. Dry, refrigerated and freeze raw products are stored in the busiest rooms. The surrounding areas, like the corridor and the expedition zone have a refrigerated environment to reduce possible oscillations in temperature.

The most significant fungal isolates were identified by RT-PCR and are discriminated in Table 3. These microorganisms were selected accordingly to their ubiquity and colony morphology. Although other fungi were found, they were not sequenced. The factory mycobiota is very dynamic and strictly correlated with the processing operations and areas. For example, activities that generate humidity in combination with the obstruction of venting contribute to mould growth (Dijksterhuis *et al.* 2013). Microorganisms can be introduced into the plant by cross-contamination, by the raw materials, by their packaging such as paper board, wood pallets or containers that had been in contact with many different surfaces. Actually, cross-contamination is the

most likely form of microbial load (Kornacki 2010). Microorganisms can also enter via worker's skin and garments.

Table 3. Fungi identified in the several departments of the Tortosendo unit. (-) means that no microorganism was found in any of the plates. More information about the identified fungi can be found in the appendix, Table A.1.

Section	Occurring fungi
Production	*Cladosporium* sp.
Fruit preparation	*Candida sake*, *Penicillium* sp.,
Weighing of dry raw products	Presence of yeasts and moulds not identified by RT-PCR
SIP	-
CIP	*Cladosporium* sp., *Penicillium* sp., *Phoma* sp.
Washing zone	*Penicillium* sp.
Refrigerated storage	*Penicillium spinulosum*
Freezing storage	*Phoma pinodella*
Dry raw materials storage	*Cladosporium* sp. *Epicoccum nigrum*, *Penicillium* sp.,

Penicillium, *Cladosporium* and *Phoma* were the most recurring genus of fungi found in the houseplant. Fungi can grow on almost all natural and synthetic materials, especially if they are hygroscopic or wet (Haleem Khan & Mohan Karuppayil 2012). Wood pallets are highly hygroscopic and are a big source of moulds, being easily infested by *Cladosporium* and *Penicillium* species. This fact can explain the presence of *Penicillium sp.* in the refrigerated, freezing and dry raw materials storage room, where the presence of wood pallets and paper board packaging can induce the contamination. The presence of *Cladosporium* sp. in the production zone can be related to its proximity to the weighing of dried raw products room, where there are paperboard bags with dried raw materials retained on pallets. As *Cladosporium* spores are very prone to aerial dispersal, it is possible that they can easily be spread to other areas. The occurrence of *Epicoccum nigrum* in the dry raw products storage room might be related with the fact of this soil fungi usually attacks seeds. The packages stored in this section might, eventually, had been in contact with soil, or the raw materials themselves were a source of contamination.

Large metallic containers are cleaned by CIP operations. These containers are used in the expedition and then return to be reused. As the outer area of these containers may have been in contact with a wide variety of surfaces, the presence of soil fungi, such as *Phoma sp.* can be easily explained. The presence of *Phoma pinodella* in the freezing storage room, can be explained by its ability to grow at low temperatures, 1 to 5 ºC (Pitt

& Hocking 2009a). Although the freezing storage room is maintained at -20 ºC, the continuous opening and closing of the gates, can create zones near the entrance with oscillations of temperature, providing conditions for its growth.

In the refrigerated storage room, raw materials such as cereals and cereal flours are stored at 4 ºC. *Penicillium spinulosum*, found in this space, is a fast growing fungi that occurs on wheat and flours (Pitt & Hocking 2009a). As *Penicillium* species can grow at low temperatures such as the freezing storage room temperature, flours and cereals can be a source of contamination. Moreover, *Penicillium* spp. can be introduced into the plant by the fruits, as this mould usually constitutes fruits microflora, and then, be spread over the plant via air by dissemination of its spores.

In terms of yeasts, they were predominantly found in the fruit preparation room. The presence of yeasts in this room can be easily explained by their presence in fruits microflora (Fleet 2003). As fruits have high water activity, high sugar content, and pH around 4, they are very prone to yeast contamination, especially in this case, in which the fruits are milled and sliced (Fleet 2003). The higher number of yeasts and low number of moulds in this room, can be explained by the ability of osmophilic yeasts such as *Cryptococcus*, *Rhodotorula* and *Saccharomyces* to grow faster than the moulds (Kalia & Gupta 2006).

Analysing the PDA plates collected in each section gives an idea of the quantity and types of predominant fungi. No correlation was found between the plates placed in different heights and the number and/or genus of the occurring fungi. Based on the number and diversity of colonies grown in each plate, the production and the weighing of dried raw materials room are the less critical areas. In the SIP section, no growth of any species of fungi was observed. The fruit preparation room was the most critical in terms of yeasts, and the storage of dried raw materials room, the most critical in terms of moulds.

3.4 Conclusions

The work developed in this chapter is an important step to assess the predominant fungi associated with each raw material and with each department of the Tortosendo unit. The results obtained give an idea of the most recurring fungi, that can be used as standard microorganisms in future studies developed by FRULACT.

In terms of raw materials, the most critical in terms of microbial load were raspberry, blackberry and wheat. It was possible to observe that *Cladosporium* sp., *Cryptococcus* sp., *Penicillium* sp. and *Mucor piriformis* are the prevalent species among the tested raw materials. In the Tortosendo factory, the most predominant fungi were also *Penicillium* and *Cladosporium* genus. The microorganisms identified in this chapter will be used in the next chapters as standard spoilage fungi.

Chapter 4.

Antifungal activity of plant extracts rich in polyphenols

4.1   Introduction

Fruit-based preparations and cereal-based preparations produced by FRULACT are mainly addressed to dairy industries, specifically to yogurt applications. These preparations are prone to be contaminated by yeasts and moulds due to their low pH and high content in fermentable sugars. In FRULACT, microbiological safety of these products is assured by proper pasteurization in closed processing and packaging lines. However, if these products are not properly handled by the consuming industries, they can be easily contaminated, for example, by yeasts and moulds, spoiling and shortening its shelf-life. To avoid this situation, FRULACT incorporates potassium sorbate (E202) in its products when it is required by the client. Potassium sorbate is a fungi inhibitor and however it is a naturally occurring unsaturated fatty acid, most of it is made synthetically (Alrabadi *et al.* 2013). Nowadays, the consumers' demand for products free of synthetic chemicals impels the search for alternatives able to maintain products' properties and microbiological safety. One alternative is using natural antimicrobial compounds from plants. Plants produce a myriad of secondary metabolites involved in functions such as natural protection against microorganisms, insects and herbivores being also responsible for plant pigment, odor and flavor (Cowan 1999). Many of these compounds have been used for food or medical applications in the form of whole plants or plant extracts (Wallace 2004). Among these products, polyphenols, that are one of the largest groups of secondary metabolites act as antioxidants preventing food spoilage and browning of fruits in fruit preparations.

In this chapter, several plant extracts rich in polyphenols were screened for their *in vitro* antifungal activity against the fungi identified in the previous chapter. These microorganisms were isolated from the raw materials and from the Tortosendo factory. As the aim of this study is to found a natural antimicrobial compound able to prevent food spoilage in the final product, when it is handled by the client, for a more rigorous analysis, the extracts should have been tested against fungi isolated from the client's factories. However, as that was not possible, the panoply of fungi collected in the previous chapter

can be used to test the antimicrobial properties of selected extracts. Actually, they were mostly ubiquitous and air-borne fungi that would probably be present in the client's factory.

4.2 Materials and Methods

4.2.1 Tested microorganisms

The microorganisms used to test the antimicrobial activity were the ones isolated from the raw materials and from the environmental air of Tortosendo factory. They were stored in PDA plates at 4 ºC, with 7 days old, and renewed every two weeks. The fungi tested were the following: *Aspergillus* sp., *Candida sake*, *Chaetomoium* sp., *Cladosporium* sp., *Cryptococcus* sp. *Epicoccum nigrum*, *Mucor piriformis, Penicillium* sp. (several species isolated from different sources: wheat, barley and fruit preparation room) *Penicillium spinulosum, Phoma pinodella* and *Peyronellaea glomerata*. The bacteria isolated in the previous chapter *Pseudomonas* sp. was also tested.

4.2.2 Raw Materials

The substances tested for their antifungal activity against the selected microorganisms are discriminated in Table 4. Fungal activity of potassium sorbate used by FRULACT was also tested for comparison.

Table 4. Substances and extracts screened for their antifungal activity, their specifications and supplier. Due to confidential issues supplier A and supplier B names can't be revealed.

Extract	Specifications	Supplier
Acai	3% polyphenols	Supplier A
Cranberry	2% proanthocyanidins	Supplier A
Grape seed	87.3% polyphenols: 16.3% condensed tannins, 49.6% polymeric procyanidins	Supplier A
Grape seed	95% proanthocyanidins	Supplier B
Green coffee	19.3% chlorogenic acids	Supplier A
Green tea	97.3% polyphenols: 9% catechins	Supplier A
Hibiscus	12.9% polyphenols	Supplier A
Olive	2.94% hydroxytyrosol	Supplier A
Vanillin	99%	Sigma-Aldrich

4.2.3 Preparation of extract stock solutions

Stock solutions of each extract were prepared by mixing 2.5 g of extract with 50 mL of ethanol/water solution (1:10). The stock solutions were stored at room temperature in the dark until used.

4.2.4 Antifungal activity

The level of growth inhibition induced by the plant extracts was tested by the poisoned growing media technique (Brenes *et al.* 2011). After sterilization, the PDA medium was cooled to approximately 55 ºC and the extracts stock solutions were mixed with molten medium to obtain the final concentrations: 0.05, 0.075, 0.1, 0.2 and 0.5% (w/v). Approximately 15 mL of each medium containing different concentrations was poured into 90 mm sterile Petri plates and inoculated with approximately 5 mm plugs from 7-day-old cultures, in the case of filamentous fungi, or, in the case of yeasts, scratched with biomass using a sterile loop. The plates were incubated at room temperature and the growth was monitored after day 3, 5 and 7. Both the negative control, without culture, as well as the positive control, without extract were prepared to assess the extracts fungal load and for comparison with the effective samples. Medium with ethanol was also prepared at the concentrations used in the extract plates (0.1, 0.15, 0.2, 0.4 and 1%) in order to check its role on the microorganisms' growth. The MIC was determined as the lowest concentration of extract preventing growth of macroscopically visible colonies on extract containing plates, when there was visible growth on the extract-free control plates. The minimum concentration that retarded the growth of fungi, in other words the minimum concentration that inhibit mycelia growth, when compared with the control, was also determined. Whenever possible, the % of Mycelial Growth Inhibition (MGI) was calculated in terms of colony diameter reduction, by comparing with the control, after 7 days of incubation, and by the following equation suggested by Pandey *et al.* (1982):

$$\%MGI = \frac{d_c - d_e}{d_c} \times 100 \tag{1}$$

Where d_c corresponds to the average diameter of fungal colony in the extract-free PDA plate and d_e to the average diameter of the colony in PDA plates with extract.

4.3 Results and discussion

The addition of ethanol into the culture media did not influence the growth of the tested microorganisms. In terms of the extracts fungal load, no colonies were observed for the plates containing grape seed (both Supplier A and Supplier B extracts), cranberry and green tea extract and vanillin. Yeasts were present in plates containing acai, olive and green coffee extracts, and moulds were present in hibiscus and green coffee extracts. The antifungal activity of selected compounds are discriminated in Table 5.

Table 5. Antifungal activity of selected extracts against isolated microorganisms. MIC means "minimum inhibitory concentration", and the value in brackets corresponds to the minimum concentration tested, at which no growth was observed. RG means "retarded growth" and the value in brackets means the minimum concentration in which mycelial growth inhibition was observed when compared with the control. NA means that the compounds tested did not exert any antimicrobial activity against the tested microorganisms, under the maximum concentration evaluated (0.5%).

*includes all species of *Penicillium* because similar results were obtained.

Extract	*Aspergillus* sp.	*C. sake*	*Chaetomoium* sp.	*Cladosporium* sp.	*Cryptococcus* sp.	*E. nigrum*	*M. piriformis*	*Penicillium* *	*P. glomerata*	*P. pinodella*	*Pseudomonas* sp.
Acai	NA	NA	RG (0.5%)	NA	NA	NA	NA	NA	NA	NA	NA
Cranberry	NA	NA	RG (0.5%)	NA	NA	NA	NA	NA	NA	NA	NA
Grape seed (supplier A)	NA	NA	RG (0.2%) MIC (0.5%)	NA	MIC (0.5%)	RG (0.5%)	RG (0.1%)	NA	RG (0.5%)	NA	MIC (0.5%)
Grape seed (supplier B)	NA	NA	RG (0.2%) MIC (0.5%)	NA	MIC (0.5%)	RG (0.5%)	RG (0.5%)	NA	RG (0.5%)	NA	NA
Green coffee	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Green tea	NA	NA	RG (0.2%) MIC (0.5%)	NA	NA	NA	RG (0.5%)	NA	RG (0.5%)	NA	MIC (0.5%)
Hibiscus	NA	NA	RG (0.5%)	NA	NA	RG (0.5%)	RG (0.5%)	NA	RG (0.5%)	RG (0.5%)	NA
Olive	NA	NA	RG (0.5%)	NA	NA	RG (0.2%)	RG (0.5%)	NA	NA	RG (0.5%)	NA
Vanillin	RG (0.05%) MIC (0.2%)	MIC (0.2%)	RG (0.075%) MIC (0.1%)	RG (0.075%) MIC (0.2%)	MIC (0.1%)	RG (0.075%) MIC (0.2%)	RG (0.05%) MIC (0.2%)	RG (0.1%) MIC (0.2%)	RG (0.05%) MIC (0.2%)	RG (0.05%) MIC (0.2%)	MIC (0.2%)
Potassium sorbate	MIC (0.05%)	MIC (0.05%)	MIC (0.05%)	MIC (0.05%)	MIC (0.05%)	RG (0.05%) MIC (0.5%)	RG (0.05%) MIC (0.1%)	MIC (0.5%)	RG (0.05%) MIC (0.1%)	RG (0.05%) MIC (0.2%)	MIC (0.5%)

It is important to refer that the RG values give an idea of the compound antifungal activity. However, to FRULACT, only the MIC value is relevant, since the antimicrobial compound should completely inhibit the presence of fungi in the food product.

The acai, cranberry and green coffee extracts showed the weaker antifungal activity. Acai and cranberry extract were just able to inhibit mycelial growth of *Chaetomoium* sp. by approximately 40 and 60%, respectively, at a concentration of 0.5%. Anthocyanins are abundant in acai fruit, and these compounds have been shown to have antifungal properties against fruit-rot fungi (Schaefer *et al.* 2008). However, the used extract contained only 3% of polyphenols, which can explain its poor results in terms of antifungal activity. The same can be said for cranberry extract containing just 2% of proanthocyanidins.

As for green coffee extract, the tested sample contained approximately 20% of chlorogenic acids. Suárez- Quiroz *et al.* (2013) studied the effect of green coffee chlorogenic acids (GCCA) on the growth of *A. flavus* and *A. ochraceus* and observed a reducing effect on the growth rate of both fungi, at a minimum concentration of 0.1% in the case of *A. ochraceus,* and 0.3% in the case of *A. flavus.* They also found that starting from a concentration of 0.75mg/disc, using the disc diffusion method, GCCA had antimicrobial activity against *P. fluorescens*. As the maximum concentration of the tested extract was 0.5% and considering that the extract contain 20% of chlorogenic acids, this means that a maximum concentrations of 0.1% GCCA was tested, which according to the examples found in the literature, might be too low to exert any antimicrobial effect.

In relation to green tea extract, it completely inhibited the growth of *Chaetomoium* sp. and *Pseudomonas* sp., at a concentration of 0.5%. Additionally, it also inhibited the mycelial growth of *P. glomerata* and *M. piriformis* by approximately 12.5 and 14%, respectively, at a concentration of 0.5%. Tea polyphenols have already been demonstrated to have antifungal activity against post-harvest fruit pathogens such as *Botrytis cinerea, Monilinia fructicola* and *Rizophus stolonifer* (Yang *et al.* 2013; Chen *et al.* 2013; Yang & Jiang 2015), thus, its antifungal activity against these species of fungi is not surprising. Yang and Jiang (2015) have studied the antifungal activity of tea polyphenols against *R. stolonifer*, and found a MIC value of 0.4% (w/v), which is in between the range of the tested concentrations. Green tea extract did not exert any kind of antifungal activity against both of the tested yeasts. Cheruiyot (2015) reported green tea antifungal activity against some *Candida* and *Cryptococcus* species with MIC values

ranging from 0.3 to 2.5%. So, an increased green tea concentration for a value within this range, would probably be able to induce some antifungal activity against the tested yeasts.

Green tea extract was not able to inhibit *Aspergillus* sp.*, Cladosporium* sp. or *Penicillium* sp. at the tested concentrations. These results are in accordance with the studies of Sakanaka *et al.* (1997), in which they discovered that green tea extract is not able to inhibit the growth of Aspergillus *niger*, *P. citrinum* and *P. chrysogenum* at concentrations of 0.4% in culture media, using the same method that was used in this work to assess the antifungal activity.

Hibiscus extract showed mycelial growth inhibition of 50, 40, 30, 45 and 20% against *Chaetomoium* sp., *E. nigrum, M. piriformis, P. glomerata* and *P. pinodella*, respectively, at a concentration of 0.5%. Its antimicrobial activity is thought to be due to its content in flavonoids and tocopherol (Abd-ulgadir *et al.* 2015). This extract did not show any antimicrobial activity against *Pseudomonas* sp. or against the tested yeasts. Nevertheless, hibiscus leaves extract has been shown to exert antimicrobial activity against *Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans*, with MIC values of 1.25, 2.5 and 0.625%, respectively (Abd-ulgadir *et al.* 2015). These values are greater than the tested values, which can explain their lack of activity against some of the species.

The olive extract tested in this work contained approximately 3% of hydroxytyrosol, which means that a maximum concentration of 0.015% was tested. This concentration was able to inhibit the mycelial growth by approximately 20% of *M. piriformis*, and 30% of *E. nigrum*, and *P. pinodella*. A final concentration of 0.006% was able to inhibit the growth of *Chaetomoium* sp. by 20%. Antifungal activity of olive extracts has been regarded to be due to the presence of glutaraldehyde-like compounds such as hydroxytyrosol (Vagelas *et al.* 2009; Yangui *et al.* 2009). Medina *et al.* (2013) studied the antifungal activity of aqueous solution from table olives, which contain a high amount of antimicrobial phenolic compounds such as hydroxytyrosol. They found that this solution was able to inhibit the mycelial growth of *Alternaria* spp, *Botrytis cinerea* and other phytopathogenic fungi, even when diluted up to 50%. However, the concentration of phenolic compounds in the solutions were not assessed. The antifungal activity against these fungi is not surprising, since plants produce compounds to defend against plant pathogens and soil fungi. In terms of yeasts, olive extract was not able to inhibit the growth of *Cryptococcus* sp. neither *C. sake*. Some studies have pointed out the antifungal activity of olive leaf extracts against yeasts like *S. cerevisiae¸ C. oleophila,*

Metschnikowia fructicola and others, obtaining MIC values between 0.001 and 0.0028% (w/v) of extract (Korukluoglu *et al.* 2006). These values are much smaller than the ones tested in this thesis, which can be mainly explained by (1) the type of extract; (2) concentration of active compounds; (3) the solvent used; (4) or by the microorganisms themselves.

The grape seed extracts from supplier A and from supplier B, had very similar results. Both extracts have high content in polyphenols, 87.3 and 95%, respectively. Their slightly differences in terms of antifungal activity may have to be due to (1) extraction procedures, (2) to the grape species from which the grape seed was extracted, (3) or to the type of polyphenols present in each of them. Grape seed extract from supplier A had the best results. It completely inhibited the growth of *Chaetomoium* sp., *Cryptococcus sp.* and *Pseudomonas* sp. at a concentration of 0.5%. Besides, it inhibited mycelial growth of *M. piriformis* in a dose dependent manner, starting exerting effect at a minimum concentration of 0.1%. It also retarded the growth of *P. glomerata* and *E. nigrum* at a minimum concentration of 0.5%. No references on the literature were found regarding grape seed extract antifungal activity. However, the results presented in this work regarding this sort of extract are very promising, which can be something to be further investigated in the future.

The tested vanillin, which was not an extract, but a pure molecule, showed the greater antifungal activity against selected microorganisms. It was able to completely inhibit the growth of all tested microorganisms at concentrations of 0.2% or below. Vanillin inhibited the mycelial growth of filamentous fungi in a dose dependent manner, as can be seen in Figure 5 for *M. piriformis.* It was the only substance tested that was able to inhibit the growth of the spore producing moulds *Penicillium* sp., *Aspergillus* sp. and *Cladosporium* sp., and the growth of *C. sake*. Additionally, it had the lower minimum inhibitory concentration against *Pseudomonas* sp., which was 0.2%. Vanillin has reported to have antifungal activity against *Aspergillus* species with MIC values around 0.2% in fruit agar media (López-Malo *et al.* 1995).

Due to vanillin's type of formulation, no interfering agents were present, which probably means that it was more bioavailable than the active molecules in the extracts, thus exerting higher antimicrobial activity. Since the tested vanillin was of analytical grade, in an economical point of view, it would be very expensive to use it industrially. However, it has been reported that vanillin and potassium sorbate have a synergistic

activity against several species of *Penicillium*, which could reduce the concentration of both molecules in the final product (Matamoros-León *et al.* 1999). Nevertheless, more studies are needed in this area, especially regarding the use of food-grade vanillin as a preservative.
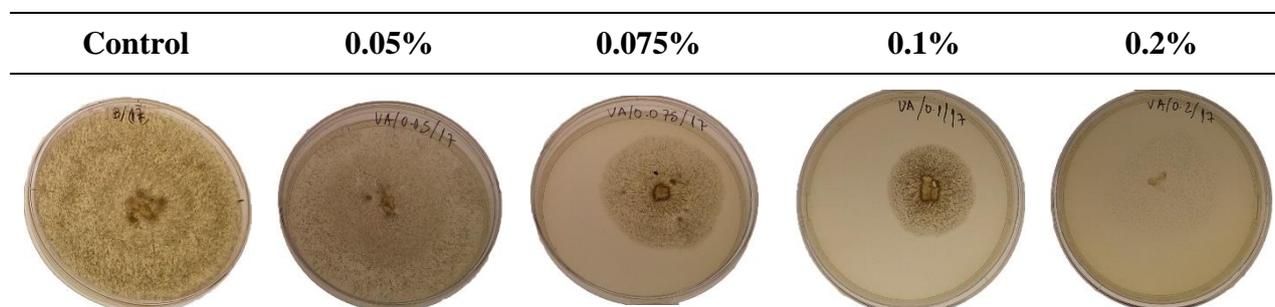
Figure 5. Mycelial growth inhibition of *M. piriformis* by different concentrations of vanillin after 7 days of incubation.

As a preservative, potassium sorbate is said to inhibit the growth of food-related yeasts and moulds such as: *Candida, Cryptococcus, Rhodotorula, Saccharomyces* and *Alternaria, Aspergillus, Mucor, Penicillium,* among others (Sofos & Busta 1993). This preservative has also shown to have antimicrobial activity against several *Pseudomonas* species (Moustafa *et al.* 1969; Robach 1978). The results presented in Table 5 show that potassium sorbate has antifungal activity against all the tested isolates as well as antimicrobial activity against *Pseudomonas* sp*.* The results presented show that vanillin could potentially substitute potassium sorbate. Generally, vanillin showed higher MIC values than potassium sorbate, with exception of *E. nigrum*, *Penicillium* species, and *Pseudomonas* sp. This means that vanillin would need to be applied in food products at a higher concentration than potassium sorbate to obtain the same preserving effect. Hence, taking into account the obtained MIC values, vanillin would be likely effective as a preservative if applied at a concentration of 0.2%, though further studies to evaluate the performance of vanillin in food-products are needed. Vanillin is added to many foods as a flavoring agent at concentrations ranging from 0.015% to 0.4% (Hocking 1997), thus the 0.2% here suggested would probably be organoleptically accepted.

4.4   Conclusion

Several factors can influence the antifungal activity of selected extracts: (1) the species of the fungi; (2) the type of extract; (3) the classes; (4) concentrations; (5) and bioavailability of polyphenols present in the extract.

None of the tested extracts was able to inhibit the growth of all isolated microorganisms at concentrations ranging from 0.05 to 0.5%. The most resistant species to extracts, in the studied conditions, were mainly environmental moulds such as *Aspergillus* sp, *Cladosporium* sp., *Penicillium* species, as well as the yeasts *C. sake* and *Cryptococcus* sp. Nonetheless, grape seed extract and green tea extract showed the better results, among the tested extracts, in terms of antifungal activity. The molecule vanillin was also tested and showed a higher antifungal activity, being able to inhibit the growth of all tested microorganisms at a concentration of 0.2% or below.

Giving the obtained results, grape seed and green tea extracts, as well as vanillin were further studied for their performance as preservatives in food products. This subject is addressed in chapter 5.

Chapter 5.

Effect of selected extracts on the final product

5.1 Introduction

Using natural antimicrobials such as plant extracts as an alternative to synthetic antimicrobials to prevent food spoilage is an increasing trend in the food industry (Perumalla & Hettiarachchy 2011). The major substances in plant extracts with bioactive activity are polyphenols. Besides their antimicrobial properties, they also contribute to health benefits, which can make them ingredients in functional foods. The selection of these natural antimicrobials and their further application in food products depends on (1) their functional properties; (2) availability; (3) cost effectiveness; (4) consumer awareness; (5) and their effect on the organoleptic properties of the final product (Perumalla & Hettiarachchy 2011). In this chapter, the tested extracts were green tea and grape seed (from supplier A) extracts and the molecule vanillin. These substances were chosen accordingly to their antifungal activity against previously isolated spoilage fungi (see chapter 4).

Besides their antifungal activity, the selected substances are well known for their functional properties. Vanillin is very popular as a flavoring agent and can be extracted from vanilla beans (Tai *et al.* 2011). Vanillin may contribute to several health benefits such as antimutagenic, antiangiogenetic, anti-colitis and, anti-sickling effects, which are thought to be due to its antioxidant activity (Tai *et al.* 2011). Besides, its flavor is well accepted by the consumers, being used in a variety of products such as fruit juices, ice creams and yogurts. (Cerrutti *et al.* 1997). Accordingly, its organoleptic properties will not be an issue. Green tea and grape seed extracts are well known for their health benefits and well accepted by the consumers. Some health benefits concerning these extracts include anticancer, anti-inflammatory, protection against cardio-vascular diseases, just to mention a few (Jaziri *et al.* 2009; Perumalla & Hettiarachchy 2011). These properties have been attributed to the antioxidant activity of tea catechins and grape seed proanthocyanidins, respectively (Najgebauer-lejko 2014). It is also important to mention that green tea and grape extracts and vanillin have GRAS status by FDA. In this work, these substances are aimed to be added to preparations, whose final application is the

incorporation in yogurt. Yogurt is perceived as a healthy food due to its nutritional properties and health benefits. Regular yogurt consumption with live cultures is said to have many health benefits, such as aiding reducing serum cholesterol levels, bowel syndromes, colon cancer, among others (Vasiljevic & Shah 2006). Thus, the maintenance of yogurt natural microflora is an aspect that should not be forgotten when adding antimicrobials. The addition of plant extracts rich in polyphenols should result in a final product with functional properties and antioxidant capacity. These polyphenols should be stable in the final product over the product shelf-life, so that they could provide the above-mentioned health benefits.

In this part of the work, the influence of selected extracts in a cereal and a forest fruit preparations and in dosed yogurt will be tested. In yogurt, the stability of polyphenols and its antioxidant capacity will be monitored over time, as well as their natural microflora and the presence of possible spoilage fungi. Additionally, preparations containing selected extracts and related dosed yogurts will be monitored over time for their microbiological stability and for their ability to prevent purposive contaminations. The organoleptic properties of the novel developed yogurts will be assessed by a panel of non-expert food tasters.

5.2 Materials and Methods

5.2.1 Preparation of yogurts with extracts

The yogurts used in this part of the project were Mimosa plain yogurts without sugar. Samples of yogurt (50 g) were placed in flasks (Sarstedt, Germany) and a certain amount of extract was added to reach a final concentration of 0.1% (w/w) for green tea and grape seed extract, and 0.04% for vanillin. These concentrations were calculated by taking 20% of the concentration tested in chapter 4 that gave the best antifungal activity. In the case of yogurts, this concentration (20%) is in accordance with the maximum dosage percentage that would be incorporated in the lactic matrix, in FRULACT. For each sampling day several flasks were prepared, and each test was carried out in duplicated. The samples were stored at 4 ±1 ºC until used. Samples without extract, and with potassium sorbate (E202) in a concentration according to the legislation in force were also prepared. Not-open yogurts were also stored in the same conditions. The mixing of extracts with yogurts occurred in a non-sterile environment to mimic a worst case scenario in terms of contamination.

5.2.2 Preparation of yogurt water extract

A yogurt water extract was prepared for further chemical analysis. Water extraction was carried out according to Amirdivani & Baba (2011). A sample of plain and yogurts with extracts (10 g) were homogenized with 2.5 mL of distilled water. The pH of the yogurts was acidified to pH 4.0 with HCl (0.1 M). The samples were heated in a water bath at 45 ºC for 10 minutes followed by centrifugation (4000 g, 18 min, 4 ºC). Supernatants were adjusted to pH 7.0, followed by re-centrifugation (4000 g, 17 min, 4 ºC). The supernatant was harvested and chemically analyzed in the same day for total phenolic content and antioxidant capacity.

5.2.3 Determination of Total Phenolic Content

The TPC of yogurts water extract was estimated according to the Folin-Ciocalteu method (Singleton et $al.$ 1999) with some modifications. To 500 µL of sample solution, 2.5 mL of Folin-Ciocalteu reagent (Merck, Germany) was added (1:10 in distilled water), following the addition of 2 mL of Na_2CO_3 (Scharlau, Spain) (7.5% in aqueous solution). After, the samples were left in a water bath in the dark, at 45 ºC for 15 minutes. Then, the samples were left to cool at room temperature for 30 minutes in the dark. A smaller volume (200 µL) of this final sample was transferred to a 96-well microtiter plate and the absorbance was measured in a microtiter plate reader (Synergy HT, Biotek, United States of America), at 765 nm. A calibration curve was done using different concentrations of gallic acid (Sigma-Aldrich, Germany). The TPC was determined as gallic acid equivalents (µg GAE) /mL extract.

5.2.4 Determination of antioxidant activity

Free radical scavenging activity (RSA) was determined according to Brand-Williams et $al.$ (1995) using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), with some modifications. To 250 µL of sample, 2.75 mL of DPPH at 60 µM (Sigma-Aldrich, Germany) in ethanol (99%) (aga, Portugal) were added and the mixture was vortexed and left to stand for 30 minutes in the dark. A smaller volume (200 µL) of this mixture was transferred to a 96-well microtiter plate and the absorbance was measured in a microtiter plate reader (Synergy HT, Biotek, United States of America) at 517 nm. Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined using the following equation:

$$\%RSA = \frac{A_c - A_s}{A_c} \times 100 \qquad (2)$$

Where A_c is the absorbance of the control and A_s the absorbance of the sample. Water was used as control.

5.2.5 Enumeration of viable cell count in yogurt

Samples of yogurt (10 g) were added to 90 mL sterile BPW (Merck, Germany) and left to rest for about 30 minutes. Appropriate dilutions were made in MRD (Liofilchem, Italy). The microorganisms were cultivated using the drop technique: a drop containing 20 μL was placed into selective media MRS (Liofilchem, Italy) for the enumeration of *Lactobacillus bulgaricus* and on ST media (homemade prepared media:10 g/L triptone (Liofilchem, Italy), 10 g/L sucrose (AppliChem, Germany), 2 g/L K_2HPO_4 (AppliChem, Germany) 15 g/L agar (VWR Chemicals, Belgium) and 6 mL/L bromocresol purple for the enumeration of *Streptococcus thermophilus*. The cultures were incubated at 37 ± 1 ºC for 3 days.

5.2.6 Enumeration of yeasts and moulds

The preparation of samples and following enumeration of yeasts and moulds was carried out according to ISO 21527:2008 as described in Chapter 3, sub-sections 3.2.2, 3.2.3 and 3.2.4, except when mentioned the opposite.

5.2.7 Fruit and cereal preparations

The present work was carried out with two different preparations: one forest fruit preparation and one cereal preparation, previously developed and selected by FRULACT, and applicable in stirred yoghurt at dosages of 15 and 4%, respectively. When needed, each preparation was done considering a total weight of 1 kg. All the ingredients were mixed in a pan, until reach the pasteurization temperature and left to stand for the pasteurization time. The formulation of each preparation can be found in Table 6. The percentage of all the ingredients cannot be revealed but the combination of all those amounts to 100%. The added substances: green tea extract, grape seed extract, vanillin and potassium sorbate are not in the formulation tables. These are present when applicable at the following concentrations: 0.5, 0.5, 0.2, 0.1% (w/w), respectively. Vanillin and grape seed and green tea extract were added to the formulation after the pasteurization period at approximately 65 ºC.

Table 6. Formulations of the tested cereal and the forest fruit preparations.

Cereal preparation		Forest fruit preparation	
Ingredient	**% (w/w)**	**Ingredient**	**% (w/w)**
Water	82.56	Water	35.62
Sugar	3.00	Blackberry	30.00
Wheat bran	2.80	Raspberry	13.00
Starch	2.70	Blueberry	12.00
Cereal flavouring	-	Flavouring agents	-
Oat flour	0.88	Fruit concentrates	-
Barley flour	1.75	Sweeteners	-
Malt extract	-	Thickeners	-
Thickeners	-		
Acidity regulator	-		

5.2.8   ºBrix, pH and water activity

Several parameters of the preparations were measured. These are standard parameters used by FRULACT. ºBrix was measured using an automatic refractometer (Bellingham + Stanley, United Kingdom). The pH value was measured using a digital pH meter with automatic temperature compensation (Consort 6860 Hanna Instruments). Finally, water activity was measured with a meter Hygrolab (Rotronic, Switzerland).

5.2.9   Evaluation of organoleptic properties

A panel of 20 non-expert tasters evaluated the organoleptic properties of newly developed yogurts in terms of sweetness, acidity, astringency, and flavour when compared with a control. Several questions about their preferences for the developed yogurt or for the control were also done. The questionnaire is available at the appendix, Figure A.1.

5.2.10 Artificial contamination of preparations

Preparations were artificially contaminated with *Penicillium* sp. (isolated from barley) and *Aspergillus* sp (isolated from wheat). *Penicillium* sp. and *Aspergillus* sp. were selected because they are ubiquitous fungi (*Penicillium* was one of the most often occurring both in the raw materials and in the Tortosendo unit) that can produce mycotoxins, compromising food safety. A inoculum was prepared accordingly to Guarro *et al.* (1998) with some modifications. The strains were cultured in Petri dishes with PDA and incubated for 7 days at 25 ºC. The surface of the plates were flooded with 10 mL of sterile distilled water and the sporulated aerial mycelium was scrapped with a sterile loop.

The suspensions obtained for *Penicillium* sp. and *Aspergillus* sp. were mixed and diluted 10 times. This dilution will be referred to as the inoculum. Due to unavailability of haemocytometer or other mean for spore counting, the inoculum was characterized by culturing serial dilutions on RBC plates and estimating CFU/mL. The preparations were contaminated by mixing a certain amount of inoculum to reach a final concentration of 1% (v/w) (5 mL of inoculum to 500 g of preparation).

5.2.11 Statistical analysis

Statistical significant differences between samples was assessed using Student's *t*-test with Microsoft Excel 2013. The value of *p*, for the rejection of the null hypothesis was set to $p < 0.05$.

5.3 Results and discussion

5.3.1 Effect of selected extracts in plain yogurt

Yogurts with vanillin and grape seed and green tea extract were monitored over time to assess polyphenol stability. For this, the TPC of these yogurts was examined over time, whose results are presented in Figure 6. The antioxidant capacity of these yogurts was also monitored using DPPH radical scavenging activity assay over time, and the results are presented in the form of charts in Figure 7.
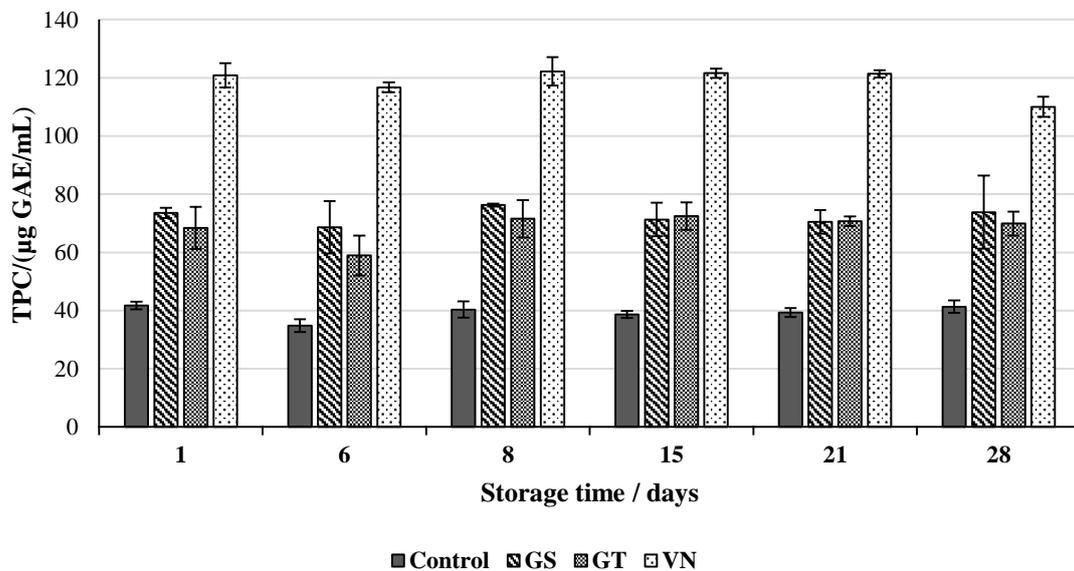

Figure 6. TPC of plain yogurt (control) and yogurts containing grape seed (GS) and green tea (GT) extracts and vanillin (VN) over 28 days of refrigerated storage at 4 ℃. Bars represent average and error bars the standard deviation.
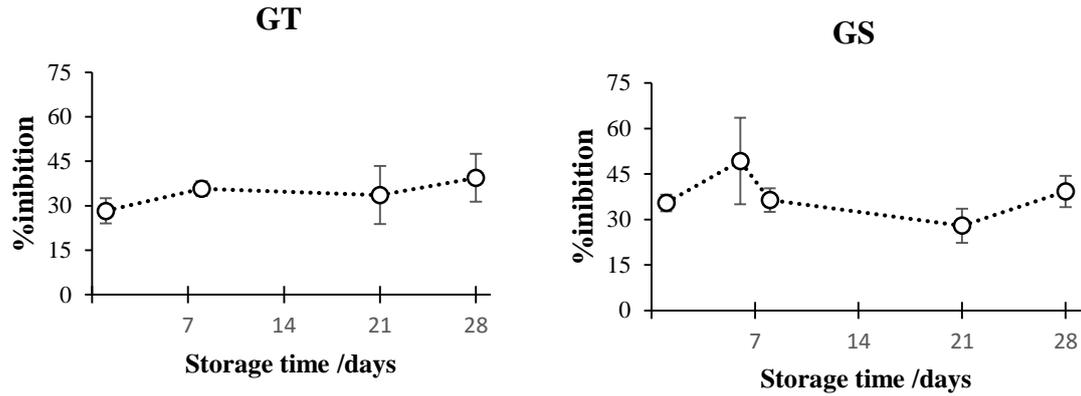
Figure 7. Antioxidant activity of yogurts containing green tea (GT) and grape seed (GS) extracts, over 28 days of refrigerated storage at 4 ºC, expressed by % of DPPH inhibition. Circles represent mean and error bars the standard deviation.

These results give an idea of polyphenols content during storage as well as their antioxidante activity. As following explained, these results should not be taken at an analytical precision level, but as a way to relatively compare the several tested samples. The Folin-Ciocalteu spectrophotometric method tends to overstimate the polyphenol content as it is non-specific and so, many other compounds may react such as proteins, vitamins, amino acids and others (Everette *et al.* 2010). It is known that polyphenols have the ability to interact with proteins, specifically the proline-rich ones, like α and β caseins by hydrophobic and hydrogen bonding (Lamothe *et al.* 2014). These interactions lead to the formation of protein-polyphenol complexes, which can decrease yogurts antioxidant activity (Arts *et al.* 2002). This happens because the formation of complexes decreases the electron donation capacity of polyphenols by reducing the number of available hydroxyl groups in the solution.

In all cases, TPC was significantly higher in all sampling days in treated yogurts when compared to the control, suggesting supplementation. Although vanillin was present in the yogurt in a lower concentration, 0.04 %, when compared with grape seed and green tea extract, 0.1% , it showed the higher TPC. The phenolic content in control samples may be due to the presence of milk polyphenols, dairy proteins or other reducing compounds that respond to the photometric total phenolic measurement (Chouchouli *et al.* 2013). Yogurts with green tea and grape seed extract didn't show significant differences ($p > 0.05$) in terms of TPC over time, during refrigerated storage, as well as regarding their radical scavenging activity, which can be an indicator of polyphenol extracts stability in yogurt over time. In relation to vanillin, a sligthly decrease of TPC at day 28 was observed ($p < 0.05$). Other authors have reported reduction in TPC in yogurts

containing green tea, ater 14 days storage (Muniandy *et al.* 2016). In another study, Karaaslan *et al.* (2011) studied the effects of the grape extracts in yogurts to enhance the phenolic content of the yogurt. The yogurts containing red grape and callus extracts possessed higher level of radical scavenging activity compared with control yogurts. However, the TPC and the antioxidant activity of the fortified yogurts, decrease during the 14-day storage period. The lowest radical scavenging capacity was detected at the last day of storage.

The DPPH assay gives information about the radical scavenging activity of the antioxidant substances present in the examined sample. Plain yogurts and yogurts with vanillin did not show any radical scavenging activity with the DPPH assay. As no additional substances were added to plain yogurt, its inability to reduce the DPPH radical was expected. However, the same can't be said to yogurts with vanillin. Studies on the antioxidant activity of vanillin are not consistent, varying greatly with the used methodology. For example, a studied carried out by Tai *et al.* (2011) have shown that vanillin show stronger antioxidant activity than ascorbic acid and Trolox in the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$\cdot^+$) scavenging assay, but show no activity in the DPPH radical assay. Considering this scenario, the results here obtained for vanillin are not surprising. To test vanillin antioxidant activity over time, another assay should have been done such as oxygen radical absorbance capacity (ORAC) or ABTS$\cdot^+$ scavenging assay (Tai *et al.* 2011).

Concerning yogurt natural microflora, using the procedures here described for the enumeration of viable cell count, no growth of *Lactobacillus bulgaricus* was reported for the yogurts used in this part, even in the control. It was possible to enumerate *Streptococcus thermophilus* for this same yogurt. The viable cell count of *S. thermophilus* as well as the yogurts' pH over time is presented in Figure 8.

In all cases, viable cell count was in between 9 and 9.3 Log(CFU/g) over time. So, it remained above the required level, 7 Log (CFU/g) (Codex Alimentarius 2011), during the 4-week cold storage. There were no significant differences between treated yogurts and control ($p > 0.05$) with exception of green tea yogurt at day 28, in which viable cell count was slightly higher, but within the same log range (9.3 Log (CFU/g)). Several studies had demonstrated that the addition of green tea extracts to yogurt did not affect the viability of the starter cultures. For example, Jaziri *et al.* (2009) studied the effect of green tea extract on yogurts and showed that *L. bulgaricus* and *S. thermophilus* were not affected

during 42 days of refrigerated storage. The same tendency was observed for *S. thermophilus* counts in the study carried out by Najgebauer-lejko (2014). A stimulating effect had also been reported in yogurt supplemented with green tea (Najgebauer-Lejko *et al.* 2011). Vanillin and grape seed extract have also been reported to not derail yogurt natural microflora (Rezazadeh *et al.* 2015; Chouchouli *et al.* 2013).
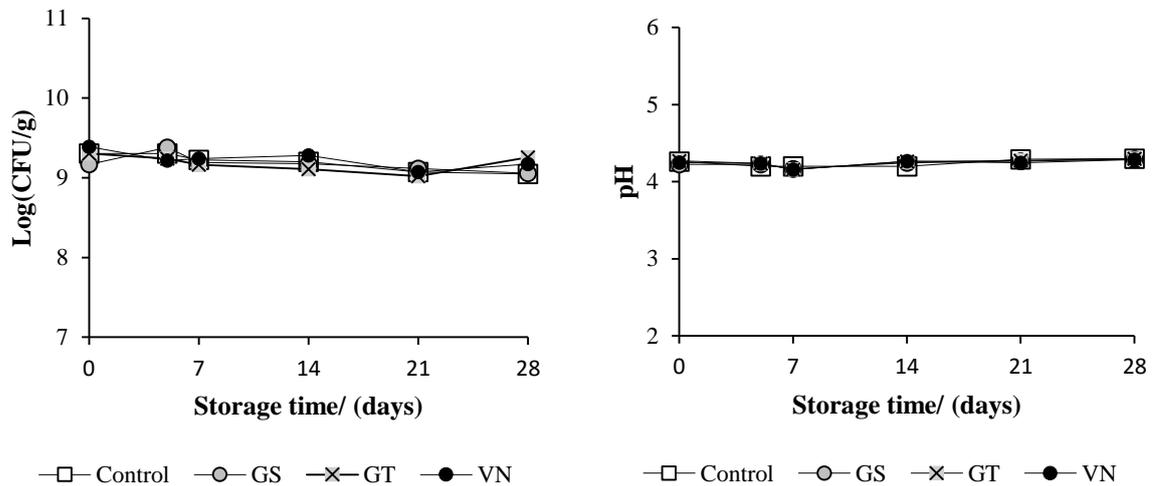

Figure 8. Viable cell count of *S. thermophilus,* at the left, and pH, at the right, for plain yogurt (control) and yogurts containing grape seed (GS) and green tea (GT) extracts and vanillin (VN), over 28 days of refrigerated storage at 4 ºC.

The pH value of treated yogurts was not affected over time when compared with control, so it provided an environment propitious for the maintenance of *S. thermophilus*. Najgebauer-Lejko *et al.* (2011) reported a maximum pH value decrease of 0.3, during 28 days of refrigerated storage in green tea infusion supplemented yogurts. Also, the pH value of yogurts supplemented with green tea were lower by 0.09-0.15 units than the yoghurts without tea. Regarding grape seed extract, Chouchouli *et al.* (2013) reported that this extract did not influenced yogurts pH values during refrigerated storage when compared with control.

The fungal load of yoghurts was also monitored over time. It is important to notice that the incorporation of extracts in yogurts was carried out in a non-sterile environment, and so, they are propitious to be contaminated by environmental fungi. Additionally, some fungal load associated with the extracts may also contaminate the yogurts. A sample without any extract was also prepared in the same conditions of other yogurts as well as a sample with potassium sorbate. The results are following presented in Figure 9.
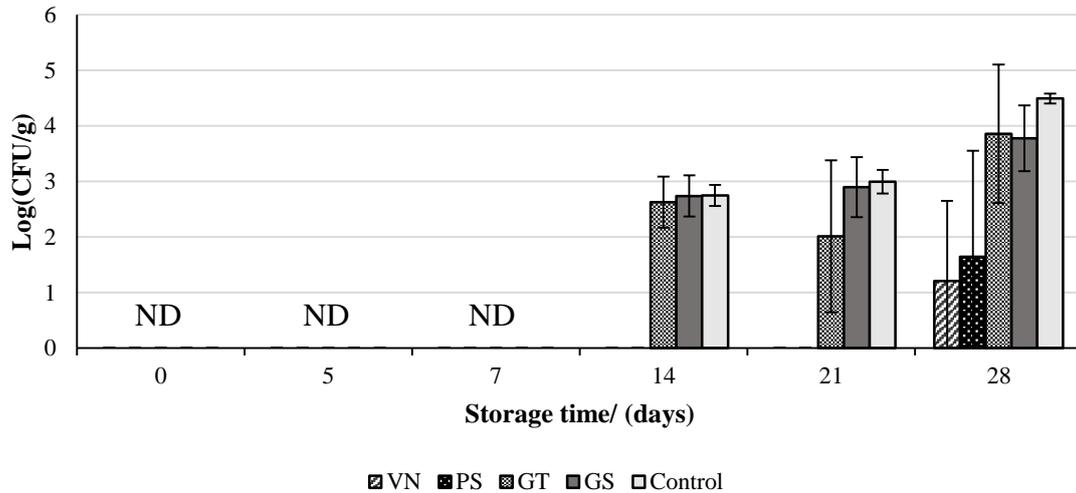
Figure 9. Fungal load of yogurts containing vanillin (VN), potassium sorbate (PS), green tea (GT) and gape seed (GS) extracts and plain yogurt (control) during 28 days of refrigerated storage at 4 ºC. ND means "not detected".

As can be observed in Figure 9, some bars have high standard deviation values. This happens mainly in cases in which Log (CFU/g) < 2. In these situations, colonies in plates are below the minimum threshold to count colonies according to the ISO 21527:2008. However, to FRULACT, it is important to know when fungi are present, even if at very low levels. This way, plates with number of colonies below the threshold were counted and the average was calculated taking into account all the replicates, even when there weren't any colony on them. For this reason, the presented results have high standard deviation values.

At day 0, 5 and 7 no detectable levels of fungal load were found in any of the yogurts tested. Yeasts and moulds started being present at detectable levels after 14 days of refrigerated storage, in yogurts containing grape seed and green tea extract and in plain yogurt, with a higher fungal load in the last day of storage. There were no significant differences between yogurts containing grape seed and green tea extract when compared with the control (plain) at day 14, 21 and 28 ($p > 0.05$). Additionally, for these extracts, the fungal load was always higher when compared with yogurt containing potassium sorbate, in which fungal load was just detected at day 28. In this day, the fungal load of green tea and grape seed extract yogurts was mainly due to yeasts, although hyphal fungi was also present. These yogurts had visible deterioration with a noticeable mould mycelium on the surface of grape seed yogurts and a yellow slime on the surface of green tea yogurts. Some studies with yogurts containing polyphenols have reported a decreased in the fungal load in polyphenol-rich yogurts in relation to control. For example, in a

study carried out by Georgakouli *et al.* (2016) the fungal load in yogurt containing olive polyphenols at 0.125% (w/w) was significantly reduced by 0.6-2.2 Log(CFU)/g compared to the control over 60 days of refrigerated storage. Fungal load in yogurts containing vanillin was just detected at the last day of storage and below the plain yogurts and yogurts containing potassium sorbate. Penney *et al.* (2004) also studied the addition of vanillin to yogurts and monitored its fungal load. They reported that minimally processed blueberry yogurts containing vanillin at 0.2% significantly suppressed the fungal growth over 3 weeks of refrigerated storage. In the case of this study, vanillin at 0.04% had a similar performance when compared with potassium sorbate at 0.02% in maintaining the yogurts fungal load at low levels. These results are in accordance with several studies showing that vanillin can increase the shelf-life of food products (Cerrutti *et al.* 1997; Fitzgerald *et al.* 2004; Tomadoni *et al.* 2016).

5.3.2   Effect of selected extracts in the microbiology of cereal and fruit preparations

Cereal and fruit preparations with extracts were monitored over time for the assessment of their fungal load. Internally, for FRULACT, in the quantification of yeasts and moulds, these should be under the detectable level. So, any preparation, whose microbiological analysis reveals the presence of a single colony should be rejected. As the preparations were subjected to proper pasteurization in laboratory assays, any prior contamination is very unlikely to be due to the raw materials fungal load, unless there are thermoresistant fungi. Possible sources of contamination include the extracts themselves, as they were added at approximately 65 ºC, or environment fungi present in the air or in the surfaces. The characteristics of the elaborated preparations are presented in Table 7.

Table 7. Parameters of the forest fruit and cereal preparations tested with potassium sorbate (PS), grape seed (GS) and green tea (GT) extracts and vanillin (VN). Plain refers to the preparations without any preservative.

	Forest Fruit Preparation					**Cereal Preparation**				
	Plain	**PS**	**GS**	**GT**	**VN**	**Plain**	**PS**	**GS**	**GT**	**VN**
ºBrix	9.6	10.0	9.8	9.8	10.0	10.2	10.1	10.2	9.9	10.0
pH	3.94	3.96	3.98	3.98	3.91	4.29	4.54	4.14	4.40	4.20
aw	0.90	0.89	0.92	0.90	0.91	0.96	0.96	0.96	0.96	0.96

Forest fruit preparation developed fungal load at detectable levels at day 14 in preparations without any preservative, at day 21 in preparations with green tea and at day 28 in preparations with grape seed. No fungal load was associated with preparations with sorbate or with vanillin, after 28 days of refrigerated storage. Figure 10 shows the aspect of fruit preparations at day 28. There were visible mycelium in preparations without potassium sorbate and with green tea. Although fungal load was detected in the sample

containing grape seed extract there weren't any visible colonies on the surface. The shape, colour and aspect of the colonies, show that the contamination probably occur with some fungi belonging to the *Penicillium* genus.

| Potassium sorbate | Plain | Grape seed | Green tea | Vanillin |

Figure 10. Aspect of forest fruit preparations after 28 days of refrigerated storage at 4 ºC.

In relation to cereal preparations, no fungal load was detected even after 28 days of refrigerated storage in all tested samples. So, the cereal preparation did not spoil even without any preservative. The parameters measured for both cereal and forest fruit preparations are very similar. Both present a high water activity, a similar value of ºBrix and a pH value around 4. Their characteristics makes them very prone to yeast contamination. In fact, at day 28, in the forest fruit preparation, although the visible mould mycelium, the fungal load was mainly due to yeasts. As these parameters are very similar, the discrepancy between each preparation fungal load must have another reason beyond these parameters. These differences could be due to the fact that fruit is perishable, while cereals can maintain its properties longer. The fruit added to the preparation contributes to the addition of fermentable sugars that propitiate ideal conditions for the growth of fungi. On the other hand, cereals are a source of carbohydrates, which are complex molecules, that aren't so bioavailable.

5.3.3 Effect of selected extracts in the organoleptic properties of dosed yogurts

A panel of 20 non-expert food tasters tested yogurts containing cereal and fruit preparations, at the doses recommended by FRULACT, containing grape seed and green tea extracts and vanillin.

Relating yogurts containing preparations with green tea extract and vanillin, the majority of tasters reported that they didn't feel any different between the novel yogurts and the standard in terms of acidity, sweetness and astringency. Relating yogurts containing forest fruit preparation with grape seed extract, more than 50% reported that the sample with the extract felt sweeter than the standard, while in the cereal preparation, the majority didn't notice any difference. With the exception of yogurt containing

vanillin forest fruit preparation, all the tasters reported that differences between the flavour of the standard and the novel yogurts was very little or imperceptible. In the case of yogurt with forest fruit preparation, 45% said that the flavour between the one containing vanillin and the standard was very perceptible. The same was not reported for the yogurt with cereal preparation because the used dose in yogurt was smaller (4%) when compared with the fruit preparation (15%).

The tasters were also asked if, in terms of flavour, they would prefer the standard or the novel yogurt. Despite the noticeable difference in terms of flavour of vanillin-containing yogurts, these were described as the ones with the pleasant flavour when compared with the control. The tasters' preferences are presented in Figure 11 for all tested yogurts.
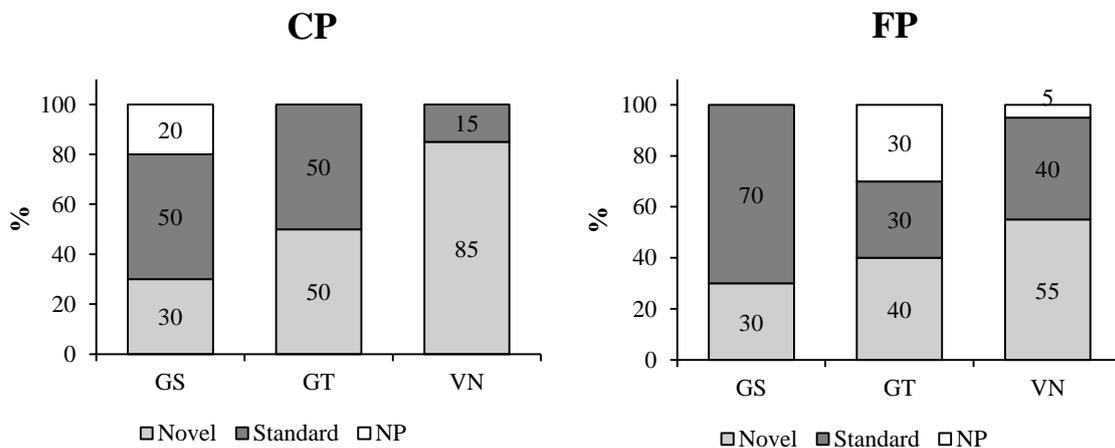

Figure 11. Preferences of the tasters when comparing the yogurts with cereal (CP) and forest fruit (FP) preparations containing grape seed (GS), green tea (GT) or vanillin (VN) with the standard. NP means "no preference". The novel yogurts refers to the one in which the aforementioned substances were added to their preparations.

5.3.4 Effect of preparations containing grape seed extracts in the yogurt natural microflora

In the section 5.3.1, the effects of selected extracts on plain yogurt were studied. The results showed that the tested substances did not affect the yogurt natural microflora, or at least *S. thermophilus* viability. In this section the effects of a cereal preparation and of a forest fruit preparation containing grape seed extract on plain yogurt are tested. The grape seed extract was selected for these tests because of their antifungal activity and organoleptic properties. Although vanillin had better results in terms of antifungal activity as well as for the organoleptic properties, it was not selected because the vanillin used in this work was chemically synthesized.

As in the previous section, the enumeration of *L. bulgaricus* was not possible, therefore a yogurt from another supplier was tested ("O meu primeiro", Danone). The results for the enumeration of *L. bulgaricus* and *S. thermophilus* in yogurts containing preparations with grape seed extract, over 21 days of refrigerated storage are shown in Figure 12. A control with just yogurt and a yogurt containing preparations with potassium sorbate were also monitored for comparison.
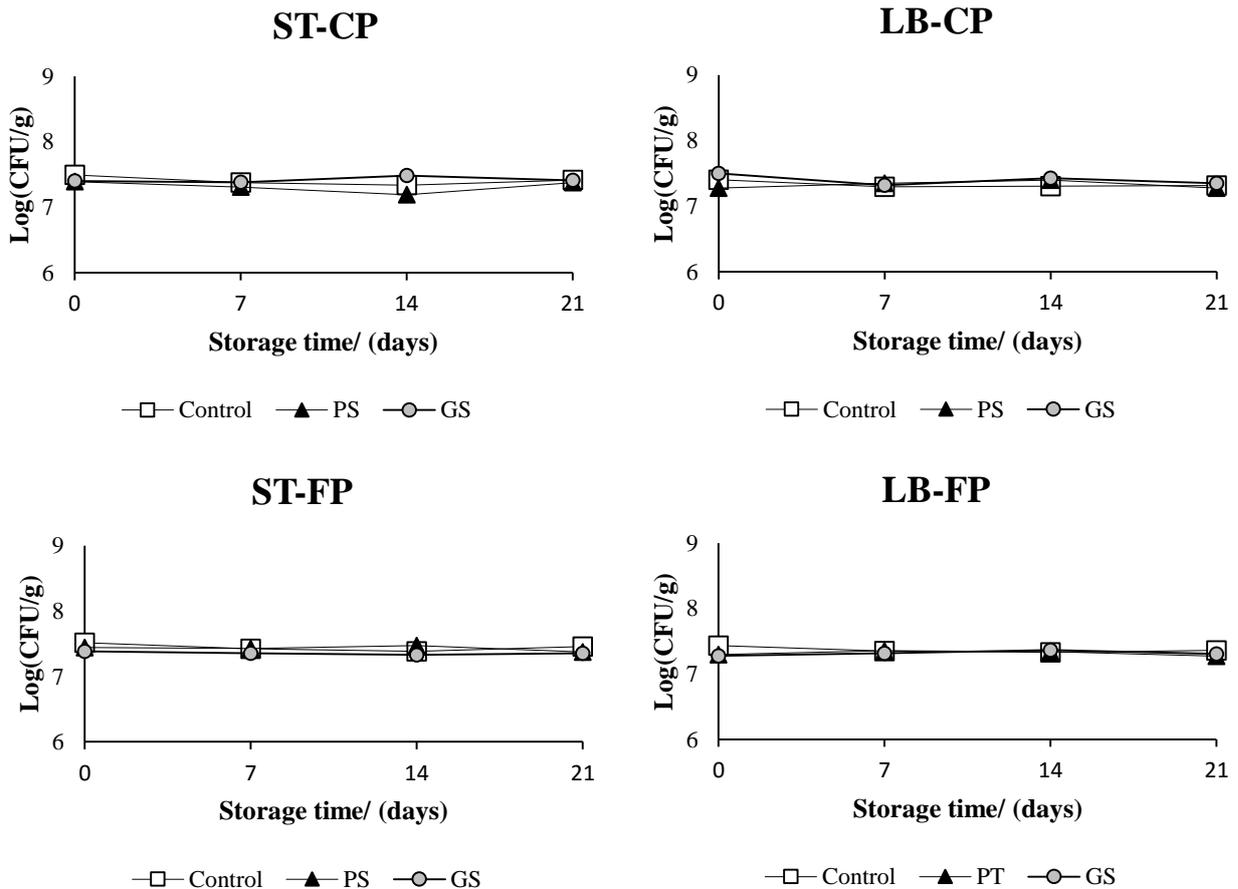

Figure 12. Viable cell count of yogurt microflora over 21 days of refrigerated storage at 4 ºC. ST-CP: counting of *S. thermophilus* for yogurts with cereal preparation; LB-CP: counting of *L. bulgaricus* for yogurts with cereal preparation; ST-FP: counting of *S. thermophilus* for yogurts with forest fruit preparation; LB-FP: counting of *L. bulgaricus* for yogurts with forest fruit preparation. Control refers to plain yogurt, PS refers to yogurts containing preparations with potassium sorbate, GS refers to yogurts containing preparations with grape seed extract.

In all cases, viable cell count of both monitored species remained higher than the required level by law, 7 Log(CFU/g), with values varying from 7.2 to 7.5 Log(CFU/g). There were no significant differences ($p > 0.05$) between the results obtained for different preparations. Comparing the results of viable cell count of *S. thermophilus* and *L. bulgaricus* with the control and with the sample containing potassium sorbate, no significant differences were found ($p > 0.05$). Thus, the preparations did not affected

yogurt natural microflora, which means that it is feasible to add them without affecting the viability of the starter cultures. As discussed earlier, in section 5.3.1 grape seed extract has already been shown to not interfere with yogurt natural microflora (Chouchouli *et al.* 2013).

These results show that the addition of these kind of preparations do not interfere with the viability of *L. bulgaricus* and *S. thermophilus* as opposed to strawberry preparation, which has shown to decrease the viability of both species, with a higher reduction of *L. bulgaricus* over 28 days of refrigerated storage (Oliveira *et al.* 2015). Since several studies show that many substances commonly used in the dairy industry such as fruit juices and flavoring-coloring agents affects the viability of yogurt microflora (Vinderola *et al.* 2002; Oliveira *et al.* 2015), it is important to test the effect of these preparations besides just testing the effect of isolated extracts.

5.3.5 Effect of grape seed extract in artificially contaminated preparations and dosed yogurts

The ability of grape seed extract to prevent a hypothetical spoilage caused by environmental fungi was tested by purposely contaminating the tested preparations. The fungi used were *Penicillium* sp. and *Aspergillus* sp. due to their ubiquity and spoilage tendency. Cereal and forest fruit preparations containing grape seed extract were tested as well as with potassium sorbate and without any preservative for comparison. The inoculum was tested to assess their concentration in terms of Log(CFU/mL). It was shown to have an average concentration of 4.5 Log(CFU/mL) (average calculated using two replicates), in which approximately 15% of the colonies corresponded to *Aspergillus* and the remaining percentage to *Penicillium* sp. The fungal load of the artificially contaminated preparations was monitored over 21 days of refrigerated storage and the results are shown in the form of charts in Figure 13. Fungal load includes both *Aspergillus* sp. and *Penicillium* sp. colonies. In both situations, all preparations started approximately with the same fungal load. Cereal preparations contained 2.6, 3.0 and 2.5 Log(CFU/g) for control, with potassium sorbate and with grape seed extract samples, respectively. The fungal load of cereal preparations containing grape seed extract increased up by 0.7 Log(CFU/g) after 21 days of refrigerated storage. When comparing these results with the control, without any preservative, no significant differences were found ($p > 0.05$). The same tendency was observed for both preparations: a slightly higher increase in the fungal load after 7 days of storage, with a tendency to stabilize to a plateau

value over the time. The cereal preparation containing potassium sorbate, started with a little higher fungal load than the other samples, but a clearly decrease is observed over time. In fact, at day 21, some plates did not contain any detectable colony, while others had just one single colony. This fact was considered for counting purposes, which explains the high standard deviation value. These results show that potassium sorbate decreased the viability of the fungal colonies, thus controlling the contamination as opposed to the other samples. If the tendency had continued, probably at day 28 no detectable fungal load would be observed in the samples containing potassium sorbate.
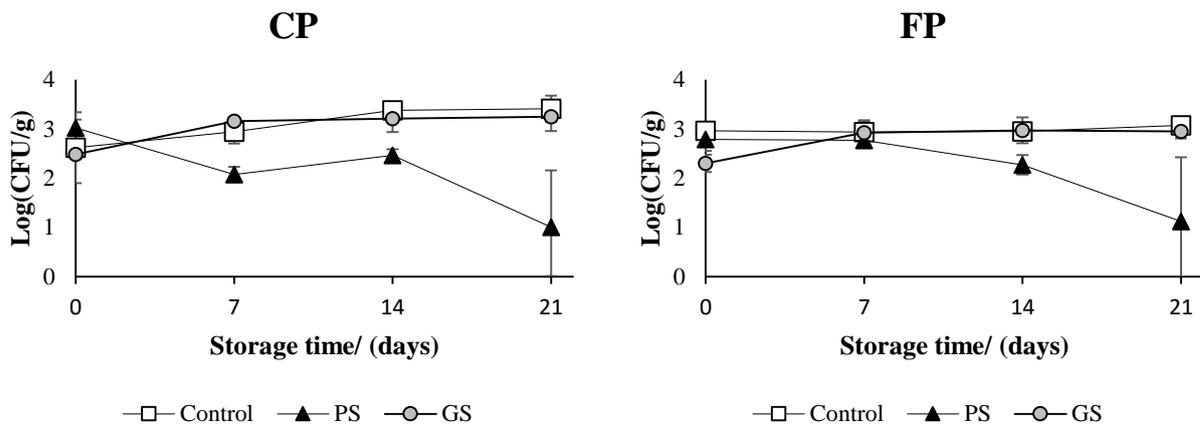

Figure 13. Fungal load of cereal (CP) and forest fruit (FP) preparations containing potassium sorbate (PS) and grape seed extract (GS) artificially contaminated, over 21 days of refrigerated storage at 4 ºC. Markers represent average and bars the standard deviation. The control is a preparation without any preservative.

As for the forest fruit preparations, these contained 3.0, 2.8 and 2.3 Log(CFU/g) for control, with potassium sorbate and grape seed extract samples, respectively, at day 0. The same tendency observed in the cereal preparations was observed here. Samples containing grape seed extract increased by approximately 0.7 Log(CFU/g) after the storage period. After 7 days of storage no significant differences were found between the grape seed extract sample and the control. Similarly to the previous case, potassium sorbate diminished the viability of the fungal colonies, which was observed by a decrease in the fungal load.

The fungal load of yogurts dosed with these contaminated preparations was also monitored over time. Results are presented in Figure 14. The same way as for the preparations, a slightly increase is observed in the fungal load of yogurts containing contaminated preparations with grape seed and without any preservative. As for samples containing potassium sorbate, a decrease is observed in cereal preparations, whereas in fruit preparations, practically no difference is observed in the fungal load over 21 days of

refrigerated storage. These differences can be probably explained by the type of preparation used, as fruit preparations provide a more propitious environment for the growth of these fungi.
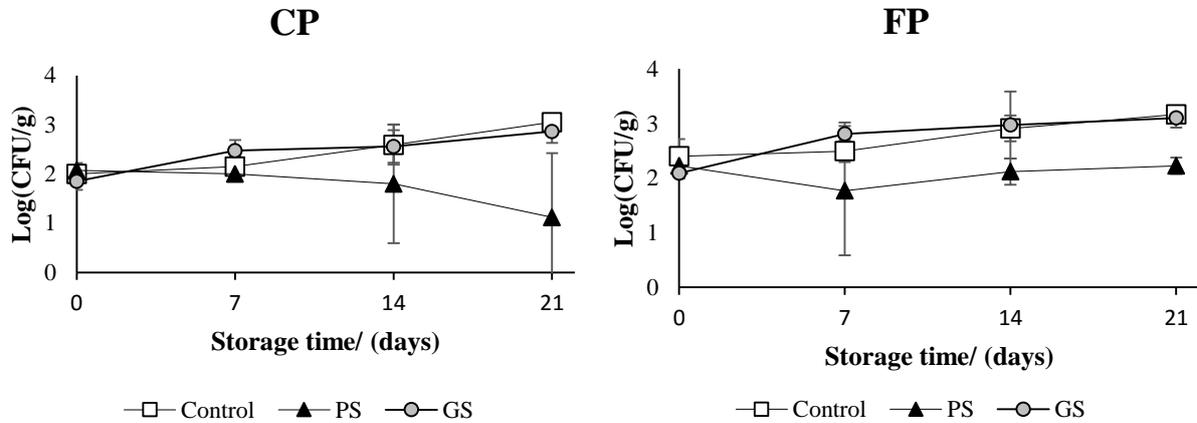

Figure 14. Fungal load of yogurts containing artificially contaminated preparations. CP refers to yogurts with cereal preparations, FP refers to yogurts with forest fruit preparations. Control is a yogurt with a preparation without any preservative, PS refers to yogurts with preparations containing potassium sorbate and GS refers to yogurts with preparations containing grape seed extract.

5.4 Conclusions

In this chapter the effect of selected extracts on yogurt and on preparations were studied. Yogurts containing green tea and grape seed extract and vanillin showed higher TPC than plain yogurt. In each yogurt, the TPC value maintained approximately equal during 28 days of refrigerated storage at 4 ºC. The addition of green tea and grape seed extract increased the radical scavenging activity of yogurt samples using the DPPH assay. These remained approximately the same over the storage period. Thus, these substances can be added to functional foods without losing activity over time. In plain yogurt, grape seed and green tea extract and vanillin did not interfere with yogurt natural microflora. However, the fungal load of these yogurts increased over time, especially the ones containing green tea and grape seed extracts, in which fungal load was detected after 14 days of storage. Yogurts with vanillin and potassium sorbate showed similar results in terms of fungal load over time, with detectable but low levels after 28 days of storage.

The effect of selected substances on preparations microbiology was also assessed during 28 days of refrigerated storage. None of the cereal preparations developed any detectable fungal load over this period. However, forest fruit preparations containing green tea and grape seed extract developed detectable levels of fungal load, with visible

mycelium on the surface of green tea extract preparations. Preparations with vanillin behave the same way as the ones containing potassium sorbate. Preparations containing grape seed extract didn't influence the yogurt natural microflora over 21 days of refrigerated storage. The ability of grape seed extract to prevent a purposive contamination in the tested preparations and corresponding yogurts was null, having very similar results to preparations that did not contain any preservative. In general, yogurts containing the preparations with the extracts were reported to have a very similar flavor when compared with a yogurt with the standard preparation. Yogurts with added vanillin were the ones more preferred over the standard.

Given the results here presented, in the tested conditions, green tea and grape seed extract were not effective as potential potassium sorbate substitutes, but still could be added as functional ingredients since their antioxidant activity remain stable over time. On the other side, vanillin had very promising results as a preservative, having very similar effects when compared to potassium sorbate. This way, further studies with vanillin from natural origin are need to prove their potential as a natural preservative.

Chapter 6.

Concluding remarks and future work

6.1 General conclusions

Plant polyphenols have been shown to exert antibacterial, antifungal and antiviral activities, as well as contributing with many health benefits when consumed, mainly due to their antioxidant activity. Due to their bioactive properties, many studies have pointed out the potential of plant extracts rich in polyphenols as natural preservatives in food products, as well as functional ingredients. However, studies on the effects of these substances in food products are scarce when comparing with *in vitro* studies. In this way, the main aim of this study was to evaluate the potential of several plant extracts rich in polyphenols to fulfill the above-mentioned characteristics.

The work developed throughout this study, showed that *Penicillium*, *Cladosporium* and *Aspergillus* are the most recurring species in the tested raw materials as well as in the environmental air of the Tortosendo unit. Generally, the antifungal activity of tested extracts was weaker when compared with potassium sorbate. These extracts were not able to inhibit the most recurring fungi species at a maximum concentration of 0.5% in laboratory media. Nevertheless, green tea and grape seed extracts were able to inhibit the mycelial growth of many other species. On the other hand, vanillin was an exception, being able to prevent the growth of all isolated fungi at a maximum concentration of 0.2%.

When added to yogurt, green tea and grape seed extracts and vanillin did not interfere with yogurt natural microflora, showing that its addition is feasible. Additionally, yogurts with these substances showed higher and stable values of total phenolic content and antioxidant activity than control yogurts, suggesting supplementation. When added to forest fruit preparation at 0.5%, the samples with green tea and grape seed extract developed a significant and detectable fungal load after 28 days of refrigerated storage. This means that these extracts were not able to prevent the growth of environmental spoilage fungi. The fruit preparation containing vanillin at 0.2% was equivalent to the one with potassium sorbate in terms of fungal load, suggesting inhibition. The cereal preparation did not develop any detectable fungal load after 28 days of storage, even in

the sample without any preservative. This suggests that this preparation is microbiologically stable, even in the absence of potassium sorbate, at the conditions tested. Grape seed extract did not have the ability to prevent a purposive contamination with *Aspergillus* sp. and *Penicillium* sp. in cereal and forest fruit preparations and respective yogurts, which is not surprising given the mentioned results.

Regarding organoleptic properties, most tasters did not perceive any or few differences between yogurts with preparations containing grape seed or green tea extract or vanillin when comparing with a standard sample. This means that the organoleptic properties of these substances are not an issue. Additionally, samples with vanillin were mainly preferred over the standards.

In conclusion, green tea and grape seed extracts, in the tested conditions, are not good potassium sorbate substitutes. Still, they have potential as functional ingredients in yogurts due to their antioxidant activity and to their harmlessness regarding yogurt natural microflora. Vanillin and potassium sorbate results were very similar, showing that vanillin has potential as a preservative. The results obtained also show vanillin potential as a functional ingredient.

6.2 Future work

Throughout this work, the antifungal potential of eight different plant extracts rich in polyphenols and vanillin against isolated fungi was tested. These fungi were isolated from the preparations' raw materials and from the environmental air of the Tortosendo factory. The main purpose of using potassium sorbate in FRULACT is to prevent spoilage of preparations, when these are handled by the client. So, for a more rigorous analysis, it is suggested to test the antifungal activity of these extracts against fungi isolated from the clients' factory, or the most recurring fungi pointed out by the client to cause spoilage.

Another important issue to address is the polyphenols content in the tested extracts. The better results in terms of antifungal activity were observed for the extracts with higher concentration of polyphenols. Most part of the tested extracts contained low levels of polyphenols, lower than 20%, which easily explains their poor results in terms of antifungal activity. This way, it is suggested to test extracts with higher polyphenol content. Besides, other extracts could be tested in the future, such as pomegranate and grapefruit seed extracts, as these are often referred in literature for their potent antifungal activity.

The results obtained during this study showed the potential of vanillin as a potassium sorbate substitute. However, the vanillin used in this work was chemically synthesized, which don't fulfil one of the main requisites of this project: natural origin. This way, it is suggested to test the effects of vanillin extracted from vanilla pods. Additionally, further tests should be taken to assess vanillin ability to prevent purposive contaminations caused by the most common spoilage fungi.

In FRULACT, in the actual processing lines, the plant extracts should be added before pasteurization. So, further studies are needed regarding polyphenols thermal stability to the pasteurization conditions.

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APPENDIX

Table A.1. Fungi isolated from the raw materials and from the air of the Tortosendo Unit identified by RT-PCR. Picture of the colonies and their origin.

Species	Picture	Isolated from
Aspergillus sp.		Wheat
Candida sake		Fruit preparation room
Chaetomoium sp.		Raspberry
Cladosporium sp.		Barley
Cryptococcus sp.		Barley
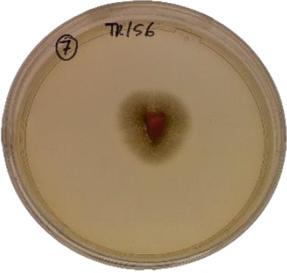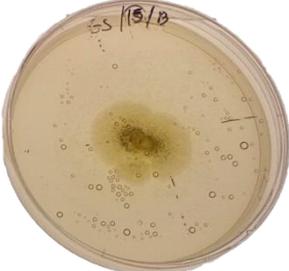
Species	Picture	Isolated from
Epicoccum nigrum		Dry raw materials storage room
Mucor piriformis		Raspberry
Penicillium sp.		Wheat
Penicillium sp.		Fruit preparation room
Penicillium sp.		Barley
Penicillium spinulosum		Refrigerated storage room
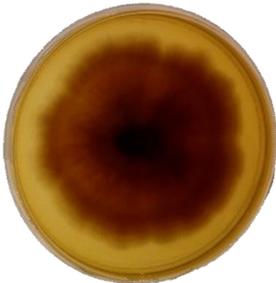
Species	Picture	Isolated from
Phoma pinodella		Freezing storage room
Peyronellaea glomerata		Blackberry
Pseudomonas sp.		Wheat

Avaliação sensorial de iogurte com frutos silvestres com **extrato de grainha de uva**

1.

Descritores	Menos (-)	Igual	Mais (+)
Acidez			
Doçura			
Adstringência			

Nota: a avaliação deve ser realizada através da comparação com o padrão

2. **Sabor em relação ao padrão:**

Igual ☐   Pouco diferente☐   Muito diferente☐

3. **Sabor mais agradável:**

Padrão ☐   Com extrato ☐   Indiferente ☐

Observações:

_____

Figure A.1. Example of the questionnaire filled by the tasters to assess the organoleptic changes caused by the addition of the antimicrobial substances. This is an example for the yogurt with forest fruit preparation with grape seed extract. The same questionnaire was done for green tea extract and vanillin regarding yogurt with forest fruit and cereal preparations.