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Pleurotus species as a source of natural preservatives: mycelia production to obtain tocopherols used as antioxidants in yogurts

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LIST OF ABBREVIATIONS

A_{DPPH}	Absorbance of DPPH solution
A_S	Absorbance of the solution
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
A. bisporus	*Agaricus bisporus*
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAT	Catalase
Cu	Copper
DMPBQ	2,3-Dimethyl-6-phytyl-1,4-benzoquinone
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl radical
Dw	Dry weight
EC_{50}	Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay
EMM	Estimated marginal means
ETS	Mitochondrial electron transport system
EU	European Union
FAD	Ferry & Das medium
FAME	Fatty acids methyl ester
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fe	Iron
Fe^{2+}	Blue ferrous form
Fe^{3+}	Yellow ferric form
FOSHU	Foods for Specified Health Use
Fw	Fresh weight
G. rutilus	*Gomphidius rutilus*
GC	Gas chromatography

GC-FID	Gas chromatography coupled to a flame ionization detector
GGPP	Geranyl geranyl diphosphate
GGR	Geranyl geranyl diphosphate reductase
GLM	General linear model
GP_x	Glutathione peroxidase
GRAS	Generally Recognized as Safe
G_{red}	Glutathione reductase
GRMP1/GRMP2	Two novel polysaccharide fractions from the mycelium of *Gomphidius rutilus*
GSH	Glutathione
HGA	Homogentisic acid
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
HPLC-FL	High performance liquid chromatography coupled to a fluorescence detector
HPLC-RI	High performance liquid chromatography coupled to a refraction index detector
HPLC-UV	High performance liquid chromatography coupled to an ultraviolet detector
HPP	*p-Hydroxyphenylpyruvic acid*
HPPD	4-Hydroxyphenylpyruvate dioxygenase
HPT	Homogentisic acid phytyl transferase
IAEA	International Atomic Energy Agency
IS	Internal standard
LDA	linear discriminant analysis
LP	Lipid peroxidation
MGGBQ	2-Methyl-6-geranylgeranylplastoquinol
mMMN	Modified Melin–Norkans medium
MMN	Melin–Norkans medium
Mn	Manganese
MPBQ	2-Methyl-6-phytyl-benzoquinol
NDGA	Nordihydroguaiaretic acid
$NO^{\bullet}$	Nitric oxide
$O_2^{\bullet -}$	Superoxide anion

$OH^{\bullet}$	Hydroxyl radical
OH^{-}	Hydroxyl ion
PACH	Pachlewski medium
PDA	Potato dextrose agar medium
PDB	Potato dextrose broth
PDP	Phytyldiphosphate
P. eryngii	*Pleurotus eryngii*
pH	Potential of hydrogen
P. ostreatus	*Pleurotus ostreatus*
$R^{\bullet}$	Fatty acid radical
RH	Polyunsaturated lipid
$ROO^{\bullet}$	Peroxyl radical
$ROOH$	Hydroperoxide
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RSA	Radical scavenging activity
Se	Selenium
SOD	Superoxide dismutase
ST	Storage time
US	United States
UV	Ultraviolet
Vit. C	Vitamin C
Vit. $C^{\bullet}$	Vitamin C radical
Vit. E	Vitamin E
Vit. $E^{\bullet}$	Vitamin E radical
WHO	World Health Organization
YF	Yogurt formulations
Zn	Zinc
$\alpha - TOH$	$\alpha -$ Tocopherol
$\alpha - TO^{\bullet}$	Tocopheroxyl radical

µg	Microgram
µl	Microliter

ABSTRACT

Mushrooms are consumed worldwide not only as a part of the normal diet, but also as a delicacy due to their highly desirable taste and aroma. In addition to their nutritional value, mushrooms have been considered functional foods and even as adjuvants in some therapies, namely chemotherapy, because they are rich in bioactive molecules, such as phenolic compounds, tocopherols, ascorbic acid, or carotenoids. In particular, tocopherols are powerful antioxidants that can be explored as natural food preservatives, in order to replace the widely used synthetic counterparts.

With these aspects in mind, and given the evidence that the *in vitro* culture of mushroom mycelia promotes the production of tocopherols, the present work aimed to sub-culture two edible species, *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm. and *Pleurotus eryngii* (DC.) Quél., in different culture media (i.e., Melin–Norkans medium, MMN; modified Melin–Norkans medium, MMNm; and Potato dextrose agar medium, PDA) in order to enhance the production of tocopherols, and use the mycelia as sources of preservative ingredients for foodstuff. Since the best growth was obtained on PDA medium, both species were cultivated in Potato dextrose broth liquid medium (PDB), in order to obtain the amount of biomass required for the assays. The tocopherols content was evaluated by high performance liquid chromatography coupled to a fluorescence detector (HPLC-fluorescence). A formulation enriched in tocopherols was incorporated in yogurts and its preservative capacity was assessed, through the evaluation of the antioxidant properties before and after incorporation in the foodstuff. The results were compared with commercial available and used preservatives (i.e., α-tocopherol - natural antioxidant (E307) and potassium sorbate - synthetic preservative (E202)).

The results showed that the mycelium content of tocopherols was particularly high in β-tocopherol and, in the case of *P. ostreatus*, it was higher than in *P. eryngii*. Despite the similarity showed in the tocopherol profile for both mushrooms, *P. ostreatus* mycelium revealed a slightly high DPPH radical scavenging activity, while *P. eryngii* showed higher reducing power. The DPPH radical scavenging activity measured in yogurts fortified with *P. ostreatus* and *P. eryngii* extracts was similar to that achieved in yogurts prepared with potassium sorbate, despite not as strong as yogurts with α-tocopherol. Regarding the reducing

power, yogurts with *Pleurotus* species extracts revealed approximate results of those obtained in yogurts with α-tocopherol, reaching best results than those obtained in yogurts containing potassium sorbate. In neither case were observed significant changes induced by the storage time.

RESUMO

Os cogumelos são consumidos no mundo inteiro, não só como parte da dieta normal, mas também como uma iguaria devido ao seu gosto e aroma altamente desejáveis. Além do seu valor nutricional, os cogumelos são considerados alimentos funcionais, sendo mesmo adjuvantes em algumas terapias, nomeadamente a quimioterapia, uma vez que são ricos em moléculas bioativas, como compostos fenólicos, tocoferóis, ácido ascórbico ou carotenóides. Em particular, os tocoferóis são poderosos antioxidantes que podem ser explorados como conservantes de alimentos naturais, podendo substituir os equivalentes conservantes sintéticos utilizados atualmente.

Tendo em conta estes aspetos, e a evidência de que a cultura *in vitro* de micélio de cogumelos promove a produção de tocoferóis, o presente trabalho pretende sub-cultivar duas espécies comestíveis, *Pleurotus ostreatus* (Jacq. ex P.) P. Kumm. e *Pleurotus eryngii* (DC.) Quél. em diferentes meios de cultura (isto é, meio Melin-Norkans, MMN; meio Melin-Norkans modificado, MMNm; e meio de agar de dextrose de batata, PDA). Uma vez que o melhor crescimento foi obtido no meio PDA, as duas espécies foram cultivadas em meio líquido de dextrose de batata (PDB), de forma a obter a quantidade de biomassa necessária para a realização dos ensaios. O teor de tocoferóis foi avaliado por cromatografia líquida de alta performance acoplada a um detector de fluorescência (HPLC-fluorescência). Uma formulação enriquecida em tocoferóis foi incorporada em iogurtes e sua capacidade de conservação foi avaliada através da avaliação das propriedades antioxidantes antes e depois da incorporação no género alimentício. Os resultados foram comparados com os conservantes comercialmente disponíveis e utilizados (nomeadamente, α-tocoferol - antioxidante natural (E307) e sorbato de potássio - conservante sintético (E202)).

Os resultados mostraram que o perfil de tocoferóis no micélio era praticamente β-tocoferol e, no caso de *P. ostreatus*, a concentração era maior do que em *P. eryngii*. Apesar da semelhança evidenciada no perfil de tocoferóis em ambos os cogumelos, o micélio de *P. ostreatus* foi o que revelou uma actividade captadora de radicais DPPH ligeiramente superior, enquanto o micélio de *P. eryngii* apresentou maior poder redutor. A atividade captadora de radicais DPPH medida em iogurtes fortificados com extratos de *P. ostreatus* e *P. eryngii* foi semelhante à obtida em iogurtes preparados com sorbato de potássio, embora não tão elevada quanto em iogurtes com α-tocoferol. Em relação ao poder redutor, os iogurtes com extratos de

espécies de *Pleurotus* revelaram resultados aproximados aos obtidos em iogurtes com α-tocoferol, atingindo melhores resultados do que os obtidos em iogurtes contendo sorbato de potássio. Em nenhum dos casos foram observadas alterações significativas induzidas pelo tempo de armazenamento.

CHAPTER 1

1. INTRODUCTION

1.1. Tocopherols in macrofungi

The consumption of mushrooms has been increasing over the years. As a consequence, the world mushroom production enlarged significantly, being this increase tenfold from 1969 to 2009 (Zhang et al., 2014).

Therefore, nowadays, mushrooms occupy a special place in the human daily diet, being a product of significant economic value. In addition to their highly desirable taste and aroma, mushrooms have a great nutritional value, since they are poor in fat, cholesterol, sodium, and calories, while they are rich in proteins, vitamins (B1, B2, B12, C, D, and E), fiber, carbohydrates and essential amino acids (Kalač, 2009 and 2013; Mattila et al., 2001).

Mushrooms have also become interesting as functional foods because they are a source of bioactive compounds such as phenolic compounds, tocopherols, ascorbic acid or carotenoids, which confer them antioxidant, antimicrobial, anti-inflammatory, or cytotoxic properties (Alves et al., 2013 ; Barreira et al., 2014 ; Boonsong et al., 2016 ; Taofiq et al., 2015).

Since mushrooms are rich in such bioactive compounds, they can also be studied as a source of molecules that can be used as food additives, namely additives with antioxidant potential. Although synthetic antioxidants are the most commonly used as food additives, they may have a restricted use in the food industry. For example, two of the most widely used antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been identified as the trigger for irritation in a case report of two patients with chronic urticaria (Randhawa & Bahna, 1987). Also nordihydroguaiaretic acid (NDGA), another antioxidant used as food additive, causes renal cystic disease in rodents (Evan & Gardner, 1979). Therefore, in recent years, there has been an increased interest in the development of natural antioxidants. Given the antioxidant potential that mushrooms have shown (Ferreira et al., 2009; Sánchez, 2017), these have become an option, along with plants. Moreover, these natural matrices present a significant advantage for the extraction of antioxidant compounds since, besides the fruiting bodies, it is possible to cultivate the mycelium of certain species,

and, in a relatively short period of time, manipulate / optimize the conditions to obtain higher quantities of interesting compounds (Sánchez, 2017).

The term "vitamin E" does not refer to a single compound, but it is rather used to name a family of eight tocochromanols. Four of these compounds are termed tocopherols and the other four are coined as tocotrienols (Yin et al., 2011). These compounds are chemically related. They contain a common structure with a polar chromanol head group remaining at the membrane surface and a hydrophobic isoprenic side chain associated with membrane lipids which are synthesized in plastid membranes (Chaudhary & Khurana, 2009). All the members of vitamin E are amphiphilic molecules produced by the same biosynthetic pathway. Depending on both the saturation degree of their hydrophobic tails and the number and position of the methyl groups on the chromanol head, they can be classified as α-, β-, γ- and δ-isoforms, (**Figure 1**) (DellaPenna, 2005). Up to now, although there are not many studies reporting the presence of tocotrienols, α-, β-, γ-, and δ-tocopherols have been identified and quantified in cultivated and wild mushrooms (Ferreira et al., 2009 ; Kozarski et al., 2015).

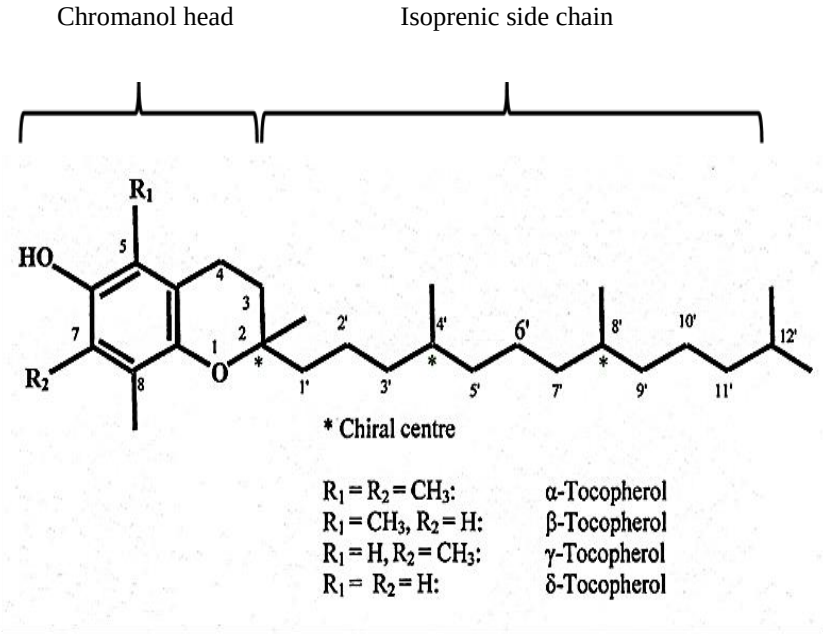


Figure 1. Chemical structure of tocopherols (Panfili et al., 2003).

Differences on the number and position of the methyl groups in the aromatic head are indicated, with α-tocopherol having three methyl groups, β- and γ-tocopherol two methyl groups, and δ-tocopherol only one methyl group.

1.1.1. Biosynthetic pathway

The biosynthetic pathway of tocopherols was determined due to the identification of the full genome DNA sequences for several photosynthetic organisms, and the availability of reverse genetic resources (Li et al., 2011).

The biosynthesis of tocopherols is based on two compounds derived from different metabolic pathways, which are considered the precursors. They include the homogentisic acid (HGA), for the synthesis of the head group, and phytyldiphosphate (PDP), for the synthesis of the hydrophobic tail of tocopherols (Sattler et al., 2003). The PDP, incorporated into tocopherols, is produced by two different sources. It was originally proposed to result exclusively from the stepwise reduction of geranyl geranyl diphosphate (GGPP) by a geranyl geranyl diphosphate reductase (GGR). However, some studies demonstrated that a free phytol, derived from chlorophyll degradation, can recycle to produce PDP. Actually, recent studies have proposed that the recycling of phytol present the major way of PDP production used for tocopherol synthesis (Mène-Saffrané & DellaPenna, 2010).

Tocochromanols synthesis is initiated by the production of the aromatic head group by an oxidative decarboxylation reaction, catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD), in which *p*-hydroxyphenylpyruvic acid (HPP) is converted into HGA. This reaction is irreversible, as shown in **Figure 2** (reaction 1) (Jiang et al., 2017). The tocochromanol biosynthetic pathway diverges at the second reaction, in which HGA is subjected to condensation with PDP or GGPP. This reaction is catalyzed by the homogentisic acid phytyl transferase (HPT), to form the first intermediates of all tocopherols and tocotrienols, 2-methyl-6-phytyl-benzoquinol (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ) (Mène-Saffrané & DellaPenna, 2010). Once MPBQ and MGGBQ are formed, the substrate specificity of HPT is responsible for determining if tocopherols, tocotrienols or all tocochromanols are synthesized in an organism. The next steps in the synthesis processes are ring methylation and ring cyclization. δ-Tocopherol is formed by a direct cyclization of MPBQ by tocopherol cyclase, whereas methylation of MPBQ at the ring position C-3, produce the precursor of γ-tocopherol: 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ). α- and β-tocopherols are generated by a methylation reaction, catalyzed by tocopherol methyltransferase. It consists in the addition of a methyl group in the chromanol head of δ- and γ-tocopherols, respectively (DellaPenna, 2005).

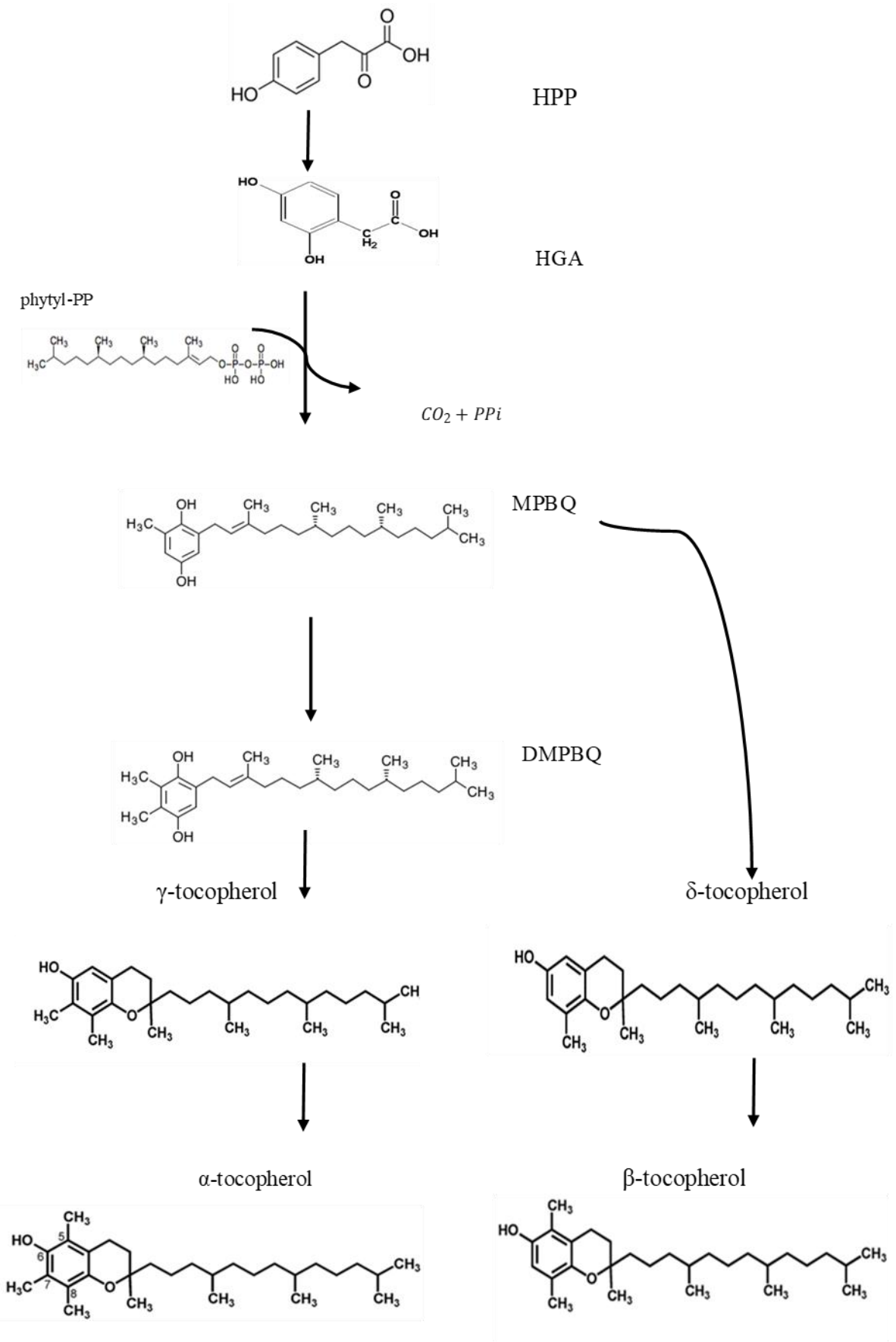


Figure 2. Biosynthetic pathway of tocopherols (based on Mène-Saffrané & DellaPenna, 2010).

1.1.2. Antioxidant and antimicrobial properties

1.1.2.1. Antioxidant properties

Photosynthetic organisms are largely responsible for the production and maintenance of the oxygen content on the Earth's atmosphere (Sánchez, 2017).

The partial reduction of oxygen can generate reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl ion (OH^-), hydroxyl radical ($OH^\bullet$), nitric oxide ($NO^\bullet$) and hydrogen peroxide (H_2O_2). These free radicals and non-radical molecular forms are produced during the normal metabolism of aerobic cells (Kozarski et al., 2015).

Free radicals represent the most important class of radical species generated in living systems. A free radical can be defined as a chemical compound containing one or more unpaired electrons in atomic or molecular orbitals. Oxidation is the process used by the human body to convert nutrients such as carbohydrates, fats, and proteins into energy. In normal physiological conditions ROS are produced during oxidation to maintain the normal cell functions (Sánchez, 2017). According to Cederbaum and collaborators (2009), ROS are derived from a variety of different sources: cellular and environmental sources (**Figure 3**).

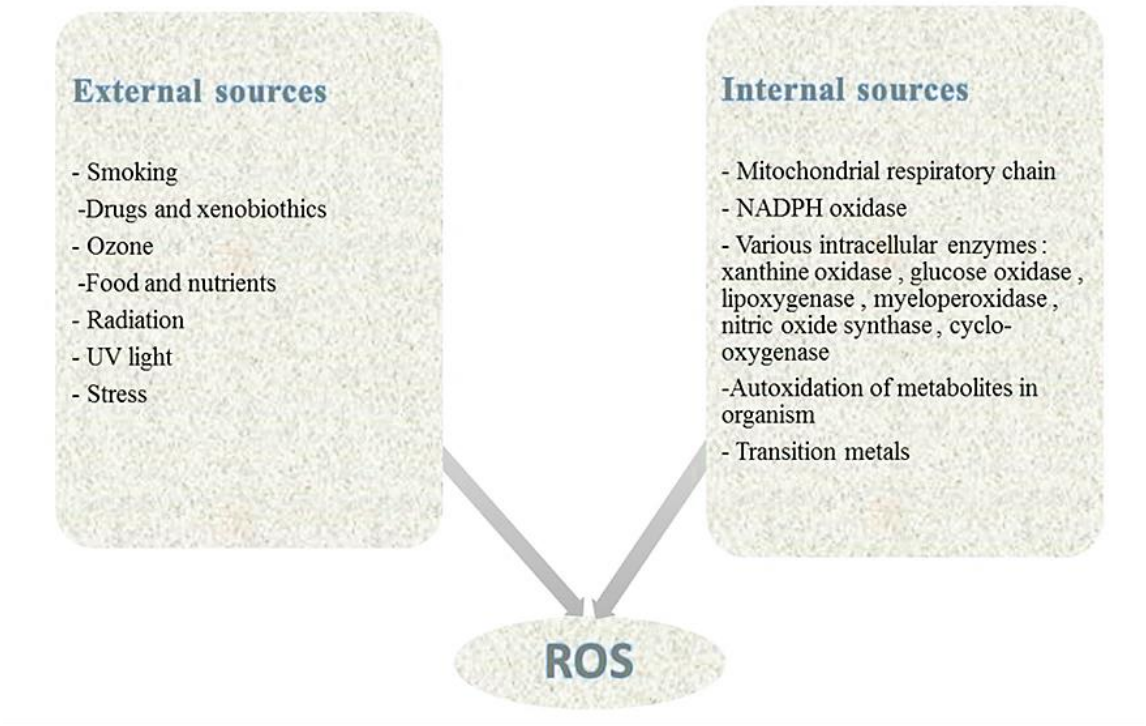


Figure 3. Internal and external inducers of ROS production (Based on Kozarski et al., 2015).

Mitochondria produce more than 90% of the ROS in eukaryotic cells via the continuous leakage of electrons from the mitochondrial electron transport system (ETS) (Kozarski et al., 2015). In the biological systems, the oxidative stress is related to the physiological disturbance of the equilibrium between the ROS production and the capability of the body to remove them and repair the damage (Gupta et al., 2014; Touyz, 2004). When ROS levels in the human body increase dramatically, it becomes toxic to the organism. Since free radicals need to pair up their electrons, they "attack" nearby chemical compounds, causing changes in their chemical structure and changing or causing a loss of their function. ROS "attack" most cellular macromolecules by the inactivation of some enzymes, modification of nucleic acids, denaturation of proteins and causing DNA damage (Cederbaum et al., 2009). This cellular damage contributes for more than one hundred diseases such as cancer, cardiovascular diseases, cataracts, immune system decline, liver diseases, diabetes *mellitus*, inflammation, renal failure, or brain dysfunction (Ferreira et al., 2009).

The molecules that protect the human body from the oxidative stress have been extensively studied for their positive influence on human health. For the scavenging of free radicals two compounds are involved: i) primary free radical scavengers, which react directly with ROS, and ii) secondary antioxidants, which react with species producers of free radicals, preventing the production of ROS. The latter, are responsible for metal atoms chelation (such as copper) (Martínez & Reina, 2017). Actually, the oxidation of an organic compound by a free radical, produce another radical as first compound. This can lead to chain reactions of oxidation by low molecular weight antioxidants. This process can be illustrated by the example of lipid peroxidation (Fridovich, 1999).

According to Ferreira and collaborators (2009), lipid peroxidation (or autoxidation) is a cascade of reactions which proceeds in three phases: i) initiation, begins with the removal of an hydrogen atom from a polyunsaturated lipid (RH), through the action of reactive species being produced a fatty acid radical ($R^{\bullet}$); ii) propagation, a readily reaction of fatty acid radicals with molecular oxygen, which is not a very stable molecule, thereby creating an unstable species , the peroxyl radical ($ROO^{\bullet}$). This radical reacts with another polyunsaturated lipid molecules and produce a hydroperoxide ($ROOH$) with a new fatty acid radical that is converted into another peroxyl radical. This propagation continues and can become a “leak” process, by a consumption of valuable polyunsaturated fatty acids and production of a corresponding quantity of hydroperoxide ($ROOH$); iii) termination, non-radical species (inactive products) are produced; (**Figure 4**) (Burton & Traber, 1990).

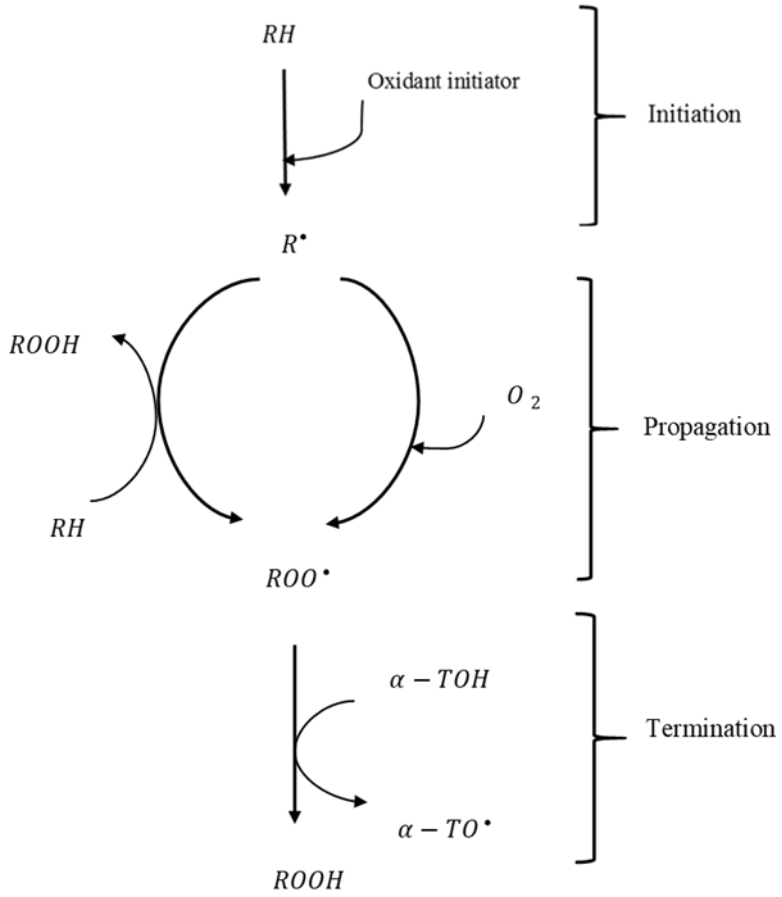


Figure 4. Chain reactions of lipid peroxidation (Based on Burton & Traber, 1990).

The cellular antioxidant defenses can be enzymatic or non-enzymatic. The endogenous enzymatic antioxidants, which can fight free radical formation and their propagation in the organism, include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_x), and glutathione reductase (G_{red}). The non-enzymatic defences may be endogenous such as glutathione (GSH) and melatonin, or obtained through the diet. These include proteins and low molecular weight antioxidants such as vitamins C, E and A, carotenoids, polyphenols or uric acid; and large molecules such as albumin, ceruloplasmin, transferrin or ferritin. In addition, zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), and selenium (Se) exert also antioxidant effects (Kozarski et al., 2015).

As previously referred, vitamin E is widely known for its antioxidant potential. α-tocopherol is known as the most biologically active compound among tocochromanols, being an important natural antioxidant in food. Since tocopherols are fat soluble compounds, they can be embedded within the cell membrane, exerting protective effects (Ferreira et al., 2009).

The health benefits of tocopherols as bioactive compounds are well described in several studies. Tocopherols act as a free radical scavenger of ROS and they are considered as the first line of defense against lipid peroxidation (LP), acting as a chain breaking antioxidants in cell membranes (Burton & Traber, 1990).

Vitamin E and vitamin C can also exhibit a synergistic action. Vitamin E reacts with ROS (hydroxyl radicals, peroxyl radicals, etc.) generating a reactive phenolic radical (vit.E•). Vitamin C then interacts with vit.E• to regenerate the membrane-bound oxidized vitamin E and producing the vitamin C radical (vit.C•) noted that both radicals (vit.E• and vit.C•) are poorly reactive species (**Figure 5**) (Carocho & Ferreira, 2013).

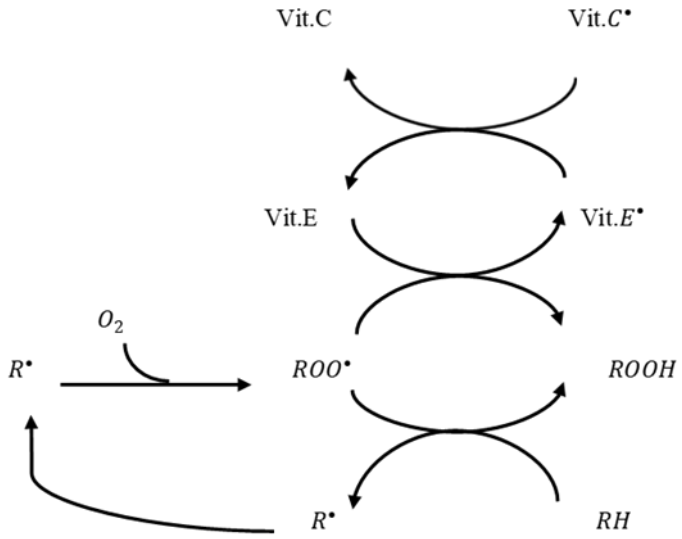


Figure 5. Cooperative interactions among vitamin C and vitamin E (Based on Ferreira et al., 2009).

1.1.2.2. Antimicrobial properties

Despite their simplicity, bacteria contain a well-developed cell structure. It is composed of a cytoplasmic membrane which is an essential structure surrounding the cytoplasm and being responsible for several functions including DNA replication, secretion of enzymes, biosynthesis of components, transport of solutes and energy production. Another structure, on the outside of the cell membrane, is the cell wall which confers rigidity to bacteria. According to its permeability properties and composition, bacteria are divided into two classes: gram-positive and gram-negative (Alves et al., 2013).

Bacterial infections have become a dominant public health disease worldwide due to the significant increase of bacterial resistance caused by the increased consumption of antibiotics (Lima et al., 2016). Therefore, antimicrobial resistance presents a clear risk since it is responsible for increasing rates of mortality and morbidity, prolonged hospital stays and additional health costs (Davey & Marwick, 2008).

Staphylococcus aureus is the common cause of infections due to its resistance to several antibiotics. It is responsible for a range of diseases, from a variety of skin infections, to life-threatening diseases such as different types of intoxications, osteomyelitis, pneumonia, abscesses, endocarditis and bacteremia (Andrade et al., 2014). *Pseudomonas aeruginosa* is the major cause of hospital acquired infections, causing damage to the skin, urinary tract, ears and eyes. In addition to its resistance to antibiotics, this bacterium is very virulent due to toxins and enzymes present in its structure. *Escherichia coli* is the most known species of the genus *Escherichia*. It is related to severe infections of the urinary tract, meningitis and gastroenteritis (Lima et al., 2016).

The World Health Organization (WHO) has identified the antibacterial resistance as one of the three biggest threats to human health (Gilbert et al., 2010). This problem usually happens after an excessive misuse of antimicrobials or after acquisition of mutations in the bacterial genome, which leads to an adaptation and resistance of the bacteria (Ren et al., 2014).

It is clear that the treatment against these microorganisms by the available drugs is difficult. So the development of new drugs from other sources and maybe with different mechanisms of action must be performed.

To survive in their environment, mushrooms require the production of compounds with antibacterial activity. Therefore, it is not surprising that it is possible to extract compounds with such properties from these natural sources (Lindequist et al., 2005).

Liposoluble compounds can exert antimicrobial activity due to their capacity to alter the fluidity of the bacterial cytoplasmic membrane, making it permeable to some substances, namely antibiotics. This way, bacteria lost the resistance capacity (Andrade et al., 2014). A study performed in 2004 by Meydani and collaborators about the effect of vitamin E against infection diseases, suggested that the immunostimulatory effect of vitamin E is associated with resistance to infections.

In 2014, Andrade and collaborators described for the first time the modulatory effect of α-tocopherol in multiresistant bacteria. Due to its liposoluble nature, α-tocopherol changes the fluidity of the bacterial membrane by modifying some crucial elements for its integrity. This leads to the decrease in the membrane potential and loss of ions, cytochrome C, proteins and radicals, followed by the collapse of proton pumps and decrease in ATP. This caused the permeability of the membrane to various substances, particularly antibiotics (Andrade et al., 2014).

1.2. Food additives

1.2.1. Overview of the use of food additives

Food is undoubtedly vital and beneficial for mankind (Carocho et al., 2015). We should consume it not just because we are hungry, but for health reasons too. The German dictum «man ist was man isst» means “you are what you eat” clearly illustrate the relation between food and health (Atkins & Bowler, 2016).

Despite the enormous variability in food supply, consumers have become increasingly concerned about the type and the quality of the consumed food (Atkins & Bowler, 2016). Consequently, healthy foods with physiologically active components become the major interest of consumers. These foods are called “functional foods” (Pang et al., 2012).

In the mid-1980s, Japan was the first country that introduced the term “functional food”, which was defined as transformed food, containing some nutritional ingredients with specific

bodily functions (Hasler, 1998). In 1988, Japan was the only country that has formulated a specific regulatory approval process for functional foods known as Foods for Specified Health Use (FOSHU). The definition of functional food by the Institute of Medicine's Food and Nutrition Board (IOM/FNB, 1994) is "any food or food ingredient that promotes a positive health impact" (Hasler, 1998).

In modern industrial countries, consumers expect a wide range of foodstuffs available throughout the year. Supermarket shelves are stocked with different products such as fresh fruits and vegetables, or meat and fish exported from many different countries. They are also stocked with prepared salads, breads, preserved meats, spreads, quick-to-prepare dishes, sauces, drinks and chilled and frozen meals which just need for re-heating. Moreover, these ready-made foods should have enough shelf-life for consumer satisfaction (Saltmarsh , 2013).

The use of food additives to preserve food is not novel; it is an old-age need. In fact, the modern techniques that are used are canning, refrigeration/freezing and drying. The two most basic functions of additives are the preservation of food from bacteria or other chemical reactions which have an impact on its quality (Saltmarsh , 2013; Parke & Lewis, 1992).

Nowadays, food additives have been vilified in the press, becoming almost synonym of adulteration, which leads consumers to avoid certain food products. Consumers usually associate food additives with intolerance, hyperactivity and long term chronic diseases. This misunderstanding between food additives and chemicals was sufficiently enough to their consideration as nasty.

Food additives are defined by the *Codex Alimentarius* as "any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result, (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include "contaminants" or substances added to food for maintaining or improving nutritional qualities" *(Codex Alimentarius*).

The European Union (formally the European Economic Community), has developed the E system which provides a listing of several commonly used additives. The list, which is

updated on a regular basis, includes those additives that are generally recognized as safe and reliable within the member states, and allows the transport of food from country to country within the European Union (Branen et al., 2001).

1.2.2. The class of preservatives: natural *versus* synthetic agents

Within the EU, food additives are divided into 26 functional classes, depending on their function in food: sweeteners, colorants, preservatives, antioxidants, carriers, acids, acidity regulators, anticaking agents, antifoaming agents, bulking agents, emulsifiers, emulsifying salts, firming agents, flavor enhancers, foaming agents, gelling agents, glazing agents, humectants, modified starches, packaging gases, propellants, raising agents, sequestrants, stabilizers, thickeners, and flour treatment agents (Council Regulation (EC) 1333/2008).

According to the Food and Drug Administration (FDA) of the United States (US), there are more than 3000 food additives. Additives can be divided into six major categories: preservatives, nutritional additives, coloring agents, flavoring agents, texturizing agents, and miscellaneous agents (**Figure 6**). Furthermore, the preservatives group is divided into 3 subgroups, even some additives may exert one or more functions in foods: antimicrobials, antioxidants, and anti-browning agents. The flavoring agents group, composed by 3 subgroups: the sweeteners, the natural or synthetic flavors, and the flavor enhancers. The texturizing agents comprise emulsifiers and stabilizers and the coloring agents encompass the azo compounds, the chinophthalon derivatives, the triarylmethane compounds, the xanthenes and the indigos. Finally, the miscellaneous agents are composed of many classes: chelating agents, enzymes, antifoaming agents, surface finishing agents, catalysts, solvents, lubricants, and propellants (Carocho et al., 2014).

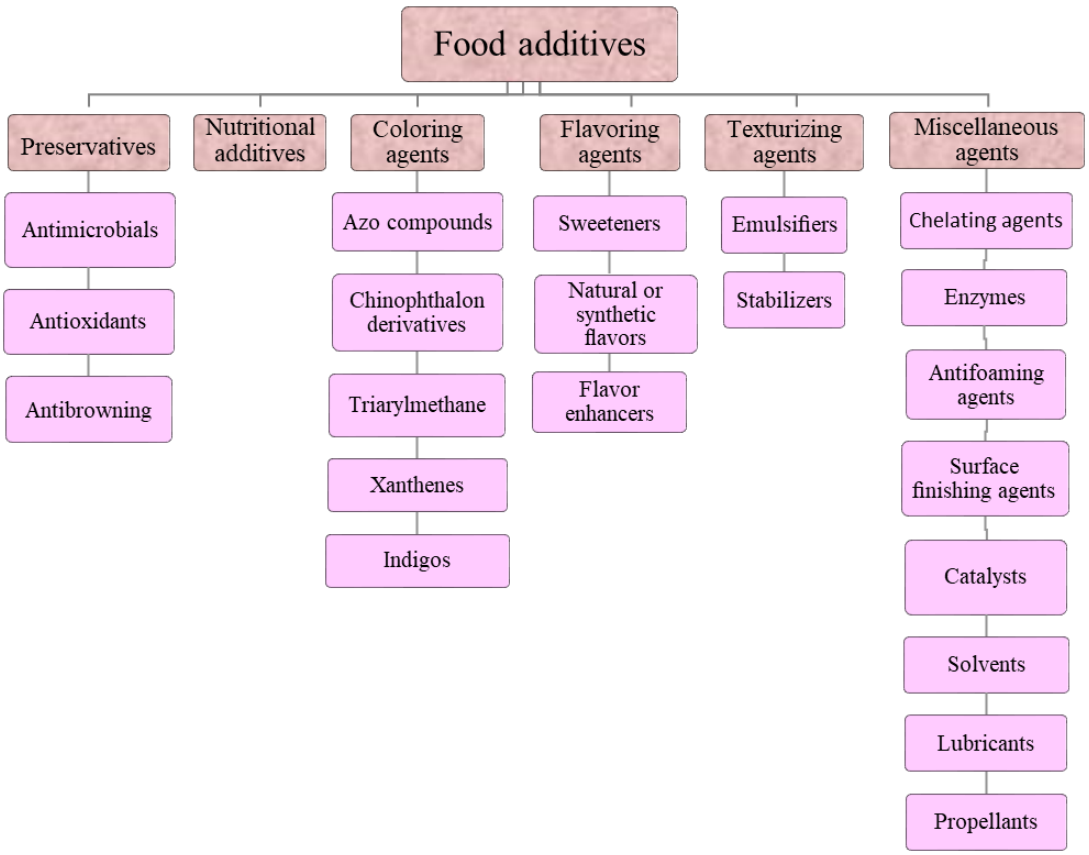


Figure 6. Groups and sub-groups of food additives (adapted from Carocho et al., 2014).

Despite the diverse classes of additives and the various classifications used, we can distinguish 4 fundamental groups of food additives with regard to their origin and manufacture: natural additives (direct extracted from animals or plants); similar to natural additives (synthetic production mimicking natural ones); modified from natural (chemical modification of natural additives); and finally artificial additives (synthetic compounds) (Carocho et al., 2014).

Some studies have demonstrated that consumers have recently become more well-informed about food additives and they are likely to choose the natural additives than their synthetic analogues (Carocho et al., 2015). The benefit of using synthetic additives in food industry is the improved characteristics and properties of processed foods. However, the consumption of

synthetic food additives in excess is linked to gastrointestinal, respiratory, dermatological, and neurological adverse reactions.

One of the most preservatives used in food industry is potassium sorbate, as antimicrobial agent, since it is efficient in the inhibition of the growth of fungi, aerobic bacteria and yeasts. Despite the safety, efficiency, and the lower toxicity compared to other preservatives, some authors take into consideration that the use of this preservative has adverse effects on human health, reporting some cases of allergic effects (urticaria and asthma), as also some cases of intolerance (Caleja et al., 2016).

The antioxidants present in plants, algae and mushrooms are excellent natural additives and have been presented as alternatives to synthetic additives. Vitamins, polyphenols and carotenoids are presented as natural antioxidant molecules. Polyphenols are among the most interesting and applicable natural compounds as food preservatives and bioactive ingredients, due to their high antioxidant capacity (Caleja et al., 2016). In 2016, Caleja and collaborators reported that the incorporation of aqueous extracts prepared from *Matricaria recutita L.* (chamomile) and *Foeniculum vulgare Mill.* (fennel) in yogurts, improved the antioxidant activity and revealed higher potential compared to the synthetic additive potassium sorbate. Furthermore, the authors demonstrated that the use of these decoctions did not significantly alter the nutritional profile, external appearance, pH and individual fatty acids of the matrix.

In fact, the incorporation of the natural extracts in food may provide benefits for consumers, due to their antioxidant and antimicrobial activity, as well as the maintenance of the nutritional profile of food.

1.2.3. Tocopherols as natural food preservers

Natural antioxidants are very useful as food additives to prevent off-flavors caused by fats oxidation, and therefore stopping the initiation and propagation phases of lipid peroxidation. There are 5 types of antioxidants as referred above in section 1.1.2. First the radical scavengers or chain breaking antioxidants named as the primary antioxidants; second the chelators which are linked with metals and prevent them from initiating radical formation; third the quenchers which are responsible for the deactivation of high-energy oxidant species; fourth the oxygen scavengers, that scavenge oxygen from the systems to avoid their destabilization; and finally the antioxidant regenerators, which are capable of regenerating

other antioxidants when these become radicalized. Antioxidants are mostly used as food additives in meats, oils, fried foods, dressings, dairy products, baked goods and extruded snacks (Carocho et al., 2015).

Tocopherols are considered as the major natural antioxidants present in vegetable oil. Furthermore, they are widely used in bacon, fats and poultry, as natural antioxidant preservatives and are considered as "Generally Recognized as Safe" (GRAS). In animal studies, α-tocopherol proved to be a non-mutagenic and non-carcinogenic compound. However, the excessive intake of α-tocopherol turns of the opposite and can induce haemorrhage. Clinical studies mention that generally, an intake < 720 mg/day of tocopherols does not cause any adverse effect in man (WHO, 1987).

1.3. *In vitro* production of mushroom's mycelium

1.3.1. Brief historical perspective and advantages of *in vitro* culture

Mushrooms belong to the kingdom Fungi. Actually, the structure called "mushroom" refers to the fruiting body of the fungus which can be seen with naked eye and purchase by hands. Mycelium is the vegetative part formed by a network of interconnected cells responsible, under some favorable conditions, for the production of the fruiting body structure (Stamets, 1993).In 1994, international food institutions such as Food and Agriculture Organization (FAO), recommended the *in vitro* culture as a methodology to produce natural compounds for use in food (Dal Toso & Melandri, 2011). The term *in vitro* culture, which literally means in glass, appeared for the first time due to the use of glass vessels in the culture (Pierik, 1997). Referring to the report published by FAO in association with the International Atomic Energy Agency (IAEA), in 2002, the *in vitro* culture technology aimed at growing cells, tissues and organs under an aseptic environment and optimum conditions of temperature, humidity and nutrition. This technique is being widely used for quick multiplication of clones and for the production of bioactive compounds (Ahloowalia et al., 2002).

Miles & Chang, 2004 defined the indoors growth of mushrooms as another development of mushrooms cultivation. It consists in the *in vitro* production of mushroom's mycelium. *Agaricus bisporus* (button mushroom) is the most cultivated mushroom worldwide. It was cultivated for the first time in France during the seventeenth century, followed by *Lentinus*

edodes, *Pleurotus spp*, *Auricularia auricular*, *Flamulina velutipes* and *Volvariella volvacea* (Aida et al., 2009).

The simplicity and the promptness of the *in vitro* culture of mycelia, compared to the longest cultivation of the fruiting bodies in plastic bags, is the reason of the substitution of fruiting bodies production by mycelium production in terms of obtaining natural compounds of interest (Saltarelli et al., 2009).

Distinct advantages are related to the *in vitro* culture of mushrooms. We can mention some benefits cited by Stamets (1993), which include the use of agar in the culture, which provides a rapid multiplication of mycelia using the smallest fragments of tissue. Moreover, the cultivation of mushrooms in an isolated environment provides a sufficient quantity of nutrients. Besides, this technique of cultivating mushrooms in a culture medium is one of the best ways to follow mushroom life cycle and detect contaminations. Thus, from an ecological point of view, the *in vitro* culture is presented as an ideal methodology for the sustainable conservation of biodiversity (Pinto et al., 2013). Ultimately, a considerable advantage of the *in vitro* culture technique is its potential, under a highly controlled microenvironment and regardless the geo-climatic conditions, to guarantee a continuous, reliable, predictive and sustainable production of bioactive compounds, increasing their economic value (Anand, 2010; Dias et al., 2016). In addition, the extraction of the compounds produced *in vitro* is quick and efficient compared to the *in vivo* culture (Anand, 2010).

1.3.2. Tocopherols in mushrooms' mycelium

As previously referred, mushrooms have been recognized as functional foods and a source of bioactive compounds, which may be useful for the development of medicines and nutraceuticals. Not only fruiting bodies, but also mycelia accumulate several bioactive metabolites that could be isolated to be used in pharmaceutical and/or food industries (Pinto et al., 2013; Reis et al., 2011).

Referring to Pinto and collaborators (2013), the production of bioactive compounds by mycelia requires specific media. For β- and γ-tocopherols, PDA (Potato Dextrose Agar medium), PACH (Pachlewski medium) and FAD (Ferry & Das medium) can be used. For glucose production, PACH proved to be the best choice. MMN (Melin-Norkans medium) seems to promote phenolic compounds production, and mMMN proved to increase the

production of other compounds with antioxidant properties (Pinto et al., 2013). In 2004, Mau and collaborators have analyzed the antioxidant properties of the methanolic extracts from two kinds of *Antrodia camphorata* mycelium. They determined that tocopherols were the main natural compounds found in red and white mycelium of this species. γ-Tocopherol was produced in higher quantities by both mycelia. The contents of bioactive compounds in the mycelia was tocopherols > total phenols > ascorbic acid > β-carotene (Mau et al., 2004).

In 2011, a comparative study of tocopherols composition and the antioxidant properties of *in vivo* (fruiting bodies) and *in vitro* (mycelia) ectomycorrhizal fungi: *Paxillus involutus* and *Pisolithus arhizus* was performed by Reis et al. The authors have shown that the total tocopherols content in *Pisolithus arhizus* mycelium was higher than in *Paxillus involutus*, due to the great contribution of γ-tocopherol (154 μg/g dry weight). Moreover, this isoform prevailed on both species (mycelia and fruiting bodies), over the others. For both species a significant higher total content of tocopherols was present in mycelium, compared to fruiting bodies and culture media (Reis et al., 2011).

Regarding the techniques for tocopherol isoforms analysis and quantification, the same methodology (*in vitro* culture) was used, followed by saponification (for the extraction), and HPLC coupled to an UV detector for compounds separation and subsequent analysis. However, Barros and collaborators have proposed a new methodology without saponification, by adding an antioxidant to prevent tocopherols oxidation, and protecting samples from heat and light. The separation and detection was carried out by HPLC coupled to a fluorescence detector (Barros et al., 2008).

1.3.3. The use of *in vitro* culture to improve the biosynthesis of specific compounds

The *in vitro* culture methodology for mycelium production is related with the improvement of bioactive compounds production. Several factors influence the increase of their production, namely the optimization of the environmental conditions, control of the final product; production of pure compounds; and reduction of undesired compounds (Chattopadhyay et al., 2002; Verpoorte et al., 1999). *In vitro* culture provides the required pH, temperature and light conditions that allow the increase of natural compounds production (Dias et al., 2016).The improvement on production yields due to the connection between the biochemistry and the biotechnology engineering processes proved that the *in vitro* culture is considered as a

favorable methodology used for the production of bioactive compounds (Zhou & Wu, 2006). For these reasons, the *in vitro* culture became an attractive methodology for the production of different bioactive compounds.

Heleno and collaborators (2012) studied the antioxidant activity of phenolic and polysaccharide extracts from *Ganoderma lucidum* (Curtis) P. Karst. The authors proved that the highest levels of total polysaccharides and individual sugars were produced by the species obtained by *in vitro* culture, especially the mycelia sub-cultured in solid culture medium (Heleno et al., 2012).

In 2013, Gao and collaborators have isolated two novel polysaccharide fractions from the mycelium of *Gomphidius rutilus*, which is a traditional Chinese medicinal and edible fungus, often found beneath pine trees. These fractions, designated as GRMP1 and GRMP2, proved the benefit of the *in vitro* culture for obtaining mycelium with high quantities of polysaccharides compared to the cultivation of *G. rutilus* for obtaining fruiting bodies (which make the production very low) (Gao et al., 2013).

1.4. Working plan

1.4.1. Target mushroom species

Mushrooms have been found in a fossilized wood of around 3000 million years old, being assumed that pre-historic man collected and used wild mushrooms as food (Cheung, 2008).

The origin of the term “mushroom” is very ancient. It may be derived from the Latin word *mucus* (slime) (Baker, 1989). It has been largely used in diverse countries in several ways and at different times. The word “mushroom” embraces all large fungi, or all fungi contain stalks and caps or all large fleshy fungi. A more restricted use includes just some larger fungi that are considered as edible and/or proved their medicinal value (Miles & Chang, 2004). A mushroom is broadly defined as a macro fungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (underground) and large enough to be seen with the naked eye and to be picked by hand. According to this definition, it became known that mushrooms are not only edible Basidiomycetes, fleshy, and aerial. In fact, they can be Ascomycetes, inedible, with a no fleshy texture, and with an underground growth (Miles & Chang, 2004 ; Sánchez, 2017).

Mushrooms have two distinct phases of growth; the reproductive phase (fruiting bodies) and the vegetative phase (mycelia growth) (Sánchez, 2017).

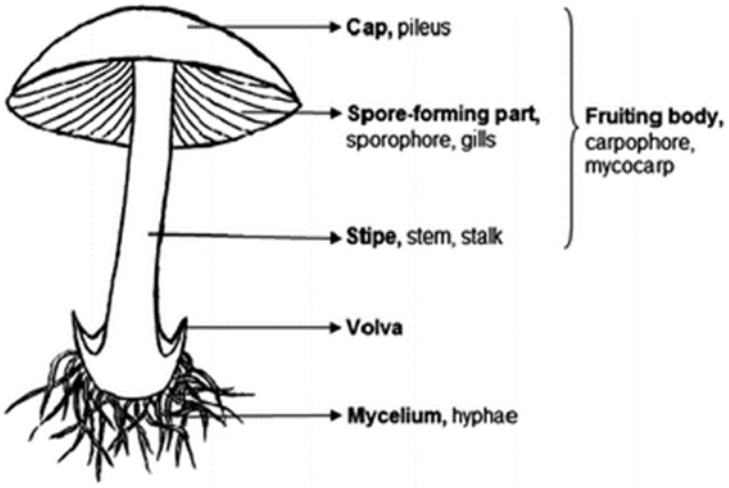


Figure 7. Schematic representation of mushrooms (Ren et al., 2012).

In 1997, the genus *Pleurotus* was considered as the third most popular cultivated edible mushrooms after *A. bisporus* (J.E.Lange) Imbach and *Lentinula edodes* (Berk.) Pegler. In 2002, *Pleurotus* production in China was estimated to be 2,594,000 MT leading all cultivated edible mushrooms. *Pleurotus* species are popularly known as oyster mushrooms according to the pileus which is shell-like, spatulate, and the stipe eccentric or lateral. For oyster mushrooms, more than 1000 species have been described throughout the world, in more than 25 related genera. However, only approximately 50 valid species are recognized in the genus *Pleurotus* (Miles & Chang, 2004).

The most common species of *Pleurotus* genus are: *P. ostreatus* (oyster mushroom), *P. djamor* (pink oyster mushroom), *P. citrinopileatus* (golden oyster mushroom), *P. eryngii* (king oyster mushroom), *P. tuber-regium* (king tuber oyster mushroom), *P. pulmonarius* (phoenix oyster mushroom), *P. nebrodensis* (white ferula mushroom), *P. cystidiosus* (abalone mushroom), *P. cornucopiae* (branched oyster mushroom), and *P. sajor-caju* (grey abalone oyster mushroom), which recently has been inserted on the *Lentinus* genus (Carrasco-González et al., 2017). Actually, *Pleurotus spp.* have been considered one of the most prodigal fungi genus to offer species of edible mushrooms.

Table 1. Taxonomic classification of *Pleurotus* (based on The Encyclopedia of Life, 2007).

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Pleurotaceae
Genus	Pleurotus

Considering the fact that their cultivation is easy and cheap, and there is a wide choice of species available for cultivation under various climatic conditions, *Pleurotus* become now widely consumed. A year-round production can provide a continuous production of *Pleurotus*. Therefore, there is no doubt about the global impact on the mushroom producing industry, (Dias, 2010; Miles & Chang, 2004).

In addition to the high culinary value of mushrooms, *Pleurotus* also featured an easy and quick cultivation not only for their fast growing but also because they are capable of colonizing crude substrates such as tree logs, cereal straw and other agricultural residues. In contrast to *Agaricus* mushrooms cultivation, *Pleurotus* species do not need compost. Due to their bland flavor and the preparation process, these mushrooms became very interesting for people unaccustomed to eat them. Among the different species of the genus *Pleurotus* in the entire world, *Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm, also known as shimeji in Brazil, is the most well studied and widely known species (Dias, 2010; Miles & Chang, 2004).

In Brazil, two strains of *P. ostreatus* are cultivated preferentially producing mushrooms with different colors. The first strain produces grey mushrooms, known as “shimeji”, and is harvested in the primordium stage; the second one produces white mushrooms, known as “hiratake", and harvested with well-developed pileus. The cultivation of these species (and others belonging to this genus) takes place in the colder seasons of the year since they require a cold environment (usually under 20ºC) to begin fructification. Thus a controlled temperature environment is needed to guarantee a continuous production (Dias, 2010).

Figure 8. *Pleurotus ostreatus* (cake and commerce/2008/10/the oyster mushrooms are Back in my Chicago alley html).

Pleurotus eryngii, (DC.) Quél. known as "king oyster mushroom" or as “cardoncello” in English-speaking countries, is another species that need a cold environment for its fructification (Dias, 2010).

P. eryngii is a crucial edible mushroom with a very pleasant smell and a sweet taste. It contains whitish gills, generally linked to thistle roots (parasitic) that takes place during spring and autumn. Its margin is very wound initially, and after maturity it becomes more corrugated (Rodríguez, 2012).

In general, mushrooms are composed of 90% of water and 10% of dry matter. They have an attractive chemical composition from the nutritional point of view which can be compared to the nutritional value of eggs, milk, and meat. Mushrooms are rich in vitamins such as thiamine, riboflavin, ascorbic acid, ergosterol and niacin as well as an abundance of essential amino acids, they have proteins, fats, ash, and glycosides. Furthermore they contain volatiles oils, tocopherols, phenolic compounds, flavonoids, carotenoids, folates, organic acids, etc. The mushroom caps have a total energetic value between 250 and 350 cal/kg of fresh mushrooms (Sánchez, 2017).

Figure 9. *Pleurotus eryngii* (Wikipedia).

A study from Reis and collaborators (2012) contributed to the elaboration of nutritional databases of the most consumed species as fresh cultivated mushrooms worldwide : *A. bisporus* (white and brown mushrooms), *P. ostreatus* (oyster mushroom), *P. eryngii* (king oyster mushroom), *Lentinula edodes* (shiitake) and *Flammulina velutipes* (golden needle mushroom), allowing the comparison between them. Shiitake and brown mushroom presented the highest content of tocopherols (11 µg/100 g), the first species with the highest levels of α- (0.92 µg/100 g) and δ- (4.36 µg/100 g) isoforms, and the second species with the highest γ-tocopherol levels (7.63 µg/100 g). β-tocopherol was found only in white, brown and king oyster mushrooms. In fact, *P. eryngii* presented the highest concentration of β-tocopherol (2.16 lg/100 g). β-Tocopherol was not detected in *P. ostreatus*; however it revealed high levels of α-tocopherol (0.59 µg/100 g), compared to brown mushroom (0.28 µg/100 g) and *P. eryngii* (0.25 µg/100 g). *Agaricus* and *Pleurotus* species revealed similar total tocopherols contents, but with some difference in the levels of the individual isoforms (Reis et al., 2012).
The work developed by Reis and collaborators (2014), focuses on chemical characterization of *P. eryngii*, *Agaricus albertii* and *Agaricus urinascens* var. *excellens*, demonstrated that the four vitamin E isoforms (α-, β-, γ-, and δ-tocopherol) were only found in *P. eryngii*, with a higher concentration of β-tocopherol (48.24 µg/100 g dw). In fact, this can be due to the high content in total fat of *P. eryngii* compared to *Agaricus* species. Overall, *P. eryngii* seems to be the most attracting species, since it contain the highest levels of macronutrients (unless proteins), as well as organic acids and tocopherols. Furthermore, it revealed the highest reducing power and radical-scavenging activity.

With these aspects in mind, and taking these studies as the base, for the present dissertation the objectives described in the next section were proposed.

1.4.2. Main objectives

As mentioned before, mushrooms have been used worldwide as food and have been described as having an excellent nutritional value, as well as high contents of compounds with antioxidant and antimicrobial potential. Since synthetic antioxidants may have a restricted use in food, we notice an increased interest in the development of natural antioxidants extracted from natural sources, such as mushrooms, to be used as preservatives. *P. ostreatus* and *P. eryngii* are two of the most consumed species worldwide as traditional food and are characterized by their biological properties. The *in vitro* culture may be used as methodology to improve the production of mycelium and consequently natural compounds from it, and use these compounds, such as tocopherols, for food incorporation.

The present work aimed at:

1) Sub-culture of *P. ostreatus* and *P. eryngii*, by *in vitro* techniques. The mycelium from each of the mushrooms was cultivated in different culture media (i.e., MMN, mMMN and PDA), in order to optimize the ideal growth conditions. Since the best growth was obtained on PDA medium, both species were cultivated in liquid medium (PDB), in order to obtain the amount of biomass required for the assays.

2) Evaluation of the tocopherols content by high performance liquid chromatography coupled with a fluorescence detector (HPLC-FL).

3) Incorporation of the mycelium extracts, enriched in tocopherols, in yogurts and evaluation of their preservative capacity, through the screening of the antioxidant properties before and after incorporation in the foodstuff. Moreover, nutritional parameters were assessed, in order to confirm the maintenance of the nutritional properties of yogurts after incorporation. The results were compared with some used preservatives (i.e., α-tocopherol- E307 and potassium sorbate- E202).

CHAPTER 2

2. MATERIALS AND METHODS

2.1. Standards and reagents

The solvents acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of high-performance liquid chromatography (HPLC) grade, obtained from Fisher Scientific (Lisbon, Portugal). α-tocopherol , as well as the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) and other individual fatty acid isomers, and sugars [D(-)-fructose, D(-)-mannitol, D(+)-raffinose pentahydrate, and D(+)-trehalose] and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA). Racemic tocol, 50 mg/ml, was supplied from Matreya (Pleasant Gap, PA, USA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Potassium sorbate was acquired from Acros Organics (Geel, Belgium). Methanol and all other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Mycelia *in vitro* production

P. eryngii (DC.) Quél. fruiting bodies were collected in Bragança (Northeast of Portugal) during November 2015. *P. ostreatus* (Jacq. ex Fr.) P. Kumm fruiting bodies were obtained after preparation of the inoculum in wheat grains and subsequent cultivation in bales of straw at the School of Agriculture of the Polytechnic Institute of Bragança.

More information about the studied *Pleurotus* species is provided in **Table 2** (Rajarathnam et al., 2009). The taxonomic identification of the sporocarps was made according to several authors (*e.g.*, Courtecuisse & Duhem, 2005; Kirk et al., 2001).

Table 2. Information about the mushroom species studied.

Scientific name	***Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm**	***Pleurotus eryngii* (DC.) Quél.**
English name	oyster mushroom	King oyster mushroom; King trumpet mushroom; French horn mushroom; King brown mushroom; Boletus of the steppes; Trumpet royal
Edibility	Edible	Edible
Substrate	Saprotrophic	Saprotrophic (sometimes parasite on the roots of herbaceous plants)

One of the major problems inherent in the cultivation of mushrooms is the maintenance of the sterilization / aseptic conditions. Therefore, a laminar flow chamber is essential for this laboratory work.

The mycelia used for the experiments were previously isolated from the sporocarps of both mushroom species (*P. eryngii* and *P. ostreatus*) and sub-cultured and maintained in solid culture medium in the Biology and Biotechnology Laboratory of the School of Agriculture of Bragança.

For the present work, the mycelia of these pre-existing petri dishes cultures were sub-cultured in order to maintain the quantities of inoculum necessary for the development of the study (**Figure 10**). Firstly, the culture medium suitable for the optimum growth of the species was tested. Thus, three different solid culture media were used: i) Potato Dextrose Agar (PDA) pH 3.5 (39g/L); ii) Melin-Norkrans (MMN) pH 6.6 (NaCl 0.025 g/L, $(NH_4)2HPO_4$ 0.250 g/L, KH_2PO_4 0.500 g/L; $FeCl_3$ 0.005 g/L, $CaCl_2$ 0.050 g/L, $MgSO_4.7H_2O$ 0.15 g/L, thiamine 100 µg/mL g/L, glucose 10.0 g/L, agar 20 g/L, malt extract 5 g/L, casaminoacids 1g/L); and iii) Modified Melin-Norkrans (mMMN) pH 6.6 (NaCl 0.025 g/L, $(NH_4)2HPO_4$ 0.250 g/L, KH_2PO_4 0.500 g/L, $FeCl_3$ 0.005 g/L, $CaCl_2$ 0.050 g/L, $MgSO_4.7H_2O$ 0.15 g/L, thiamine 100 µg/L, glucose 10 g/L, agar 20 g/L) (Marx, 1969).

One litter of solid media can abundantly fill thirty 100 mm x 15 mm Petri dishes. After sub-culture the mycelia, their radial growth was measured and registered from the moment of inoculation until the maximum growth was reached (covering all available area in the Petri dish), approximately 22 days for both *Pleurotus* species. The better results for the mycelia growth were obtained for the PDA medium. Therefore, in order to obtain the necessary biomass for the subsequent analysis and to simplify the collection of the mycelium, both species were sub-cultured in flasks containing Potato Dextrose Broth (PDB) liquid medium pH 5.1 ± 0.2 (27g/L). One litter of PDB medium can generously fill 33 flasks with 30 mL medium each. Both *P. eryngii* and *P. ostreatus* were sub-cultured and allowed to grow for a period of 30 days. Afterwards, the mycelium was collected, weighed (to know the obtained fresh weight – fw), and then frozen and lyophilized (freeze 4.5 FreeZone model 7750031, Labconco, Kansas, USA). After lyophilisation, the samples were weighted and the dry weight (dw) was calculated. Finally, the samples were reduced to a fine powder (20 mesh), mixed to obtain homogenous samples, and stored in a desiccator, protected from light, until further analysis.

Figure 10. Growth of (A) *P. ostreatus* and (B) *P. eryngii* mycelium after 5 days of inoculation in MMN medium.

2.3. Obtainment of mycelia tocopherol extracts

2.3.1. Extraction procedure

The extracts were prepared following a procedure previously described by Barros et al., 2008. Briefly, BHT (butylhydroxytoluene) (100 µL) and tocol (internal standard (IS) solution) (250 µL) were added to the samples before the extraction procedure itself. The samples (≈ 500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Afterwards, hexane (4 mL) was added and the mixture was vortex again (1 min). Saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000g) and the clear upper layer was transferred to a vial. The samples were re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, and i) re-dissolved in 1 mL of *n*-hexane, dehydrated with anhydrous sodium sulphate, filtered through a 0.22 µm disposable LC filter disk and transferred into a dark injection vial for the analysis by HPLC; or ii) re-dissolved in an amount of methanol required to obtain a stock solution of 10 mg/mL.

Figure 11. Step of solvent removal in the preparation of the extracts.

2.3.2. Tocopherols analysis

Tocopherols analysis was made by HPLC following a procedure previously optimized and described by Heleno et al., 2010. The equipment consisted of an integrated system with a Smartline pump 1000 (Knauer, Germany), a degasser system Smartline manager 5000, an AS-2057 auto-sampler and a 2500 UV detector at 295 nm (Knauer, Germany) connected in series with a FP-2020 fluorescence detector (Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm. Data were analyzed using the Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters (Japan) operating at 30ºC (7971 R Grace oven). The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards.

The quantification was based on the fluorescence signal response of each standard using the internal standard method (tocol) and by using calibration curves obtained from commercial standards of each compound. Tocopherol contents in mycelium samples were expressed in micrograms per gram of dry weight.

Figure 12. HPLC system used in tocopherols analysis.

2.3.3. Evaluation of antioxidant activity

For the evaluation of the antioxidant activity of biological material, around 11 *in vitro* methods have been used by the scientific community (Sánchez, 2017). Since the response of antioxidants to different radical or oxidant sources may be different, there is no single assay

which accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system (Prior et al., 2005).

The most commonly used methods to measure mushrooms antioxidant properties are those involving chromogen compounds of radical nature that stimulate the reductive oxygen species (*e.g.*, DPPH assay). In the present work, two different assays for testing the reducing capacity of the studied species, the DPPH assay and the reducing power assay.

2.3.3.1. DPPH radical scavenging activity assay

The DPPH assay is based on the scavenging of the deep violet chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) by the antioxidants/reducing compounds present in the tested samples. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, the purple chromogen radical is reduced by the antioxidants / reducing compounds to the corresponding pale yellow, hydrazine, according to the following equation:

$$Z^{\bullet} + AH \rightarrow ZH + A^{\bullet}$$

where $Z^{\bullet}$ represents the DPPH radical and AH the donor molecule. In the present reaction, ZH is the reduced form and $A^{\bullet}$ the free radical produced in this first step. This second radical will subsequently undergo further reactions according to the general stoichiometry, i.e. the number of reduced (discolored) DPPH molecules by one molecule of the reducing agent (Molyneux, 2004). This reduction could be monitored measuring the absorbance decrease at 515 – 528 nm until the absorbance remains stable in organic media (Karadag et al., 2009), and the free radical scavenging activity can be determined by the discoloration of the DPPH solution (Ndhlala et al., 2010).

Therefore, this methodology was performed using a Microplate Reader ELX800 (Bio-Tek Instruments, Inc., Winooski, VT, USA). The reaction mixture in each of the 96 wells consisted of different solutions of the extracts (30 µL) to which was added a methanolic solution (270 µL) containing DPPH radical scavenging (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorbance at 515 nm (**Figure 13**).

The radical scavenging activity (RSA) was calculated as the percentage of discoloration of the DPPH solution using the formula: % RSA = $[(A_{DPPH} - A_S) / A_{DPPH}] \times 100$, where A_S is the

absorbance of the solution containing a given extract concentration, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentrations providing 50% of antioxidant activity (EC_{50}) were calculated from the graphs of antioxidant activity percentages (RSA percentage graphs) against sample concentrations. Trolox was used as standard (Fernandes, 2010).

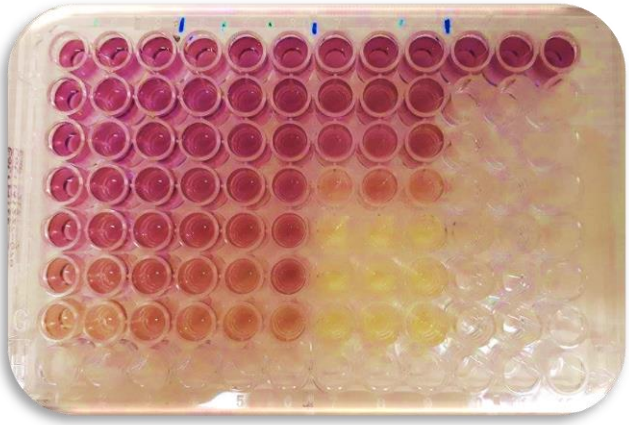


Figure 13. Example of a testing 96-well plate used for the evaluation of the DPPH radical-scavenging activity.

2.3.3.2. Reducing power assay

This methodology is based on the ability of phenolics to reduce the yellow ferric form (Fe^{3+}) to blue ferrous form (Fe^{2+}) by the action of electron-donating antioxidants (Benzie et al., 1999). When the antioxidant species is either Fe (III) or $Fe(CN)_6^{3-}$ in the composite ferricyanide reagent, either Fe(II) or $Fe(CN)_6^{4-}$ is formed as the reduction product with the antioxidant, and combines with the other reagent component to produce Prussian blue, $KFe[Fe(CN)_6]$, as the colored product. Thus, when Fe^{3+} is used along with $Fe(CN)_6^{3-}$ as the oxidizing agent, either one of the two reaction pairs occur, both ending up with the same colored product (Berker et al., 2007):

$$Fe^{3+} + \text{antioxidant} \rightarrow Fe^{2+} + \text{oxidized antioxidant},$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftarrows Fe[Fe(CN)_6]^-$$

or

$$Fe(CN)_6^{3-} + \text{antioxidant} \rightleftarrows Fe(CN)_6^{4-} + \text{oxidized antioxidant},$$

$$Fe(CN)_6^{4-} + Fe^{3+} \rightleftarrows Fe[Fe(CN)_6]^-$$

The resulting blue color could be measured spectrophotometrically at 700 nm and it is taken as linearly related to the total reducing capacity of electron-donating antioxidants (Huang et al., 2005). The reducing power assay was performed using the Microplate Reader described above. Different concentrations of the methanolic extracts (0.5 mL), sodium phosphate buffer (0.5 mL, 200 mmol/L, pH 6.6) and potassium ferricyanide (0.5 mL, 1% w/v) were mixed in eppendorf tubes. The tubes were incubated at 50°C for 20 min and trichloroacetic acid (0.5 mL, 10% w/v) was added to stop the reaction. The mixture (0.8 mL) was transferred into 48 well microplates and deionized water (0.8 mL) and ferric chloride (0.16 mL, 0.1% w/v) were added to each well (**Figure 14**). The absorbance was then measured at 690 nm. The extract concentrations providing 0.5 of absorbance (EC_{50}) were calculated from the graphs of absorbance at 690 nm against sample concentration. Trolox was used as standard (Fernandes, 2010).

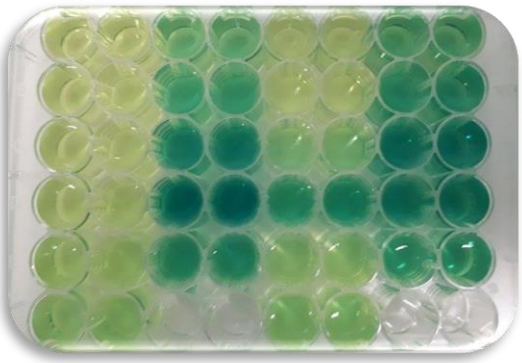


Figure 14. Example of a testing 48-well plate used for the evaluation of the reducing power.

2.4. Applicability studies of mycelia tocopherol extracts as natural antioxidants

2.4.1. Incorporation in natural yogurts

In order to prepare the tocopherol extracts of *P. eryngii* and *P. ostreatus*, the samples were extracted as described above (section 2.3.1) and the extracts obtained were used as natural

additives (**Figure 15**). The synthetic additives used as control were α-tocopherol and potassium sorbate 99% (E202) (**Figure 16**).

Four groups of samples were prepared (yogurts with 50 g each): control samples (yogurts without additives); samples with α-tocopherol; samples with potassium sorbate (E202); samples with tocopherol extract of *P. eryngii*; and samples with tocopherol extract of *P. ostreatus*. For each portion of 50 g of yogurt, a concentration corresponding to the EC_{50} obtained on the reducing power assay of the additive (natural or synthetic) was incorporated. Three groups of yogurts were prepared in duplicate, in order to study different parameters along the shelf-life of the matrix. Therefore, two storage times were tested: time zero (immediately after the incorporation), 7 days of storage at 4ºC. The tested natural yogurts were purchased at the local market, in order to use commercial products already appreciated by the consumers. After fortification with the different additives, the samples were lyophilized according to the storage time group, and tested for their tocopherols content, maintenance of the nutritional properties and antioxidant potential.

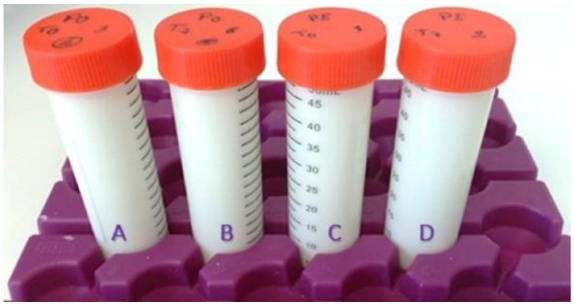


Figure 15. Yogurt samples with tocopherol extract of *P. ostreatus* (A) time zero ; (B) time 7 days; yogurt samples with tocopherol extract of *P. eryngii* (C) time zero ; (D) time 7 days.

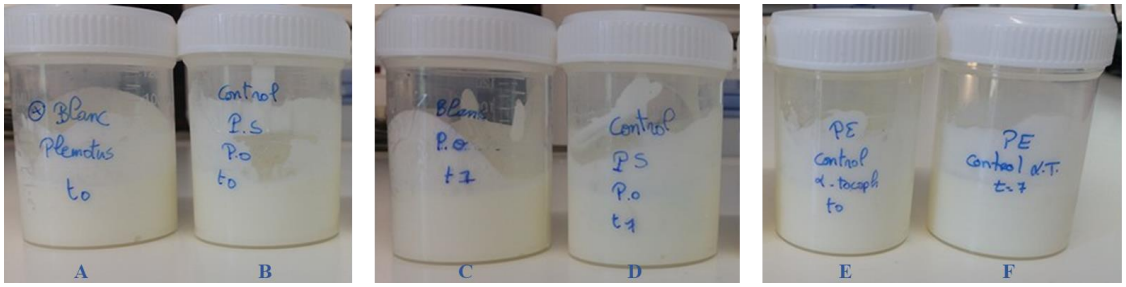


Figure 16. (A) Control yogurts, time zero; (B) yogurts with potassium sorbate, time zero; (C) Control yogurts, time 7 days ; (D) yogurts with potassium sorbate, time 7 days; (E) yogurts with α-tocopherol, time zero and (F) yogurts with α-tocopherol, time 7 days.

2.4.2. Nutritional composition of the fortified yogurts

All the samples were analyzed for the proximate composition (moisture, protein, fat, ash and carbohydrates) measured relying on AOAC procedures (AOAC, 2012). The crude protein content (N × 4.38) of the samples was estimated by the Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15ºC and total carbohydrates were calculated by difference. Energy was calculated according to the Regulation (EC) No. 1169/ 2011 of the European Parliament and of the Council, of 25 October 2011, on the provision of food information to consumers, following the equation Energy (kcal/100g dw) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

Soluble sugars were detected by HPLC (equipment described above) coupled to a refraction index detector (HPLC-RI). The lyophilized samples (≈ 1 g) were spiked with raffinose as internal standard (IS, 5 mg/mL) and were extracted with 40 mL of 80% aqueous ethanol at 80ºC for 30 min. The resulting suspension was centrifuged at 15,000g for 10 min. The supernatant was concentrated at 60ºC and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm nylon filters for analysis by HPLC-RI, as previously optimized by (Heleno et al., 2009). As above mentioned, the HPLC equipment consisted on the integrated system previously referred for tocopherols analysis, coupled to a RI detector (Knauer Smartline 2300). Data were analyzed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30ºC. The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. Sugars identification was made by comparing the relative retention times of the sample peaks with standards. Quantification was made by the internal standard method and the results were expressed in g per 100 g of fresh weight (fw).

Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol: sulfuric acid 95%:toluene 2:1:1 (v/v/v) for, at least, 12 h in a bath at 50ºC and 160 rpm. In order to obtain a phase separation, 3 mL of deionised water were added. The fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 mL of diethyl ether, and

to the upper phase was added anhydrous sodium sulphate to eliminate the water. The sample was recovered in a vial with Teflon and filtered through a 0.2 µm nylon filter.

Fatty acids were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID). The GC equipment was composed by a gas chromatograph (DANI 1000, Contone, Switzerland), equipped with a split/splitless injector and a FID detector. Separation was achieved using a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm df). The oven temperature program was as follows: the initial temperature of the column was 50ºC, held for 2 min, then a 30ºC/min ramp to 125ºC, 5ºC/min ramp to 160ºC, 20ºC/ min ramp to 180ºC, 3ºC/min ramp to 200ºC, 20ºC/min ramp to 220ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50ºC. Split injection (1:40) was carried out at 250ºC. The identification was carried out by comparing the relative retention times of the FAME of the samples with commercial standards. The quantification was made using the Clarity 4.0.1.7 Software (DataApex), being the results expressed as relative percentages of each fatty acid (Reis et al., 2012).

2.4.3. Antioxidant activity of the fortified yogurts

The antioxidant activity of the yogurts was evaluated through the DPPH radical-scavenging activity assay and reducing power, as described in section 2.3.3.

2.5. Statistical analysis

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA). Data were expressed as mean± standard deviation, maintaining the significant numbers allowed by the magnitude of the standard deviation.

The results obtained in the antioxidant activity and tocopherol contents of *P. ostreatus* and *P. eryngii* were compared by t-Student test, while an analysis of variance (ANOVA) with type III sums of squares was performed using the general linear model (GLM) procedure, to compare the parameters evaluated in the prepared yogurts. The dependent variables were analyzed using 2-way ANOVA with the factors “yogurt formulation” (YF) and “storage time” (ST). When a statistically significant interaction was detected among these two factors, they

were evaluated simultaneously by the estimated marginal means plots for all levels of each factor. On the contrary, if no statistical significant interaction was found, means were compared using Tukey's multiple comparison test, after checking the equality of variances through a Levene's test.

In addition, a linear discriminant analysis (LDA) was used to compare the effect of YF over the assayed parameters. A stepwise technique was applied, considering the Wilks' ʎ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. This procedure is based in sequential forward selection and backward elimination steps, where the inclusion of a new variable requires verifying the significance of all previously selected variables (Zielinski et al., 2014). In general, it was intended to estimate the relationship between single categorical dependent variables (yogurt formulations) and the quantitative independent variables (results obtained in the laboratorial assays). The LDA outputs allowed determining which independent variables contributed more to the differences in the average score profiles of the different yogurt formulations. To verify the significance of the canonical discriminating functions, Wilk's ʎ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

CHAPTER 3

3. RESULTS AND DISCUSSION

Mushrooms play an important role in the environment in which they develop. They recycle organic matter by returning nutrients to the ecosystem. In addition to their ecological role, mushrooms are also recognized for their nutritional and medicinal properties (Stamets, 1993). Edible mushrooms have been greatly investigated due to the increase of their consumption over the years. These have become highly prized for their taste, aroma and nutritional value. Moreover, confirmation of the existence of a variety of bioactive compounds, with antioxidant, immunomodulatory, or antitumor activities, among others, led to the introduction of these foods into the daily diet (Lindequist et al., 2005; Shi et al, 2013).

The medicinal applications of mushrooms have a very long tradition in the Asian countries, whereas only since the last decades, their use has been slightly increasing in the Western countries.

The great majority of the studies reporting the nutritional and health benefits of mushrooms are conducted on the fruiting bodies. However, the present study focuses on mushroom mycelia obtained by *in vitro* techniques. In addition to the *in vitro* culture, this work also evaluated the antioxidant activity and the effectiveness of the incorporation of tocopherols rich extracts (obtained from the cultured mycelia) in natural yogurts.

3.1. Mycelia production

P. ostreatus (Jacq. ex Fr.) P. Kumm. and *P. eryngii* (DC.) Quél. mycelia obtained after 30 days of culture on different solid media (PDA , MMN and mMMN) have an appearance depigmented and cottony (**Figure 17** and **18**). In PDA medium, mycelia appeared as a thin layer. Both mycelia showed a considerable growth from the first 2 weeks on both PDA and MMN media, whereas they showed low growth in mMMN.

In order to obtain a higher biomass for all the performed assays, both mycelia were then cultivated in Potato Dextrose Broth (PDB) medium.

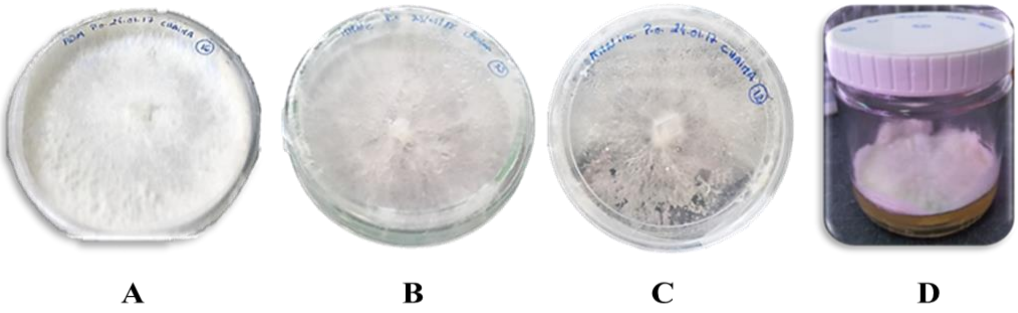


Figure 17. *In vitro* cultivation of *P. ostreatus* mycelia in: A- PDA medium; B-MMN medium; C- mMMN medium; and D- PDB medium.

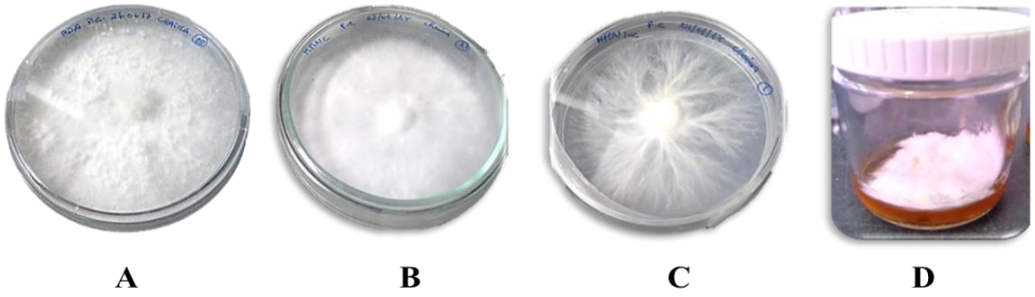
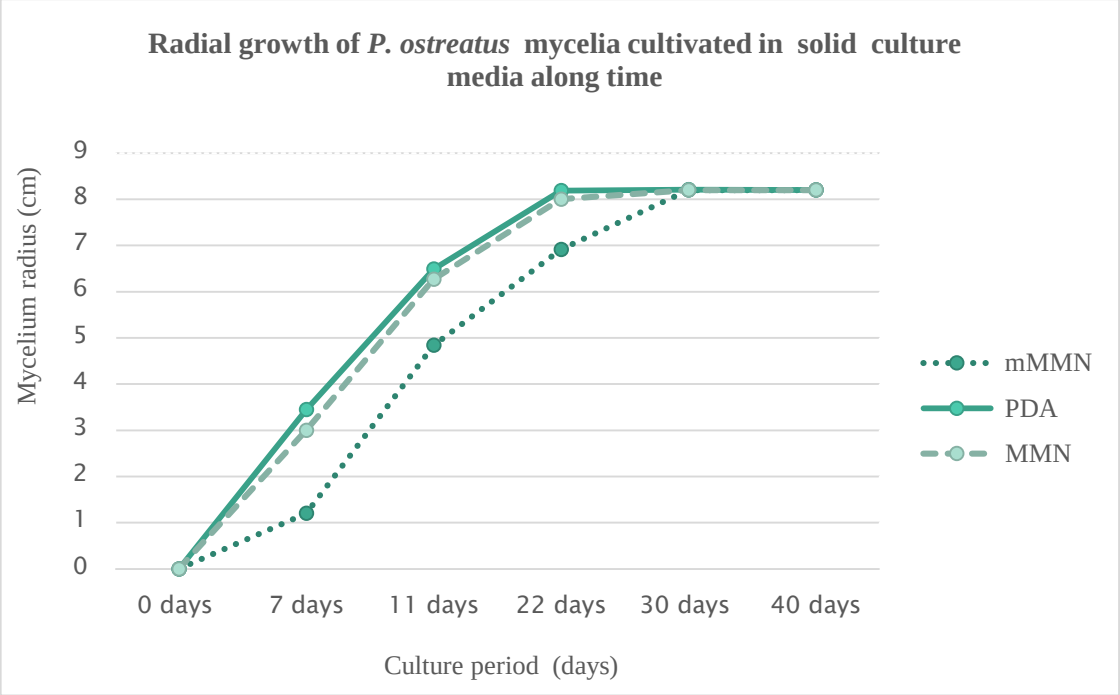


Figure 18. *In vitro* cultivation of *P. eryngii* mycelium in: A-PDA medium; B-MMN medium; C- mMMN medium; and D- PDB medium.

The growth rate and yielded biomass of mycelia are of great importance, since these parameters are used as reference to determine the industrial interest for production. Accordingly, both indicators are presented in **Figures 19** and **20**.

A



B

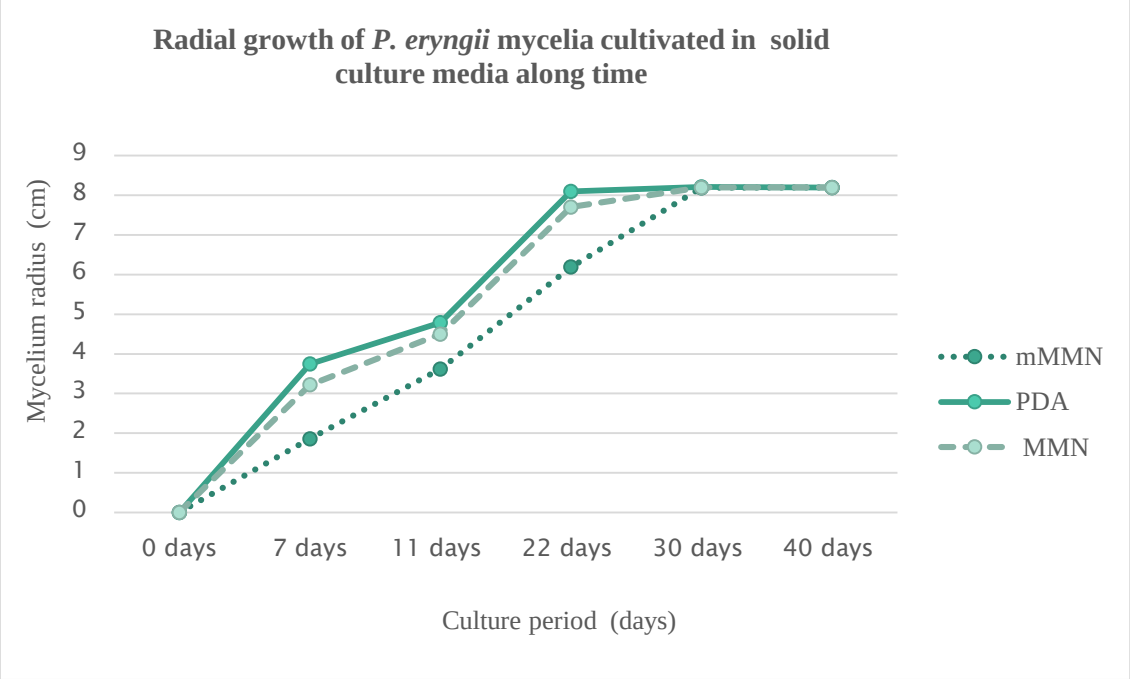


Figure 19. Radial growth of (A*) P. ostreatus* and (B) *P. eryngii* mycelia cultivated in solid culture media throughout time.

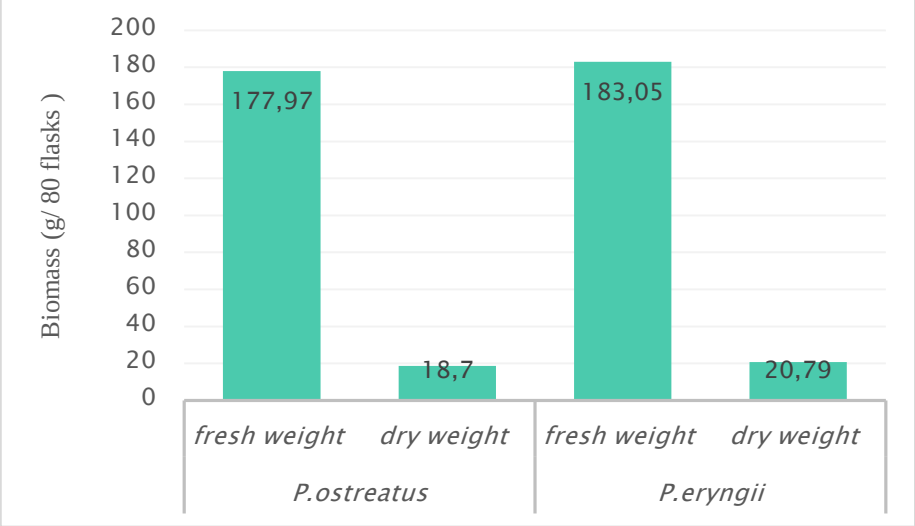


Figure 20. Total biomass of *P. ostreatus* and *P.eryngii* mycelia cultivated in liquid media.

P. ostreatus and *P. eryngii* mycelia grew for 30 days after the inoculation time. On both media (PDA and MMN), they started to grow after 7 days, with a continuous increase over the time. So, at the end of 3 weeks the entire plate was already covered with mycelia and the growth remained constant. In mMMN medium, the species showed a low growth, comparing with the other two media. The difference of growth between MMN and mMMN can be explained by the importance of the malt extract and casaminoacids for mycelia growth. *P. ostreatus* presented a faster radial growth comparing with *P. eryngii*.

Under the same culture conditions, we can consider that the best growth results for both *Pleurotus* species is verified in PDA medium. These results are in agreement with the already known better growth of nonmycorrhizal fungi in PDA medium when compared with MMN medium (Marx, 1969).

Since the best growth was obtained in PDA medium, both species were cultivated in PDB liquid medium. In PDB medium, mycelia started to grow 7 days after the inoculation time, and presented the same characteristics than in solid media. In four weeks, the mycelia already reached the optimal growth (**Figure 17** and **18 D**). Higher amounts of biomass were obtained for *P. eryngii*, comparing with *P. ostreatus* (**Figure 20**).

Both mycelia, from *P. ostreatus* and *P. eryngii*, were analyzed for their content in tocopherols, and their tocopherols rich extract was studied for its antioxidant potential, being incorporated in natural yogurt to test its effectiveness as a natural antioxidant additive.

3.2. Antioxidant activity

The interest in edible mushrooms is mainly attributed to their organoleptic quality, nutritional value and bioactive properties, which result from active substances like polysaccharides, lipids (e.g., sterols), peptides or fiber (Cheung, 2010; Ferreira et al., 2009; Öztürk et al., 2015; Reis et al., 2014; Xu et al., 2011; Zhang et al., 2016). The *Pleurotus* genus include some of the most consumed mushrooms species, thereby being extensively studied as well (Akyuz & Kirbag, 2009; Li et al., 2013; Mariga, et al., 2014a; Mariga, et al., 2014b; Xue et al., 2015). Recently, in addition to the more commonly studied fruiting bodies, the mycelia cultivated in laboratory have also been studied as potential sources of bioactive compounds (Liu et al., 2010; Ren et al., 2016; Souilem et al., 2017).
In a previous study, our research group evaluated the effects of incorporating hydrophilic extracts (decoctions of *Matricaria recutita* and *Foeniculum vulgare Mill.*) in the overall quality of yogurt (Caleja et al., 2016).

In the study reported herein, it was intended to evaluate the potentially positive effects of incorporating lipophilic antioxidant (specifically tocopherols) in the same food matrix, which has been tested in several fortification assays, owing to its high worldwide dissemination and consumption patterns (Ghorbanzade et al., 2017; Karaaslan et al., 2011; Karam et al., 2013; Santillán-Urquiza et al., 2017; Singh & Muthukumarappan, 2008).

As a first step, the antioxidant potential of *P. ostreatus* and *P. eryngii* mycelia alone was evaluated by performing DPPH scavenging activity and reducing power assays (**Table 3**). Despite the similarity showed by the results for both mushrooms, *P. ostreatus* mycelium was slightly more active against DPPH scavenging, while *P. eryngii* had higher reducing power. Considering that it was intended to specifically fortify yogurt with vitamin E, the tocopherol profiles were also characterized (**Table 3**). The first result to be highlighted is the dissimilarity with the profiles of the corresponding fruiting bodies. In fact, while the tocopherol profile of *P. eryngii* fruiting body is composed by 5% α-tocopherol, 44% β-tocopherol, 38% γ-tocopherol and 13% δ-tocopherol (Reis et al., 2012), the mycelium is almost exclusively (97%) composed by β-tocopherol. Likewise, the tocopherol profile of *P.*

ostreatus fruiting body contains 16% α-tocopherol, no β-tocopherol, 40% γ-tocopherol and 44% δ-tocopherol (Reis et al., 2012), whilst its mycelium presents 99% of β-tocopherol and only minor contents of the remaining isoforms (**Table 3**).

The second outcome of the characterized profiles is the potential utility of the mycelia of both mushrooms as sources of these lipophilic antioxidants (473 μg of tocopherols/g of extract, in the case of *P. eryngii* and 687 μg of tocopherols/g of extract, for *P. ostreatus*), particularly β-tocopherol. To achieve a comprehensive understanding of the improving effect resulting from incorporating mycelia extracts in yogurt, additional yogurt formulations were prepared by including typical commercial antioxidants (potassium sorbate E202 and α-tocopherol) and further comparing the results.

3.3. Characterization of different fortified yogurts

In addition to the previously highlighted suitability of yogurt to perform this type of studies, the increasing interest of consumers in food products prepared with natural additives instead of synthetic compounds, is also noteworthy (Carocho et al., 2014). Furthermore, besides the expected improvement in potential health effects, fortified yogurts usually present better rheological and technological properties (Caleja et al., 2016; Santillán-Urquiza et al., 2017).

Herein, five yogurt formulations (YF) were prepared: i) control (yogurt without any type fortifying agent); ii) yogurt with potassium sorbate E202 ; iii) yogurt with α-tocopherol; iv) yogurt with *P. ostreatus* mycelium extract and v) yogurt with *P. eryngii* mycelium extract; Besides evaluating the effects of incorporating agent in the same day yogurts were prepared, the same comparison was performed after 7 days of proper storage, in order to assess the possible influence of storage time (ST).

In order to obtain a clear understanding of each factor's (YF and ST) influence, their interaction (YF×ST) was also evaluated to assess possible cooperative effects (i.e., does the effect of ST over a determined parameters depended on the utilized fortifying agent?). When a significant interaction was found ($p<0.050$), no multiple comparisons could be performed. In those cases, some overall trends were tentatively indicated by analyzing the corresponding estimated marginal means (EMM) plots.

Regarding nutritional composition (**Table 4**), the interaction among factors was significant for fat, protein, lactose and energy, indicating that the effects of ST over these parameters were

depended on the YF. Therefore, the statistical classification could only be indicated in moisture, ash and carbohydrates contents. Regarding the YF effect, significant ($p<0.050$) differences were detected in all parameters, except moisture content. *P.ostreatus* presented the lowest fat content and also a tendency towards lower energy levels; the yogurt prepared with potassium sorbate, on the other hand, showed the highest fat contents, while control yogurt was classified as having the lowest carbohydrate contents; lastly, yogurts prepared with *Pleurotus* extracts had slightly lower protein quantities. In what concerns ST, its effect was only statistically significant in lactose and energy parameters, which showed higher values in stored samples. In general, the proximate composition of yogurts prepared in this work was similar to that characterized in previous works (Caleja et al., 2016).

Owing to their capacity as indicators of adequate conservation processes (Barreira et al., 2010; Pereira et al., 2016), fatty acids were individually profiled (**Table 5**). In addition to the tabled fatty acids, C11:0, C13:0, C17:0, C20:0, C20:3n6, C20:4n6, C20:3n3+C21:0, C20:5n3, C22:0, C23:0 and C24:0 were also quantified, but in relative percentages lower than 0.5% (nevertheless, all fatty acids were included in the linear discriminant analysis discussed in the next section). As it might be concluded from **Table 5**, the interaction among YF and ST was significant in most cases, except C12:0, C14:0 and C15:0. In contrast, YF caused significant changes in C6:0, C12:0, C15:0, C16:0, C18:0, C18:1n9, C18:2n6, C18:3n3, MUFA and PUFA, while ST, on the contrary, produced no significant effect in any case. Conjugating the previous results, the statistical classification could only be performed for C12:0 and C15:0, both with highest percentages in yogurts incorporated with *P. ostreatus* extracts. From the EMM, some tendencies could be identified, namely the higher percentages of C16:0 and C18:0 in yogurts prepared with *P. ostreatus* extracts and α-tocopherol, respectively, and the lower percentages of C18:1n9 and MUFA in yogurts prepared with *P. ostreatus* extracts, and of C18:2n6, C18:3n3 and PUFA in yogurts prepared with α-tocopherol.

Additionally, the antioxidant activity of different YF was evaluated to compare the selected fortifying agents. As it can be analyzed in **Figure 21**, the DPPH scavenging activity measured in yogurts fortified with *P. ostreatus* and *P. eryngii* extracts was similar to that achieved in yogurts prepared with potassium sorbate, despite not as strong as that achieved in yogurts containing α-tocopherol. Regarding reducing power (**Figure 22**), the yogurts prepared with both mushroom extracts were roughly as strong as that obtained in yogurts incorporated with α-tocopherol, reaching best results than those obtained in yogurts containing potassium sorbate. In neither case were observed significant changes induced by ST.

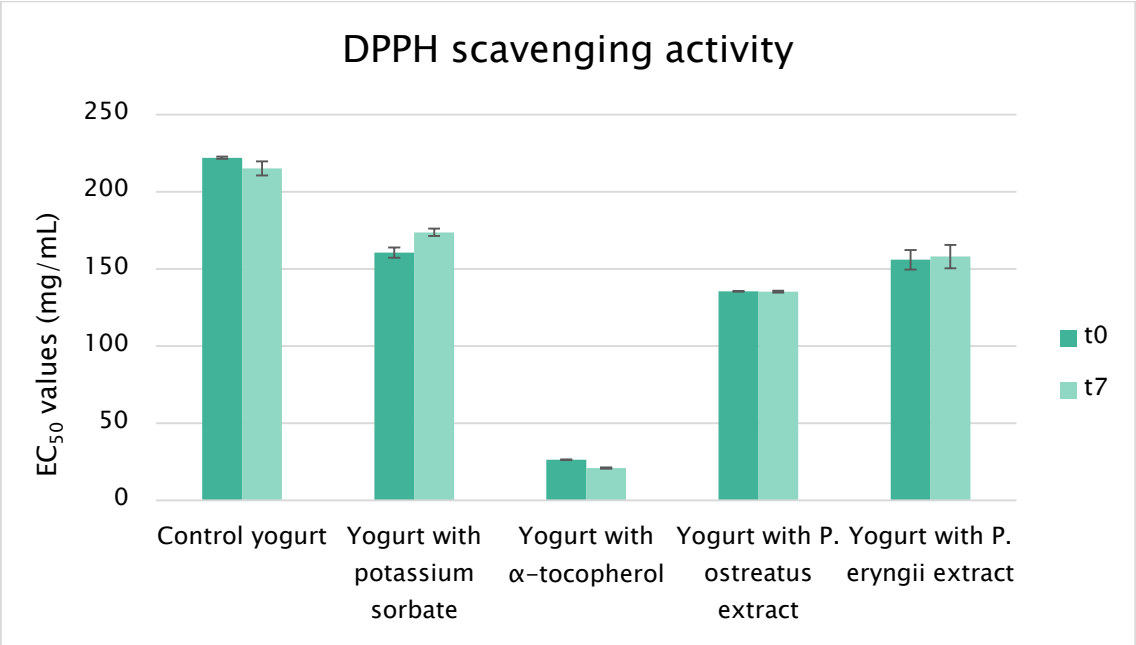


Figure 21. DPPH scavenging activity of different yogurt formulations assayed at preparation day and after 7 days of storage.

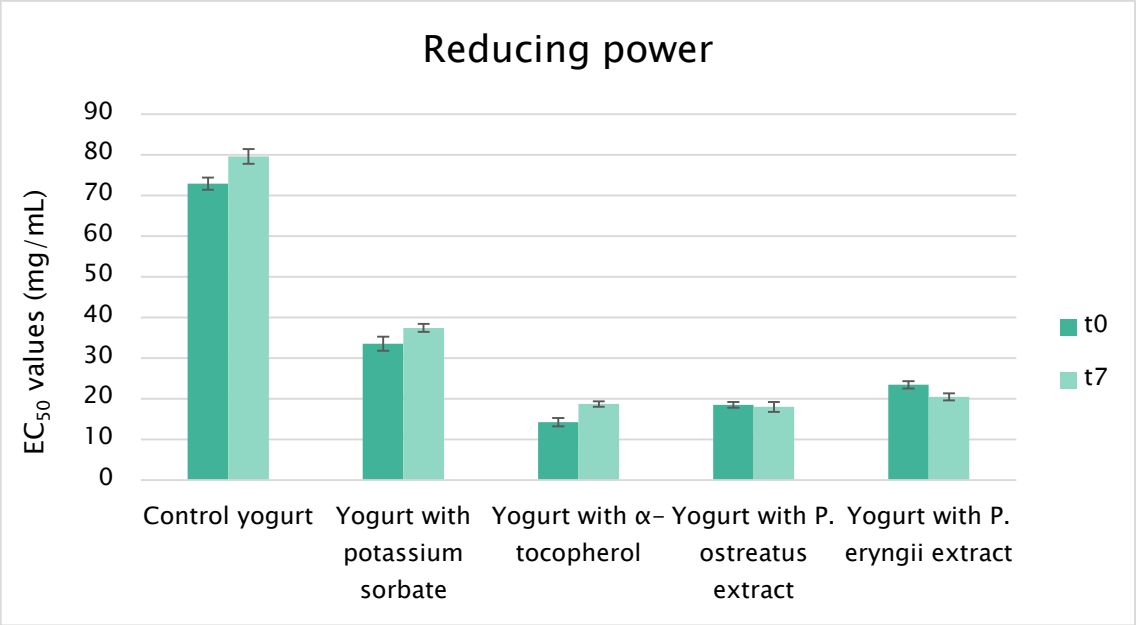


Figure 22. Reducing power of different yogurt formulations assayed at preparation day and after 7 days of storage.

Table 3. Antioxidant activity (EC_{50} values, mg/mL) and tocopherol contents (μg/g extract) in the mycelia of *P. ostreatus* and *P. eryngii*. Values are given as mean ± standard deviation.[1]

		DPPH scavenging Activity	Reducing power	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Tocopherols
P. ostreatus		13.2±0.1	0.63±0.01	17.5±0.5	664±7	nd	6.1±0.2	687±7
P. eryngii		14.2±0.3	0.56±0.01	0.6±0.1	468±6	1.8±0.1	2.1±0.1	473±6
p-value (n = 18)	Homoscedasticity[1]	<0.001	0.579	0.004	0.732	-	0.091	0.643
	t-student test[2]	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001

[1]Homoscedasticity plant species was tested by Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.050$.

[2]$p<0.050$ indicates that the mean value of both mushroom species differ from each other significantly.

Table 4. Nutritional composition and energy values for different yogurt formulations (YF) and storage times (ST).

Results are presented as mean ± standard deviation.[1]

		Moisture	Fat	Protein	Ash	Carbohydrates	Lactose	Energy
YF	Control	85±1	3.7±0.3	4.7±0.2	0.88±0.04 ab	5.7±0.2 b	4.0±0.3	75±2
	Potassium sorbate (E202)	85±1	3.6±0.2	4.6±0.2	0.89±0.04 a	5.9±0.2 a	3.8±0.3	74±2
	α-Tocopherol	85±1	3.5±0.2	4.9±0.2	0.86±0.05 abc	5.8±0.2 ab	3.6±0.5	75±1
	P. ostreatus	86±1	3.3±0.2	4.4±0.1	0.83±0.03 c	6.0±0.2 a	4.1±0.2	71±1
	P. eryngii	85±1	3.6±0.2	4.6±0.2	0.84±0.04 bc	6.0±0.2 a	4.3±0.4	75±2
	ANOVA *p*-value (n = 18)[2]	0.074	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ST	0 days	85±1	3.5±0.2	4.7±0.2	0.86±0.04	5.9±0.2	3.8±0.5	73±2
	7 days	85±1	3.6±0.3	4.6±0.3	0.86±0.05	5.9±0.2	4.1±0.2	75±2
	ANOVA *p*-value (n = 45)[3]	0.139	0.097	0.508	0.325	0.605	0.006	0.006
YF×ST	*p*-value (n = 90)[4]	0.729	0.004	<0.001	0.547	0.330	<0.001	0.017

[1]Results are reported as mean values of yogurt formulation (YF), including results from 0 and 7 days, and mean values of each storage time (ST), considering all YF in each period.

[2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one YF.

[3]If $p<0.050$, the corresponding parameter presented a significantly different value among both ST.

[4]If $p<0.050$, the interaction among factors is significant; in this case, no multiple comparisons can be performed.

Table 5. Major (detected above 0.5%) fatty acids (relative percentage) of different yogurt formulations (YF) and storage times (ST). Results are presented as mean ± standard deviation.[1]

		C4:0	C6:0	C8:0	C10:0	C12:0	C14:0	C14:1	C15:0	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n3	SFA	MUFA	PUFA
YF	Control	3.6±0.4	2.8±0.3	1.5±0.1	3.0±0.2	3.5±0.2 ab	11.2±0.3	0.8±0.1	1.4±0.1 ab	31±1	1.4±0.1	10.9±0.3	24±1	2.4±0.2	1.6±0.1	70±1	26±1	4.3±0.2
	Potassium sorbate (E202)	4.0±0.2	3.0±0.4	1.6±0.1	3.0±0.2	3.4±0.2 b	11.1±0.2	0.8±0.1	1.4±0.1 ab	30±1	1.3±0.1	10.8±0.2	23±1	2.4±0.1	1.6±0.1	70±1	26±1	4.3±0.2
	α-Tocopherol	3.8±0.1	2.8±0.2	1.5±0.1	3.0±0.1	3.5±0.2 ab	11.2±0.2	0.8±0.1	1.4±0.1 ab	31±1	1.4±0.1	11.2±0.4	24±1	2.1±0.2	1.2±0.2	71±1	26±1	3.5±0.4
	P. ostreatus	3.8±0.3	2.9±0.3	1.5±0.1	3.1±0.2	3.6±0.2 a	11.3±0.4	0.8±0.1	1.5±0.1 a	32±1	1.4±0.1	10.1±0.3	22±1	2.8±0.2	1.6±0.1	71±1	24±1	4.8±0.2
	P. eryngii	3.8±0.5	3.1±0.5	1.5±0.2	3.0±0.2	3.4±0.1 b	11.1±0.2	0.8±0.1	1.3±0.1 b	31±1	1.3±0.1	10.4±0.3	23±1	2.5±0.1	1.6±0.1	70±1	25±1	4.5±0.1
	ANOVA *p*-value (n = 18)[2]	0.064	0.042	0.107	0.402	0.014	0.132	0.519	0.021	<0.001	0.093	<0.001	<0.001	<0.001	<0.001	0.068	<0.001	<0.001
ST	0 days	3.8±0.5	2.9±0.4	1.5±0.1	3.0±0.1	3.4±0.2	11.2±0.3	0.8±0.1	1.4±0.1	31±1	1.4±0.1	10.7±0.5	23±1	2.4±0.3	1.5±0.3	70±1	25±1	4.2±0.5
	7 days	3.9±0.2	2.9±0.2	1.5±0.1	3.0±0.2	3.5±0.2	11.2±0.3	0.8±0.1	1.4±0.1	31±1	1.3±0.1	10.7±0.5	23±1	2.4±0.2	1.5±0.1	71±1	26±1	4.3±0.3
	ANOVA *p*-value (n = 45)[3]	0.143	0.701	0.146	0.161	0.130	0.748	0.318	0.523	0.302	0.672	0.894	0.363	0.846	0.135	0.523	0.378	0.489
YF×ST	*p*-value (n = 90)[4]	<0.001	<0.001	<0.001	0.009	0.542	0.614	0.007	0.159	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Results are reported as mean values of yogurt formulation (YF), including results from 0 and 7 days, and mean values of each storage time (ST), considering all YF in each period.

[2]If $p<0.050$, the corresponding parameter presented a significantly different value for at least one YF.

[3]If $p<0.050$, the corresponding parameter presented a significantly different value among both ST.

[4]If $p<0.050$, the interaction among factors is significant; in this case, no multiple comparisons can be performed

3.4. Linear discriminant analysis

In the former section, some statistically significant differences were found in specific parameters, mainly in result of changes induced by YF. To complement those results, it is now useful to identify the main characteristics of each YF by simultaneously comparing all evaluated parameters. With this purpose, a linear discriminant analysis (LDA) was performed to evaluate the correlations among YF (categorical dependent variables) and the matrix of obtained results (quantitative independent variables). The significant independent variables were selected following the stepwise method, as validated by the Wilks' λ test. Only variables with a statistically significant classification performance ($p<0.050$) were maintained by the statistical model.

The first three defined discriminant functions included 99.6% (first function: 82.3%; second function: 16.0%; third function: 1.3%) of the observed variance (**Figure 3**). From the 37 variables included in the analysis, the model selected lactose, energy, C11:0, C18:0, C18:1n9, C18:2n6, C20:4n6, C20:3n3+C21:0, C20:5n3, C22:0, C23:0, C24:0 DPPH scavenging activity and reducing power as those with the highest discriminant effect, promptly indicating that fatty acids were prone to undergone the most pronounced changes.

Function 1 was highly correlated with reducing power, effectively separating markers corresponding to control yogurts, owing to their lower activity (higher EC50 values), from all other formulations. Function 2, which was more highly correlated to C20:5n3, separated markers corresponding to yogurt prepared with α-tocopherol, which presented significantly higher percentages of that MUFA (data not shown). Finally, function 3 was particularly correlated to (ordered by correlation factor) C18:1n9, C18:2n6 and C18:0, mainly contributing to separate markers corresponding to yogurts prepared with both *Pleurotus* extracts, which showed lower percentages of C18:0 and C18:1n9, and higher percentages of C18:2n6.

In the performed LDA, the classification performance was 100% accurate, either for original grouped cases, as well as for the cross-validated grouped cases.

In conclusion, the selected *Pleurotus* extracts proved to be effective choices as lipophilic antioxidants to be employed in yogurt fortification, as validated by the similar antioxidant activities obtained with each YF. The lower reducing power of yogurts fortified with *Pleurotus* extracts, in comparison to those incorporating α-tocopherol, is probably explained by the higher bioactivity of α-tocopherol in relation to β-tocopherol, which was nearly the only tocopherol isoform present in both mycelia. Either way, the bioactivity results were highly satisfactory, particularly considering that no significant nutritional changes were detected in each YF, as indicated by the performed LDA, which did not select any nutritional variable (except lactose) as a discriminating variable.

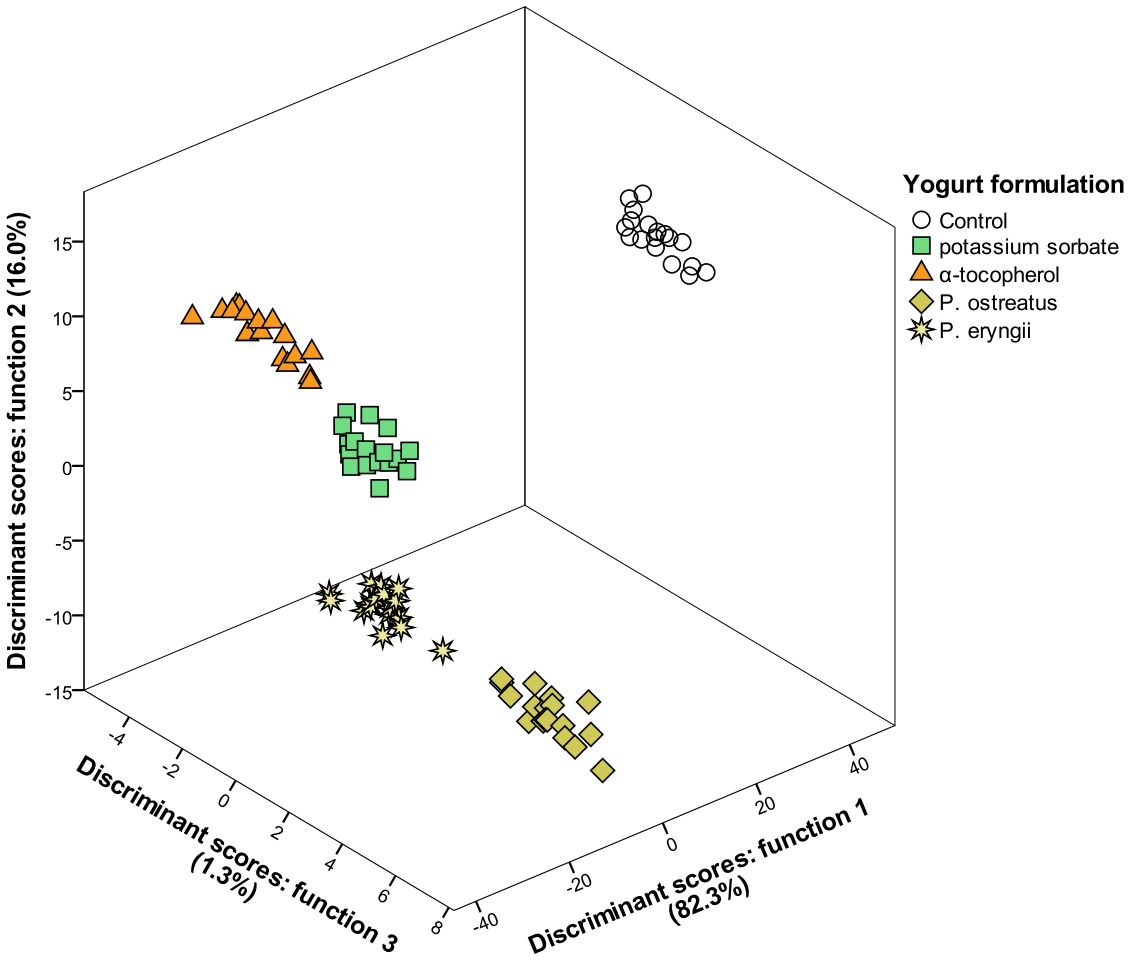


Figure 23. Canonical discriminant functions coefficients defined from the evaluated parameters to assess the overall effects of yogurt formulation.

CHAPTER 4.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

This study was initially designed to analyze the mycelia of *P. ostreatus* and *P. eryngii*, as potential alternative sources of tocopherols and to evaluate the preservative capacity of the mycelia extracts, rich in tocopherols, when incorporated in yogurts through the screening of the antioxidant properties before and after incorporation in the foodstuff. The results were compared with some used preservatives (i.e., α-tocopherol and potassium sorbate).

According to the obtained results, we have demonstrated that the growth rate of mycelia depends on the culture media. Potato dextrose agar (PDA) medium was the best one for both *Pleurotus* species, while modified Melin-Norkans medium (mMMN) revealed the poorest results. Since the best growth was obtained in PDA medium, both species were cultivated in PDB liquid medium, and they presented the same characteristics than in solid medium. *P. ostreatus* presented a faster radial growth comparing with *P. eryngii* while higher amounts of biomass were obtained for *P. eryngii*.

For the chemical analysis, this study proved that the mycelia of both mushrooms may be considered a great source of tocopherols, particularly β-tocopherol, with 473 μg of tocopherols/mL of extract, in the case of *P. eryngii*, and 687 μg of tocopherols/mL of extract, for *P. ostreatus*.

The antioxidant potential of *P. ostreatus* and *P. eryngii* mycelia, evaluated through the DPPH radical scavenging activity and the reducing power assays, showed that *P. ostreatus* mycelium was slightly more active as a DPPH radical scavenger, while *P. eryngii* showed higher reducing power.

Considering that it was intended to use the tocopherol rich extracts as preservatives, the nutritional parameters were assessed, after incorporation of both species in yogurts. *P. ostreatus* presented the lowest fat content and also a tendency towards lower energy levels; the yogurt prepared with potassium sorbate, on the other hand, showed the highest fat contents, while control yogurt was classified as having the lowest carbohydrate contents. Yogurts prepared with *Pleurotus* extracts had slightly lower protein quantities

Due to their capacity as indicators of adequate conservation processes, fatty acids were also individually profiled. Yogurts incorporated with *P. ostreatus* extracts presented a highest percentage of C12:0 and C15:0. From the EMM, some tendencies could be identified, namely the higher percentages of C16:0 and C18:0 in yogurts prepared with *P. ostreatus* extracts and α-tocopherol, respectively, and the lower percentages of C18:1n9 and MUFA in yogurts prepared with *P. ostreatus* extracts, and lower percentages of C18:2n6, C18:3n3 and PUFA in yogurts prepared with α-tocopherol.

The comparison of the antioxidant activity of the different yogurt formulations, proved that the DPPH radical scavenging activity measured in yogurts fortified with *P. ostreatus* and *P. eryngii* extracts was similar to that achieved in yogurts prepared with potassium sorbate, despite not as strong as that achieved in yogurts containing α-tocopherol. Regarding the reducing power, the yogurts prepared with both *Pleurotus* extracts were revealed similar results to those incorporated with α-tocopherol, reaching best results comparing with yogurts containing potassium sorbate. The storage time did not induce any significant change in the results.

On the basis of obtaining natural products exhibiting antioxidant properties to be used as food additives, the *in vitro* culture can be a great technique to obtain these bioactive compounds from mycelium. That is why, science tools (genomic, proteomic and metabolomic) should be used to help further *in vitro* culture, in order to develop novel natural food additives to replace the synthetic ones. Still regarding the *in vitro* culture, more work can be done, in order to promote / stimulate a greater production of certain compounds. These can be an excellent choice as alternatives to the food additives used nowadays.

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