INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

# *In vitro* production of *Ganoderma lucidum* mycelium from northeast Portugal: The antioxidant potential of tocopherols extract in the preservation of natural yogurt

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“My Lord show me right from wrong

Give me light make me strong

I know the road is long

Make me strong”

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **AdeH** | Arogenate dehydrogenase |
| **ADI** | Acceptable Daily Intake |
| **$A_{DPPH}$** | Absorbance of the DPPH solution |
| **AOAC procedures** | Official Methods of Analysis of the Association of Official Agricultural Chemists |
| **$A_S$** | Absorbance of the Solution |
| **At5g04490 (VTE5)** | **G**enes locus for the phytyl tail synthesis from chlorophyll-derived phytol in *Arabidopsis* |
| **BHA** | Butylated Hydroxyanisole |
| **BHT** | Butylated Hydroxytoluene |
| **Cyclase** | Tocopherol cyclase |
| **DMPBQ** | 2,3-Dimethyl-5-phytyl-1,4-benzoquinone |
| **DPPH** | 2,2-Diphenyl-1-picrylhydrazyl |
| **EC** | European council |
| **$EC_{50}$** | Sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay |
| **EMM** | Estimated marginal means |
| **EQ** | Ethoxyquin |
| **EU** | European Union |
| **EFSA** | European Food Safety Authority |
| **FAME** | Fatty acids methyl ester |
| **FAO** | Food and Agriculture Organization |

| | |
|---|---|
| **FDA** | Food and Drug Administration |
| **FID** | Flame ionization detector |
| **γ –TMT** | γ-Tocopherol methyltransferase |
| **GC** | Gas Chromatography |
| **GGDP** | Geranylgeranyl diphosphate |
| **GGR** | Geranylgeranyl Diphosphate Reductase |
| **GLM** | General linear model |
| **HGA** | Homogentisic Acid |
| **HPLC** | High-performance liquid chromatography |
| **HPP** | *p-Hydroxyphenylpyruvic acid* |
| **HPPD** | *p-Hydroxyphenylpyruvic acid dioxygenase* |
| **HPT** | Homogentisate Prenyl Transferases |
| **IS** | Internal standard |
| **LDA** | Linear discriminant analysis |
| **LQBA** | Laboratory of Applied Chemistry and Biochemistry |
| **MGGBQ** | *2-Methyl-6-geranylgeranylplastoquinol* |
| **MPBQ** | *2-Methyl-6-phytylplastoquinol or 2-methyl-6-phytyl-1,4- benzoquinone* |
| **MPBQ MT** | MPBQ methyltransferase |
| **NaCl** | Sodium Chloride |
| **PAT** | Prephenate amino transferase |
| **PDA** | Potato Dextrose Agar |
| **PDB** | Potato Dextrose Broth liquid medium |
| **PDP** | Phytyl Diphosphate |

| | |
|---|---|
| **PG** | Propyl Gallate |
| **PDS1** | Gene locus encoding HPPD |
| **PS** | Potassium sorbate |
| **QS** | *Quantum satis* |
| **RI** | Refraction index |
| **ROS** | Reactive Oxygen Species |
| **RNS** | Reactive Nitrogen Species |
| **RP** | Reducing power by Ferricyanide/Prussian blue assay |
| **SAM** | S-adenosyl methionine |
| **SCF** | Scientific Committee on Food |
| **SPSS** | Statistics Package for Social Sciences |
| **ST** | Storage time |
| **MMN** | Melin-Norkans medium |
| **mMMN** | Modified Melin-Norkans medium |
| **TAT** | Tyrosine amino transferase |
| **TBHQ** | *tert-Butylhydroquinone* |
| **Trolox** | 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid |
| **Tyr transaminase** | Tyrosine transaminase |
| **UL** | Tolerable Upper Intake Level |
| **WHO** | World Health Organization |
| **YF** | Yogurt formulations |

# ABSTRACT

The increase in public awareness for the direct relationship between diet and health has led to concerns related to the use of commercial food additives, promoting the search for natural alternatives. Thus, natural antioxidants are among the most searched compounds nowadays, due to the issues related with the oxidative stress and its harmful effects on both food quality and human health. Tocopherols are among the most well-known and interesting natural antioxidants because its use as a food additive has multiple benefits. These are encompassed by the designation of vitamin E, which is an essential and extremely important micronutrient for human health that should be provided by the diet, being a strong wall against oxidation, especially in cooperation with vitamin C.

The use of natural tocopherols at an industrial level depends on the sustained production, hence the importance and choice of the *in vitro* culture as an alternative to their production. The *in vitro* production of natural compounds allows a continuous year round availability of the demanded products in an independent way of environmental conditions and without ecological issues.

In recent years, mushrooms became more and more value and exploited due to their richness on valuable compounds with biotechnological interest and to their easier and faster *in vitro* production compared to plants. Moreover, the *in vitro* production of mushrooms mycelium has been explored to improve the synthesis of antioxidants, namely tocopherols.

The present work aims to evaluate the tocopherols content of the well-known medicinal mushroom *Ganoderma lucidum* (Curtis) P. Karst from the Northeast of Portugal, produced by *in vitro* culture, and comparing with its fruiting body. The tocopherol levels were determined by chromatographic techniques, namely by high performance liquid chromatography coupled to a fluorescence detector (HPLC-fluorescence). Afterwards, a tocopherol rich extract from the mycelium was incorporated in natural yogurts, and the antioxidant potential of the extract (before incorporation) and yogurts (after incorporation) was assessed, in order to study its potential as a food additive. The antioxidant activity was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the reducing power assays. Moreover, the effectiveness of the tocopherol rich extract as a food additive was evaluated for the maintenance of the nutritional properties of the studied food matrix. These studies were carried out comparing the nutritional parameters of four yogurt formulations: i) control (yogurt without any type fortifying agent); ii) yogurt with potassium sorbate - E202; iii) yogurt with α-tocopherol - E307; and iv) yogurt with *G. lucidum* mycelium tocopherol enriched extract.

Overall, with regard to the *in vitro* culture, *G. lucidum* mycelium did not show significant differences in radial growth between the two used media.

The chemical analysis showed that the *in vitro* produced mycelium of *G. lucidum* was particularly rich in δ-tocopherol (362±7 μg/g extract) and β-tocopherol (272±8 μg/g extract), among the total tocopherols content 717±12 μg tocopherols/g extract with relevant differences in comparison to the corresponding fruiting body. Besides, *G. lucidum* mycelium showed good results in the DPPH radical scavenging activity assay ($EC_{50}$ = 10.4±0.2 mg/mL), but especially good in the reducing power assay ($EC_{50}$ = 0.32±0.01 mg/mL). While, different results were registered in the tested yogurt formulations with the highest antioxidant activities observed in yogurts incorporated with α-tocopherol and *G. lucidum* extract. Besides, no particularly significant differences were observed among 0 and 7 days, especially in yogurts incorporated with tocopherol enriched extract obtained from *G. lucidum* mycelium.

The evaluation of the interaction between the yogurts formulations (YF) and the storage time (ST) on the nutritional composition of yogurts, showed a significant impact on fat, protein and lactose, indicating that the ST influenced these parameters differently for each YF. Furthermore, the effect of YF was only significant for fat, protein, carbohydrates and lactose, resulting that the statistical classification could only be indicated for carbohydrates, which reached maximum values in yogurts prepared with potassium sorbate (5.9±0.2 g/100 g). Also, significant changes in the contents of fat, protein and lactose were registered due to the ST with higher values for fat and lactose in yogurts stored during 7 days, while the protein content did not showed any identifiable tendency. Furthermore, the fatty acids profile showed that the changes induced by ST vary according to YF in most cases and that ST did not induce significant changes in the fatty acids profile, with exception for C4:0, C8:0 and C18:1n9.

According to the Linear Discriminant Analysis (LDA), it was possible to identify the most distinctive parameters of each YF, allowing to choose the functionalizing agent (potassium sorbate, α-tocopherol or the enriched extract in tocopherols obtained from *G. lucidum* mycelium), according to a specific objective (in this case preservative effect). Considering the values of the antioxidant activity, it can be concluded that the yogurt incorporated with α-tocopherol or extract enriched in tocopherols obtained from the mycelium of *G. lucidum*, presented a greater preservation potential.

Overall, the mycelium of *G. lucidum* might certainly be considered as a potential source of tocopherols to be employed as food lipophilic antioxidants.

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# RESUMO

O aumento da consciencialização pública para a relação direta entre dieta e saúde levou a um aumento da preocupação relativamente ao uso de aditivos alimentares comerciais, promovendo a busca de fontes alternativas naturais. Assim, os antioxidantes naturais estão entre os compostos mais estudados hoje em dia, devido a questões relacionadas com o stresse oxidativo e os seus efeitos nocivos para a qualidade dos alimentos e saúde humana. Os tocoferóis estão entre os antioxidantes naturais mais conhecidos e interessantes devido aos seus múltiplos efeitos benéficos. Estes são abrangidos pela designação de vitamina E, que é um micronutriente essencial e extremamente importante para a saúde humana que deve ser fornecido pela dieta, na medida em que é uma barreira protetora contra a oxidação, especialmente quando atua em cooperação com a vitamina C.

O uso de tocoferóis naturais a nível industrial depende da sua produção sustentada, daí a importância e a escolha da cultura *in vitro* como alternativa à sua produção. A produção *in vitro* de compostos naturais permite uma disponibilidade contínua durante todo o ano dos produtos desejados, de forma independente das condições ambientais e sem problemas ecológicos.

Nos últimos anos, os cogumelos tornaram-se uma mais-valia e são cada vez mais explorados devido à sua riqueza em compostos com interesse biotecnológico, e dado que a sua produção *in vitro* é mais fácil e rápida comparativamente com as plantas. Além disso, a produção *in vitro* de micélio de cogumelos já foi explorada para promover a síntese de compostos antioxidantes, nomeadamente tocoferóis.

O presente trabalho teve como objetivo avaliar o conteúdo em tocoferóis do cogumelo medicinal *Ganoderma lucidum* (Curtis) P. Karst do Nordeste de Portugal, produzido por cultura *in vitro* e comparando com o seu corpo frutífero. Os níveis de tocoferóis foram determinados por técnicas cromatográficas, nomeadamente por cromatografia líquida de alta eficiência acoplada a um detetor de fluorescência (HPLC-fluorescência). Posteriormente, o extrato enriquecido em tocoferóis obtido do micélio foi incorporado em iogurte natural avaliando-se o potencial antioxidante do extrato (antes da incorporação) e nos iogurtes (após incorporação), a fim de estudar o seu potencial como aditivo alimentar. A atividade antioxidante foi avaliada através de dois métodos: atividade captadora de radicais 2,2-difenil-1-picril-hidrazilo (DPPH) e o ensaio do poder redutor. Avaliou-se também a eficácia do extrato enriquecido em tocoferóis como aditivo alimentar, na conservação das propriedades nutricionais da matriz alimentar estudada. Estes estudos foram realizados comparando os parâmetros nutricionais de quatro formulações de iogurtes: i) controle (iogurte sem qualquer tipo de agente fortificante); ii) iogurte com sorbato de potássio - E202; iii) iogurte com α-tocoferol - E307; e iv) iogurte com extrato enriquecido em tocoferóis obtido do micélio de *G. lucidum*.

Em geral, no que diz respeito à cultura *in vitro*, o micélio da espécie em estudo não mostrou diferenças significativas no crescimento radial comparando os dois meios de cultura utilizados.

A análise química mostrou que o micélio produzido *in vitro* é particularmente rico em β-tocoferol (272 ± 8 μg/g de extrato) e δ-tocoferol (362 ± 7 μg/g de extrato), entre o total de tocoferóis 717 ± 12 μg de tocoferóis/g extrato e com diferenças relevantes em relação ao corpo de frutificação. O micélio apresentou capacidade de captação de radicais DPPH ($EC_{50}$ = 10,4 ± 0,2 mg/mL), demonstrando ainda maior eficácia para o poder redutor ($EC_{50}$ = 0,32 ± 0,01 mg/mL). As formulações de iogurte com maior atividade antioxidante pertenceram ao grupo incorporado com o aditivo α-tocoferol e com o extrato enriquecido em tocoferóis obtidos do micélio de *G. lucidum*. Contudo, não foram observadas diferenças significativas entre 0 e 7 dias, particularmente no iogurte incorporado com extrato de micélio de *G. lucidum*.

A avaliação da interação entre as formulações de iogurte (YF) e o tempo de armazenamento (ST) na composição nutricional das matrizes mostrou um impacto significativo nos teores de gordura, proteína e lactose, indicando que o ST influenciou esses parâmetros de forma diferente para cada YF. O efeito das formulações foi significativo apenas para os teores de gordura, proteína, hidratos de carbono e lactose, sendo que a classificação estatística apenas foi indicada para os hidratos de carbono, que atingiram os valores máximos nos iogurtes preparados com sorbato de potássio (5,9 ± 0,2 mg/100 g). Foram também registadas diferenças significativas nos teores de gordura, proteína e lactose devido ao ST com maiores valores nos teores de gordura e lactose em iogurtes armazenados durante 7 dias, enquanto o conteúdo de proteína não mostrou nenhuma tendência identificável. Os ácidos gordos mostraram que as alterações induzidas pelo ST variam de acordo com a YF na maioria dos casos e que o ST não induziu alterações significativas na maioria dos ácidos gordos, exceto no caso do C4:0, C8:0 e C18:1n9.

De acordo com a Análise Discriminante Linear (LDA), foi possível identificar os parâmetros mais distintivos de cada YF, permitindo escolher o agente funcional (sorbato de potássio, α-tocoferol ou extrato enriquecido em tocoferóis obtidos a partir do micélio de *G. lucidum*), de acordo com um objetivo específico, que neste caso era um efeito conservante. Atendendo aos valores da atividade antioxidante, pode concluir-se que os iogurtes incorporados com α-tocoferol ou extrato enriquecido em tocoferóis obtidos a partir do micélio de *G. lucidum*, apresentaram maior poder de conservação.

Em geral, o micélio de *G. lucidum* pode, de facto, ser considerado como uma potencial fonte de tocoferóis que podem ser introduzidos em alimentos como antioxidantes lipofílicos.

# INTRODUCTION

## 1. Food additives

### 1.1. Main classes and widespread using

The use of food additives has a long tradition. They have been widely used for years and a continuous research has been carried out in order to meet consumer demand, who nowadays has a very hectic lifestyle, but is increasingly concerned with the acquisition of healthier habits, namely the consumption of beneficial compounds through the diet. Moreover, food preservation, allowing the increase and maintenance of products shelf-life, remains a focus of the researches performed today. Indeed, over the years man has been developing methods for food preservation, as well as flavour enhancers. These include salting, sunlight and oven drying, smoking, vinegar / pickling, fermentation, or addition of herbs. Moreover, additives to alter the colour of foods making them more attractive, as well as additives that confer biological properties to food have been also developed (Carocho et al., 2015; Gilsenan, 2011; Smithers, 2016).

Since pre-history, man has been developing techniques in order to feed himself. These techniques range from improvements in methods of hunting, domestication of animals and plants, development of food preservation methods, until the introduction of food additives (Carocho et al., 2014; Smithers, 2016).

The incorporation of traditional natural additives in food in order to preserve it or enhance its flavour, has gained significant impetus due to the establishment of the population at fixed places and its continuous growth. This new era has led to an increasing need for food production and consequently to the search for new techniques of food preservation and storage (Katz, 2003). On the XVIII century, world population reached about 1 billion people (Smithers, 2016). This enormous population growth has surpassed the capacity of food production. Moreover, there was the population exodus from the countryside to the cities, which has led to major changes in lifestyles, including changes in eating habits (Carocho et al., 2014). At this stage, the food supply becomes a responsibility of the states, and the traditional small agricultural areas are replaced by large-scale production enterprises (Katz, 2003).

In this way, the food industry was born, and its main objective is to transform huge agricultural and aquaculture products into different forms of food, processed in different ways, to meet the increasing demand for processed food and ensure a safe distribution, at a national and international level. Furthermore, the food industry also aims to offer a variety of food products to meet the market challenges (Fellows, 2000). In order to achieve these objectives, the food industry needs to use a variety of additives. Given the need to increase the production levels, the costs also increased and the use of the natural additives used initially becomes unviable. Thus, there were also advances in the chemistry area that seek to fill these problems, and offer as an alternative a wide variety of chemical additives providing a wide range of colours, flavours, aromas and textures to food (Moldes, Vecino, & Cruz, 2016). Actually, the incorporation of additives has been widely disseminated, leading to an excessive use, without taking into account possible impacts on consumer health. The development of sophisticated analytical methods in 1920, together with regulatory pressure, reduces significantly these problems, ensuring the development of safer products (Carocho et al., 2014).

In the XX century, there was a breakthrough in food science and technology (Smithers, 2016), and progress was made in the area of food additives (Tomaska & Brooke-Taylor, 2014). On the current century, the world population is estimated to reach 8 billion people by 2025 and the civilization became very dependent on processed and ready-to-eat food, so food additives remain the basis of food processing (Carocho et al., 2014, 2015; Floros et al., 2010).

The definition of food additive has been updated for many times since the first proposal in 1995 by the joint panel comprised by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO). Today, food additive is defined 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*).

Nowadays, additives are added to foods with diverse purposes, namely preserving their nutritional quality, regulating acidity, preventing food from adhering surfaces, reducing foaming, improving texture, improving food's baking quality or colour, improving the organoleptic properties, or assisting in the manufacture, processing, storage and transportation (Gilsenan, 2011; Tomaska & Brooke-Taylor, 2014).

Currently, more than 2500 additives are used in food processing (Carocho et al., 2014), which are classified according to their origin and manufacture, their way of use or their function in food. According to their origin and manufacture, additives are divided in 4 groups: natural additives (obtained directly from animals or plants); similar to natural additives (produced synthetically mimicking the natural ones); modified from natural (natural additives that are then modified chemically); and artificial additives (synthetic compounds) (Carocho et al., 2014). According to their mode of use, additives are divided in 2 groups: direct (substances incorporated directly in food) and indirect (substances used in various types of applications in contact with food, such as packaging and equipment, and may be unintentionally incorporated into food) (Blekas, 2016; Gilsenan, 2011). According to their function in food, within the EU, additives are classified in 26 functional classes: 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). In the United States of America, food additives are divided into 6 groups, according to the FDA classification: preservatives, nutritional additives, colouring agents, flavouring agents, texturizing agents, and miscellaneous agents. The preservatives group is divided into 3 subgroups, though some additives may have more than 1 function in foods: antimicrobials, antioxidants, and antibrowning agents. The flavouring agents group is divided in 3 subgroups: the sweeteners, the natural or synthetic flavours, and the flavour enhancers. The texturizing agents include the emulsifiers and the stabilizers. Finally, the miscellaneous agents group comprise many classes: chelating agents, enzymes, antifoaming agents, surface finishing agents, catalysts, solvents, lubricants, and propellants (Carocho et al., 2014, 2015).

## 1.2. A special emphasis in food antioxidants

As previously referred, the use of additives for food preservation is required to maintain the quality, extend the shelf-life period, maintain the nutritional value and ensure the safety of the food products (Wedzicha, 2003). Advances in Food Science and Technology, Chemistry and Microbiology have contributed to the development of more effective preservatives and preservation technologies (Floros et al., 2010). The effectiveness of chemical preservatives depends on their concentration, food composition and on the type of microorganism or process to be inhibited (Angiolillo, Conte, & Nobile, 2014).

Food spoilage is a serious issue in food storage. Lipid peroxidation and rancidification are the prevalent type of oxidation occurred during food processing and storage. This oxidation causes undesirable off-flavours and odours, origins detrimental compounds, as well as alterations in the chemical composition and loss of the nutritional value of foodstuffs.

Oxidative reactions may also affect the food quality by changing the native colour to brown, the so called browning, in which polyphenol oxidase catalyses the conversion of polyphenols to quinones, occurring further the breakdown of these compounds and causing the darkness colour of food (Carocho et al., 2014). The browning process can also be non-enzymatic. In this type of browning occurs the production of melanoidin pigments due to various reactions such as the Maillard reaction, caramelization, chemical oxidation of phenols, and maderisation (the oxidation of a brand of wine called Madeira resulting in a darker colour and altered taste) (Carocho et al., 2014; Manzocco et al., 2001).

Antioxidant agents have been widely used in both raw and processed foodstuffs to avoid oxidation damages in food (Logan, Nienaber, & Pan, 2013). They are defined as food additives that extend the shelf-life of food by the interruption of the oxidation and peroxidation of fats (Carocho et al., 2015; Tomaska & Brooke-Taylor, 2014). These antioxidants act by donating a hydrogen atom or an electron to the oxidizing agent to be stabilized and they become reduced as stable radicals. Therefore, they preserve the *status quo* of the system and extend the shelf-life of food without any modification of food appearance or taste and without adding color (Carocho et al., 2015).

Antioxidants are divided in five groups: radical scavengers or chain-breaking antioxidants; chelators, which bind to metals and prevent them from initiating radical formation; quenchers, which deactivate high-energy oxidant species; oxygen scavengers, that remove oxygen from systems, avoiding their destabilization; and antioxidant regenerators that regenerate other antioxidants when these become radicalized (Carocho et al., 2015). Although antioxidants can be naturally present in the raw provisions, these are not considered as preservatives of the final processed food, since they can be destroyed during the food processing. Therefore, antioxidant additives are required. Several antioxidants are available for food preservation. However, their selection for food application is a serious concern, as the use of antioxidants in food is strictly controlled by legislation (Logan et al., 2013). Antioxidant additives are mainly used in meats, oils, fried foods, dressings, dairy products, baked goods and extruded snacks (Carocho et al., 2015).

The most common chemical antioxidants used are butylated hydroxyanisole (BHA, E320; Acceptable Daily Intake (ADI) 0.5 mg/kg bw), butylated hydroxytoluene (BHT, E321; ADI 0.05 mg/kg bw), propyl gallate, (PG, E310; ADI 1.4 mg/kg bw), ethoxyquin (EQ, E324; ADI 0.005 mg/kg bw), and *tert*-butylhydroquinone (TBHQ, E319; ADI 0.7 mg/kg bw).

Natural compounds are also used as alternatives, namely tocopherols, vitamin C, carotenoids and phenolic compounds (Carocho et al., 2014; Logan et al., 2015).

Overall, synthetic and natural antioxidants are used in food preservation. They must be effective at low doses, non-toxic and have no impact on flavour. For these reasons, and given the global trend for natural products, natural antioxidants are preferred for food applications (Logan et al., 2013).

## 1.3. Natural alternatives *versus* synthetic counterparts

The search for natural additives is a big challenge for the food industry nowadays. Although all food additives are submitted to safety tests and are regulated by national and international legislations (Tomaska & Brooke-Taylor, 2014), synthetic chemical additives become less desirable by consumers. This is because some studies suggested some toxicity effects, and also for lack of information about their action.

For the most common chemical antioxidants widely used (BHA, BHT, PG, EQ, and TBHQ) some contradictory effects have been published. Some studies attributed to these compounds a potential toxicity and carcinogenic effects, while others considered them tumour suppressors (Bauer et al., 2001; Botterweck et al., 2000; Carocho & Ferreira, 2013; Vandghanooni et al., 2013). Indeed, some authors reported that many synthetic additives are toxic after long-term consumption (Zhang et al., 2016).

Many food preservation technologies have been developed and improved in the last years, such as mild-heat processing, modified atmosphere packaging, vacuum packaging, and refrigeration. In addition, new others like ultraviolet radiation, ionizing radiation, pulsed-light, high pressure, among other are being studied in order to restrict the use of preservatives in food (Carocho et al., 2014). However, currently, it is impossible to think about food free of additives / preservatives. Indeed, most of these technologies are not adapted yet to be widely used at the industrial level. They are not completely effective by themselves and they are also quite expensive, being imperative the use of additives in order to complement the preservative effect. Perhaps these limitations can be solved in a few years because of the fast development of technology and continuous innovation, demonstrating whether they actually constitute alternatives to food additives or not.

For now, natural additives are the up-and-coming alternatives to the chemical synthetic ones. However, their costs, sources and effectiveness are the main limitations to their use instead of synthetic additives (Carocho et al., 2015). Indeed, at an economic level, synthetic compounds are the ideal for the food industry, markets and for consumers. Regarding natural additives, is not easy to find producers, especially able to obtain the necessary quantities of the desired compounds in a relatively short period of time, in order to meet the industrial demand. On the other hand, the production of synthetic compounds does not have these limitations. Moreover, usually, the chemical synthetic preservatives are effectives in small quantities comparing to the natural counterparts. Since higher amounts of the natural additives are required to achieve the same effectiveness, their addition to foodstuff can lead to appearance, taste, colour and texture changes. However, on food labels there is no distinction between natural and synthetic preservatives, all being regulated in the same way, under the "E" classification (Carocho et al., 2015). Given this controversy, the food industry and scientific research have accepted the challenge of seeking to dispel doubts about food safety and quality in terms of food additives.

Due to the doubts that have arisen regarding the safety of the use of chemical additives, the search for natural additives has become a hot topic. This search has included natural antimicrobials and antioxidants. In contrast to synthetic preservatives, which are either neutral or harmful to human health, some natural preservatives show additional benefits towards human health namely in the prevention and resistance of several diseases (Ćilerdžiće et al., 2014). Some preservatives, namely antioxidants, in addition to their effect on the extent of shelf-life of food, have even been investigated for the prevention and/or treatment of some diseases such as cancer, cardiovascular and brain diseases and immunological conditions (Carocho et al., 2014). In addition to plants, mushrooms and algae have also become interesting sources of food antioxidants.

As mentioned above, the well-known and the most used molecules as preservatives are vitamins, polyphenols, and carotenoids (Carocho et al., 2014).

Polyphenols are excellent antioxidants that can act as scavengers, chelators, quenchers, and as ion or hydrogen donors, being effective in food preservation. They may also assure the food safety at the microbial level. Their preservative effectiveness can be achieved when incorporated as plant extracts, where synergistic effects may exist between the compounds present in the extract, or acting as single molecules (Carocho et al., 2014, 2015; Lucera et al., 2012). They have also beneficial effects on human health against cancer, osteoporosis, cataracts, cardiovascular dysfunctions, brain diseases, inflammation and immunological problems.

Carotenoids prevent also the oxidation of food but they are limited in use due to their susceptibility to oxidation by light (Carocho et al., 2015).

Vitamins are essential elements for human body that should be provided in the diet. Their use as “green preservatives” enhances the nutritional value of food, helps to prevent food spoilage and appeal for consumption. Vitamin C, also known as ascorbic acid, is used as antibrowning agent, being also a widespread and powerful oxygen and nitrogen scavenger of a variety of ROS and RNS avoiding the oxidative stress. Vitamin E is also an antioxidant agent which encompasses 4 tocopherols (alpha, beta, gamma and delta) and 4 tocotrienols (alpha, beta, gamma and delta). This vitamin constitutes a strong wall against food oxidation, especially when acting in cooperation with vitamin C. Indeed, vitamin C recycles vitamin E by reducing the generated radicals re-establishing its antioxidant activity (Carocho et al., 2014, 2015).

An overview of the aforementioned natural food antioxidants, their contribution in terms of food safety and quality, and impact on human health, clearly shows the potential of nature as a source of bioactive compounds. Moreover, with studies being carried out in the search for new natural preservative compounds, in order to safely feed a growing population estimated at 8 billion humans by 2025 (Carocho et al., 2014), it seems inevitable to replace synthetic additives by their natural counterparts.

## 2. Tocopherols as natural preservatives

### 2.1. Antioxidant properties of tocopherols

The previously mentioned green preservative, vitamin E, is extremely important for human health as an essential micronutrient that should be provided by diet (Rizvi et al., 2014). It is widely known especially by its antioxidant activity preventing many diseases linked to the oxidative stress (Barros et al., 2008).

Vitamin E is the term used to refer to a group of eight compounds, which include both tocopherols and tocotrienols. Tocopherols are among the most well-known antioxidants (Carocho et al., 2015) that halt the propagation of the oxidation process through the stabilization of peroxyl radicals by donating their phenolic hydrogen. The oxidation of tocopherols produces powerless tocopheroxyl radicals which can be recycled to their native form (tocopherols) essentially by ascorbic acid, or undergo further reactions with others peroxyl or tocopheroxyl radicals forming more stable radicals (Barros et al., 2008; Carocho et al., 2014; Reis et al., 2010; Tomassi & Silano, 1986). Furthermore, tocopherols are quite effective in the direct neutralization of alkyl radicals when oxygen is present in low quantities and hydroperoxides are present in trace amounts. Besides, they can also react with alkoxy radicals formed in the propagation step (Reis et al., 2010).

Alpha-tocopherol is the most well-known isoform in terms of biological activity. This isoform is the one preferentially metabolized and absorbed in the human body, and due to its antioxidant potential, constitutes the first wall against cells lipid peroxidation (Barros et al., 2008; Lobo et al., 2010; Rizvi et al., 2014). Although α-tocopherol is the most recognized vitamin E isoform due to its antioxidant potential *in vivo*, γ-tocopherol have shown high effectiveness. Indeed, several studies reported that *γ*- tocopherol is more effective protecting against RNS comparing to α-tocopherol (Reis et al., 2010), and this isoform has been used for biotechnological applications such as food preservation (Boschin & Arnoldi, 2011; Tomassi & Silano, 1986).

## 2.2. The use of tocopherols as food antioxidants

It is known that the natural presence of vitamin E in vegetable oil and animal fats stabilizes them when stored under minimal light and low temperature, increasing their shelf-life (Shahidi, 2015). Regarding their lipophilic and antioxidant properties, tocopherols, especially γ-tocopherol shows an outstanding capacity, avoiding lipid peroxidation and rancidification, halting the chemical deterioration of food (Ortuño et al., 2015; Tomassi & Silano, 1986). Indeed, dietary tocopherols are among the most studied supplemented antioxidants, and it has been reported that they are among the most potent stabilizers of lipids in meat products (Logan et al., 2013). Although they are relatively stable in food, they may be oxidized when exposed to air, heat, acids, alkalis or metal ions (European Food Safety Authority (EFSA), 2015).

Tocopherols are authorized as food additives under the Annex II of Regulation (EC) No 1333/20088 on food additives. They are used as food antioxidants either individually or in combination and classified under the 'E' classification as following: tocopherol-rich extract of natural origin (E306), synthetic α-tocopherol (*all-rac*-α-tocopherol; dl-α-tocopherol; E307), synthetic γ-tocopherol (dl-γ-tocopherol; E308) and synthetic δ-tocopherol (E309). Tocopherols are included in Group I of food additives and authorised in the European Union at *quantum satis* (QS) levels in 68 food categories, at levels of 10 – 100 mg/kg in food for infants and young children (six food categories) and at a level of 200 mg/kg in fats and oils essentially free from water (only refined olive oils and only for E307). Concerning the acceptable daily intake, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) stipulated an ADI for dl-α-tocopherol of 0.15 – 2 mg/kg body weight (bw) based on clinical trials in humans and taking into account the fact that α-tocopherol is an essential nutrient. Although, the Scientific Committee on Food (SCF) concluded that the use of α-, β-, γ- and δ-tocopherols and α-tocopheryl acetate as antioxidants was acceptable, but considered that the available data were not appropriate for establishing an ADI, they establish a Tolerable Upper Intake Level (UL) of 300 mg/day for vitamin E for adults, and scaled different levels for children in the ages ranging from 1 – 3; 4 – 6; 7 – 10; 11 – 14; and 15 – 17 years to give ULs of 100, 120, 160, 220 and 260 mg/day, respectively, as well the UL is applies to pregnant and lactating women (European Food Safety Authority (EFSA), 2015).

Overall, the use of tocopherols (E306 – E309) in food preservation improves the nutritional value of food and helps to overcome losses during food processing. Better preservation effects, with extended shelf-life periods, can be achieved combining tocopherols with ascorbic acid as previously mentioned. However, it should be kept in mind that tocopherols efficiency depends on the nature of the matrices, the concentration and physical parameters, such as temperature (Tomassi & Silano, 1986).

## 3. *In vitro* culture for the production of tocopherols

### 3.1. *In vitro* production of mushrooms mycelium

The use of tocopherols in food preservation is a great advance. However, the optimization of their production in a large scale is a challenge yet. The search for potential sources, as well as tocopherols *in vitro* production, is an interesting approach (Heleno et al., 2011). Natural products constitute an excellent alternative for the *in vitro* production of compounds of interest, namely tocopherols. Moreover, the production of compounds from natural sources does not cause ecological concerns, and may have technical and economic advantages (Pinto et al., 2013). It allows a continuous year round availability of the demanded compounds in an independent way of environmental conditions (Karuppusamy, 2009). *In vitro* plant materials have been recognized as potential sources of valuable compounds, exploited for biotechnological applications (Karuppusamy, 2009), while mushrooms become an emerging alternative since they are also rich in bioactive compounds and they are quite easy to produce by *in vitro* techniques and have a quite fast growth compared to plants (Carocho et al., 2014; Ferreira et al., 2009).

Mushrooms consist on a compact association of dikaryotic mycelium shaped in the so called fruiting body or sporocarp (the reproductive form), which sprout when environmental conditions are favourable. This sporocarp has also the function of disperse teeny reproductive units called spores, which will germinate forming monokaryotic mycelium (Leiva et al., 2015). Therefore, the mycelium is the vegetative form of fungi, which is able to colonize large areas.

As abovementioned, the *in vitro* produced mycelium is considered a potential and promising source of bioactive compounds useful in the pharmaceutical and/or food industries (Reis et al., 2010). Indeed, it has been proved that the *in vitro* culture of mycelium improve the production of antioxidants namely tocopherols. A comparative study of *Cordyceps sinensis* extracts from natural and cultured mycelia showed that both have antioxidant potential, although the mycelium showed greater activity (Dong & Yao, 2007). Also, cultured ectomycorrhizal fungi mycelia showed higher total tocopherol contents, comparing with the corresponding fruiting bodies. High tocopherols levels have been registered for *Pisolithus arhizus* cultured mycelium, especially with respect to the gamma isoform (Reis et al., 2010). In the same context, the mycelium obtained from the species *Lepista nuda* by *in vitro* culture, revealed high levels of tocopherols, especially for β- and γ-isoforms (Pinto et al., 2013). Hence, the *in vitro* culture of mycelium has proven to be a promissory methodology for the production of considerable amounts of tocopherols which can meet the industrial demand.

The *in vitro* culture of mycelium can be carried out in solid and/or liquid media, which provide the nutrients necessary for its development (Leiva et al., 2015). This production method allows the adjustment of the medium in order to optimize the biomass production, as well as the quantities of the desired compounds (Ferreira et al., 2009; Heleno et al., 2011). The culture in solid medium may be performed using spores or live tissue obtained from the fruiting bodies. Both will give rise to mycelium identical to the one of origin, obtained in a sterile environment, and able to colonize the entire medium contained on a Petri dish. This is a technique that ensures the accurate preservation of the genetic material of live mushrooms (Stamets & Chilton, 1983).

Although a solid-medium culture is capable of mimicking the natural substrates at a nutritional level, mushrooms cultivation in liquid medium is an appropriate economic method for large-scale production (Heleno et al., 2011). The principle is the same as the culture in solid medium, but the yields obtained are usually higher. This can be explained by the possibility that, in the solid culture, the diffusion of the nutrients can be restricted by the agar, while the liquid medium allows a better distribution and availability of nutrients, as well as greater availability of oxygen (Heleno et al., 2011). Therefore, the fungus disperses more evenly, with increased biomass production in a short period of time. Furthermore, the biomass obtained in liquid medium has lower chances of contamination and is easier to recover (Gan et al., 2012; Heleno et al., 2011; Souilem et al., 2017; Yang & Liau, 1998; Zhang et al., 2016). It should be noted that the culture medium may also be a source of bioactive compounds, since the fungus may release them into the surrounding environment (Ma et al., 2016).

The metabolism of the species produced by *in vitro* techniques, as well as the yield obtained, vary according to the mushroom species itself, the medium composition and the culture conditions. Indeed temperature, pH, inoculum size, luminosity, incubation time etc., deeply influence the productivity in terms of biomass and produced metabolites (Yang & Liau, 1998).

Melin-Norkrans (MMN) medium, Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media, are conventional media used in mushroom culture.

### 3.2. Biosynthetic pathway of tocopherols

Tocopherols production by *in vitro* techniques, using mushrooms mycelium is a promising alternative to plants. In the recent years, numerous studies tried to elucidate the tocopherols content both in fruiting bodies and their mycelia, among other interesting bioactive compounds. Nevertheless, the literature reports that tocopherols are exclusively synthesized by photosynthetic organisms (Chen et al., 2015; DellaPenna, 2005a, 2005b; DellaPenna & Pogson, 2006; Karmowski et al., 2014), whereas mushrooms are saprophytes. Huge progress was made in the elucidation of the molecular, genetic and biochemical aspects of tocopherols synthesis in the photosynthetic models *Arabidopsis thaliana* and *Synechocystis* PCC6803 (DellaPenna, 2005a), while their biosynthetic pathway in mushrooms remains unclear.

Tocopherols are tocochromanol molecules that consist in a hydrophobic tail or apolar isoprenoid side chain associated with membrane lipids and a polar chromanol or head group remaining at the membrane surface **(Fig. 1)** (DellaPenna, 2005a; Horvath et al., 2006). Indeed, their biosynthesis is in accordance to their chemical structure. It consists of two pathways, the aromatic amino acids (shikimate) pathway, and plastidic isoprenoid deoxyxylulose phosphate (pentose) pathway (DellaPenna, 2005a, 2005b; Matkowski, 2008). Besides, the antioxidant activity of tocochromanol molecules including tocopherols depends on the number and position of methyl group of their chroman ring (Karmowski et al., 2014), which may explain the monopoly of the entire tocopherol activity by α-tocopherol **(Table 1).**

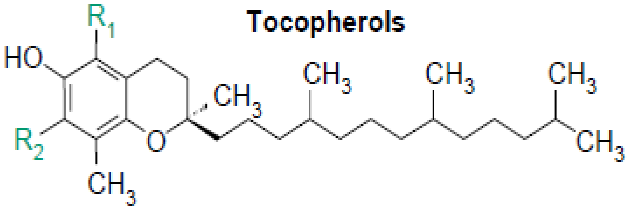

**Fig 1:** General structure of tocopherols (DellaPenna, 2005b).

**Table 1.** Tocopherols structure/antioxidant activity relationship (DellaPenna, 2005a, 2005b). The table indicates the number and position of the ring methyls in α-, β-, γ- and δ-isoforms. Relative antioxidant activity refers to the vitamin E antioxidant activity of each tocopherol with α-tocopherol being 100%.

| Isoform | R1 | R2 | Relative antioxidant activity |
|---|---|---|---|
| **α** | $CH_3$ | $CH_3$ | 100 |
| **β** | $CH_3$ | H | 25–50 |
| **γ** | H | $CH_3$ | 8–19 |
| **δ** | H | H | < 3 |

The first pathway of tocopherols biosynthesis consists in the production of the aromatic (phenolic) head group from Tyrosine (DellaPenna, 2005b; Matkowski, 2008). Indeed, a Tyr transaminase acts on tyrosine (Valentin et al., 2006) forming *p-hydroxyphenylpyruvic* acid (HPP), the substrate of p-hydroxyphenylpyruvic acid dioxygenase (HPPD) (DellaPenna, 2005a, 2005b). It is an irreversible and a complex enzymatic reaction that catalyzes the addition of two oxygen molecules, a decarboxylation and rearrangement of the side chain of HPP, resulting in homogentisic acid (HGA) production (DellaPenna, 2005a). The second pathway, results on the production of the hydrophobic tail of a tocochromanol skeleton (Matkowski, 2008). At this stage, homogentisate prenyl transferases (HPT) catalyze the prenylation of HGA with either phytyl diphosphate (PDP) or geranylgeranyl diphosphate (GGDP) yielding in 2-methyl-6-phytylplastoquinol (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ), the first intermediates in the synthesis of all tocopherols and tocotrienols, respectively (DellaPenna, 2005a, 2005b). MPBQ constitutes the substrate for tocopherol cyclase and MPBQ methyltransferase (MPBQ MT). MPBQ MT connects a second methyl group to MPBQ forming 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ). Tocopherol cyclase converts MPBQ and DMPBQ to δ- and γ-tocopherol, respectively. In the last step, γ-tocopherol methyltransferase (γ-TMT), catalyzes the methylation of the sixth position of the chromanol ring converting δ- and γ-tocopherol to β- and α-tocopherol, respectively **(Fig.2)** (DellaPenna, 2005a, 2005b).

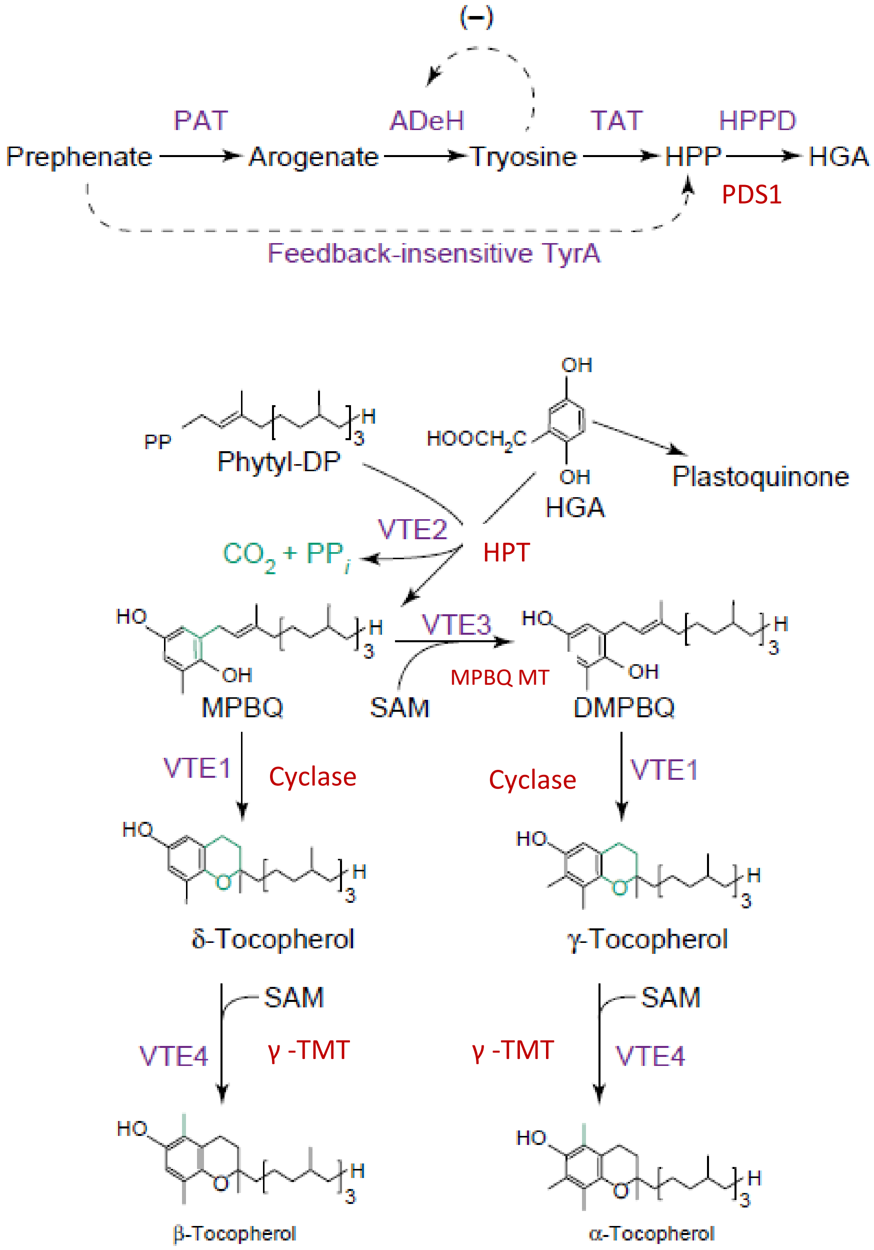

Fig 2: Tocopherol biosynthesis in plants and cyanobacteria (DellaPenna, 2005b). Compounds abbreviations: DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; HGA, homogentisic acid; HPP, *p-hydroxyphenylpyruvate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; phytyl-DP*, phytyl-diphosphate; SAM, S-adenosyl methionine. Enzyme abbreviations: AdeH, arogenate dehydrogenase; cyclase, tocopherol cyclase; HPPD, HPP dioxygenase; HPT, homogentisate phytyltransferase; MPBQ MT, MPBQ methyltransferase; PAT, prephenate amino transferase; TAT, tyrosine amino transferase; g-TMT, g-tocopherol methyltransferase.

In conclusion, HGA and PDP constitute the main precursors for tocopherols biosynthesis (Valentin, 2006). Once synthesized, tocopherols, tocotrienols or both compound classes, may be produced in an organism according to the substrate specificity of HPT (DellaPenna, 2005a). Actually, the level of tocopherols synthesis is highly dependent on the PDP availability, which, in turn, depends on the availability of its precursor. Thus, it has long been known that the hydrophobic phytyl tail is a geranylgeranyl tail because PDP is originated from the direct reduction of GGDP via geranylgeranyl diphosphate reductase (GGR) (DellaPenna & Pogson, 2006; Valentin, 2006). Although, an alternate pathway for the phytyl tail synthesis from chlorophyll-derived phytol was also demonstrated (**Fig. 3**) (DellaPenna & Pogson, 2006; Valentin, 2006).

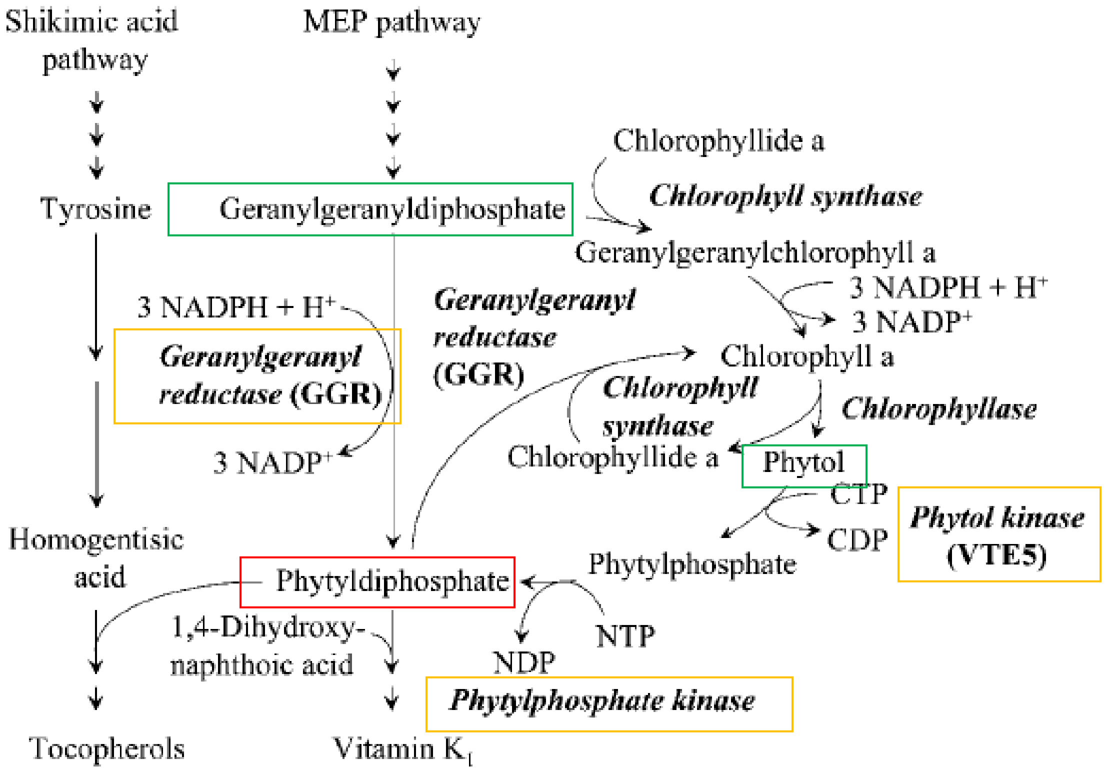

**Fig3:** Overview of tocopherols biosynthesis in plants showing the two pathways of PDP synthesis (Valentin, 2006).

Overall, a detailed biosynthetic pathway for tocopherols production has been established for photosynthetic organisms. However, it has been proved that mushrooms are a valuable source of antioxidants such as tocopherols (Heleno et al., 2009). Therefore, alternative pathway(s) of tocopherols biosynthesis occur in cellular organelles other than plastids.

## 4. Working plan

### 4.1. Mushroom species to be studied

A large variety of mushroom species from various habitats around the world has been studied for their bioactive properties, and explored as a source of interesting compounds for biotechnological applications mainly for the pharmaceutical and food industries. Indeed, filamentous fungi are already used for the production of food colorants, with the expectation that this technology will be applied for other food additives (Carocho et al., 2014), such as antioxidants for food preservation.

*Ganoderma* species, namely *Ganoderma lucidum* (Curtis) P. Karsten, are among the most cited in research publications for their cultivation, chemical analysis, pharmacology and medicinal effects (Saltarelli et al., 2009). They are the most sought medicinal mushrooms in the world market (Stojković et al., 2013) with an annual global market value over than $1.5 billion for their extracts (Ćilerdžić et al., 2014; Heleno et al., 2011, 2013). Actually, nowadays the market is growing rapidly, with a worldwide consumption estimated at thousand tons (Wachtel-Galor et al., 2011).

*G. lucidum* is a woody Basidiomycota mushroom, either parasitic of living hardwoods (especially oaks) or saprobic of deadwood from hardwoods, belonging to the Polyporales order and Ganodermataceae family (Heleno et al., 2011; Wachtel-Galor et al., 2011). It is a large, dark mushroom with a glossy exterior and a woody texture (Wachtel-Galor et al., 2011). This species constitutes a potential source of important bioactive compounds with antioxidant potential (Heleno et al., 2011). Actually, its fruiting bodies, as well as its mycelium are already used, mainly as functional foods and in nutraceutical formulations (Heleno et al., 2011; Saltarelli et al., 2009). Several studies have been carried out in order to obtain the species by *in vitro* culture, and thus to obtain compounds of interest.

Studies on *G. lucidum* have shown that its antioxidant effectiveness correlates with its content in phenolic compounds, polysaccharides, peptides as well as polysaccharide-peptide complexes, (Ćilerdžić et al., 2014; Stojković et al., 2013), although recent studies have reported polysaccharides and triterpenes as the two primary bioactive compounds (Bishop et al., 2015). However, only few reports have been published about *G. lucidum* tocopherols content. There are some studies reporting levels of 1.19 mg/g of extract in mushrooms from Taiwan (Mau et al., 2002). Other authors, studied *G. lucidum* from Serbia, reporting levels of 104.75 mg/100 g dw, consisting of 15.02 mg/100 g dw of α-tocopherol and 89.73 mg/100 g dw of δ-tocopherol (Stojković et al., 2013).

The Northeastern region of Portugal is one of the European regions with higher mycological diversity (Heleno et al., 2009). Indeed, potentially interesting Portuguese *Ganoderma* species were characterized in these recent years (Heleno et al., 2011) namely *G. lucidum*. In these studies, *G. lucidum* fruiting bodies, spores and *in vitro* produced mycelia were studied regarding their antioxidant potential (Heleno et al., 2011). However, its tocopherol content has not yet been reported.

## 4.2. Objectives

In the present work, *Ganoderma lucidum* from the Northeast of Portugal was studied regarding its tocopherols content. Furthermore, a tocopherol rich extract obtained from *in vitro* cultured mycelia was incorporated in natural yogurt, in order to test it as a potential food additive, namely an antioxidant additive. Therefore, the main objectives consist of:

- ✓ Evaluation of the tocopherols content on the *in vitro* produced mycelium from *G. lucidum*, as well as on the fruiting bodies;
- ✓ Incorporation of the tocopherol rich extract in natural yogurt, in order to assess its antioxidant capacity and its potential as food additive.

To achieve these goals, this study started with the production of the *G. lucidum* mycelium by *in vitro* culture techniques. Afterwards, the levels of tocopherols in the obtained mycelium were evaluated by chromatographic techniques, and the values were compared with those obtained for the fruiting bodies. Subsequently, a tocopherol rich extract was obtained from the mycelium, and it was incorporated in natural yogurt. After this incorporation, the studies regarding the antioxidant potential of the extract, as well as the maintenance of the nutritional properties of the studied food matrix were carried out, comparing the obtained results with a control (only yogurt without any additive), and with two food additives already used in yogurts (potassium sorbate - E202) or in other foodstuff (alpha-tocopherol – E307).

# MATERIALS AND METHODS

## 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). Tocopherols (α-, β-, γ- and δ-isoforms), as well as the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) and other individual fatty acid isomers, and sugars [lactose and D(+)-raffinose pentahydrate] 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). Thiamine, casamino acids, malt extract and agar were obtained from Panreac AppliChem (Barcelona, Spain). PDA and PDB were acquired from Oxoid microbiology products (Hampshire, United Kingdom). 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. Samples and *in vitro* mycelium production

*Ganoderma lucidum* (Curtis) P. Karsten fruiting bodies were obtained from the herbarium of the School of Agriculture from the Polytechnic Institute of Bragança, Portugal. These samples were used to analyze the tocopherols profile, since no results have been reported for *G. lucidum* from the Northeastern region of Portugal.

*Ganoderma lucidum* (Curtis) P. Karsten mycelium was obtained from previous cultures maintained in the laboratory of Biology and Biotechnology of the School of Agriculture from the Polytechnic Institute of Bragança, Portugal.

Mycelium growth was carried out in Petri dishes (9 cm diameter) with ≈ 10 ml of solid medium and in flasks (250 ml) with 30 ml of liquid medium maintained in the dark in the *in vitro* culture chamber of the laboratory mentioned above, at 23 ° C / 18 ° C during the photoperiods of day and night (16h / 8h), respectively.

In order to verify the best culture medium for the growth of the species under study, small excised pieces from the previous cultures preserved in the laboratory of Biology and Biotechnology were transferred, under sterile conditions (laminar flow hood), to new Petri dishes containing different solid media **(Fig 4):** i) Potato Dextrose Agar medium (PDA) pH 5.6 ± 0.2; ii) Melin-Norkans medium (MMN) pH 6.6 (NaCl 0.025 g/l; $(NH_4)2HPO_4$ 0.25 g/l; $KH_2PO_4$ 0.50 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; malt extract 5 g/l; casamino acids 1 g/l; glucose 10 g/l; agar 20 g/l); and iii) modified MMN medium (mMMN) pH 6.6 (NaCl 0.025 g/l; $(NH_4)2HPO_4$ 0.25 g/l; $KH_2PO_4$ 0.50 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).

**Fig 4:** Mycelium subculture under the laminar flow hood.

After 7 days from the inoculation time, the radial growth was registered every 2 days until the plate area became fully covered with mycelium (**Fig. 5**).

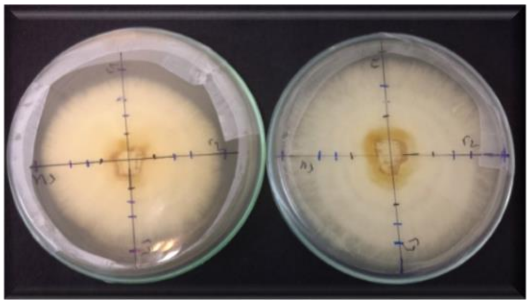

**Fig 5:** Radial growth measurements.

After reaching the maximum growth, the cultured Petri dishes were used for the inoculation of *G. lucidum* in the ideal culture medium, but in the liquid form [Potato Dextrose Broth (PDB)], in order to obtain higher yields to perform the chemical assays. Three excised fragments were inoculated in each flask with 30 ml of culture medium. The flasks were held in the above-mentioned *in vitro* culture chamber, until enough biomass was obtained for the subsequent assays (≈ 32 days) (**Fig. 6**).

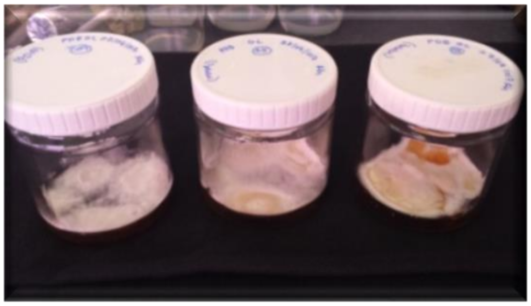

**Fig 6:** Mass production of *Ganoderma lucidum* mycelium in PDB liquid medium.

After approximately 32 days, the mycelia were recovered from the culture flasks using a sieve (particle size, 2 mm; **Fig. 7**). Afterwards, the recovered mycelia were weighted in order to obtain the fresh weight (fw), frozen and lyophilized (freeze 4.5 FreeZone model 7750031, Labconco, Kansas, USA), obtaining the corresponding dry weight (dw) (Heleno et al., 2011). The lyophilized samples were then reduced to a fine powder (20 mesh), mixed to obtain homogeneous samples and stored in a desiccator, protected from light, until further analysis.

**Fig 7:** Mycelium recovery using sieves.

## 3. Tocopherols rich extract

### 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. The samples (≈ 500 mg) were homogenized with methanol (4 ml) by vortex mixing (1 min). Subsequently, hexane (4 ml) was added and the mixture was vortex again (1 min). Saturated NaCl aqueous solution (2 ml) was added, and the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the clear upper layer was transferred to a vial wrapped in aluminum paper and placed in the ice. The samples were re-extracted twice with hexane. The combined extracts were dehydrated with anhydrous sodium sulfate and 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 the volume of methanol required to obtain a stock solution of 10 mg/ml.

### 3.2. Determination of tocopherols content

Tocopherols analysis was made by HPLC following a procedure previously optimized and described by (Heleno et al., 2010). The equipment of HPLC (**Fig. 8**) consisted of an integrated system with a Smartline 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and an FP-2020 fluorescence detector

(Jasco, Easton, MD) programmed for excitation at 290 nm and emission at 330 nm. Data were analysed using Clarity DataApex 2.4 Software. The column used was a normal-phase 250 mm × 4.6 mm i.d., 5 mm, Polyamide II, with a 10 mm × 4 mm i.d. guard column of the same material (YMCWaters, Dinslaken, Germany), operating at 30 °C. 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. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method. Tocopherols content in the samples was expressed in µg per g of dry weight (Barros et al., 2008; Heleno et al., 2009; Reis et al., 2011).

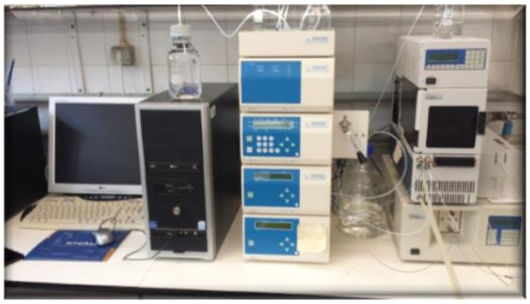

**Fig 8:** HPLC-UV equipment.

### 3.3. Evaluation of the antioxidant activity

For the evaluation of the antioxidant activity of the tocopherols rich extract, two different *in vitro* assays were performed, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the reducing power assay.

**DPPH radical scavenging activity.** DPPH is a stable radical characterized by an absorbance, in concentrated ethanol solution, at about 515 nm. This assay consists of reducing the DPPH radical, through the donation of a hydrogen (H) atom from a scavenger molecule (*i.e.*, antioxidants), resulting in the color alteration from purple to yellow, with a concomitant decrease in the absorbance at 515 nm (Mishra et al., 2012).

This methodology was performed using an ELX800 Microplate Reader (BioTek Instruments, Inc., Winooski, VT). The in each of the 96 wells **(Fig. 9)** consisted of different solutions of the extract (30 µl) with a methanolic solution (270 µl) containing DPPH radicals (6

$\times 10^5$ mol/l). The mixture left to stand in the dark for 60 min and the reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA= [(A_{DPPH}-A_S)/ A_{DPPH}] \times 100$, where $A_S$ corresponds to the absorbance of the solution containing a given extract concentration and $A_{DPPH}$ is the absorbance of the DPPH solution. The assays were carried out in duplicate and the results were expressed as $EC_{50}$ values, which correspond to the extract concentration providing 50% of radicals-scavenging activity. This value was calculated by interpolation from the graph of RSA percentage against extract concentration(Ferreira et al., 2007; Heleno et al., 2009; Reis et al., 2011). Trolox was used as standard.

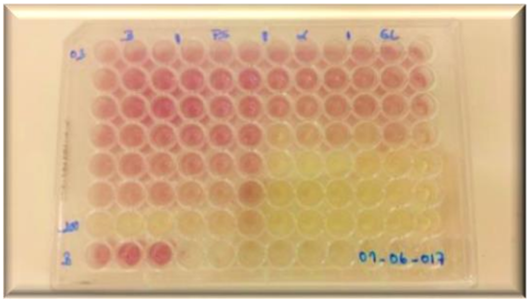

**Fig 9:** Microplates for the DPPH radical scavenging activity assay.

**Reducing power assay.** The present assay is based on the reduction of the yellow ferric form ($Fe^{3+}$) to the blue ferrous form ($Fe^{2+}$) by the action of electron-donating antioxidants. The resulting Perl's Prussian blue color could be measured spectrophotometrically at 700 nm (Ferreira et al., 2007).

This methodology was performed using the same Microplate Reader described above. In eppendorf tube, different concentrations prepared from the stock solutions (0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 °C for 20 min, and then trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the microplate wells **(Fig. 10)** with deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm (Reis et al.., 2010). The assays were carried out in duplicate and the results were expressed as $EC_{50}$ values, which correspond to the extract concentration providing 0.5 of absorbance. These $EC_{50}$ values were calculated from the graph of absorbance at 690 nm against the extract concentration. Trolox was used as standard.

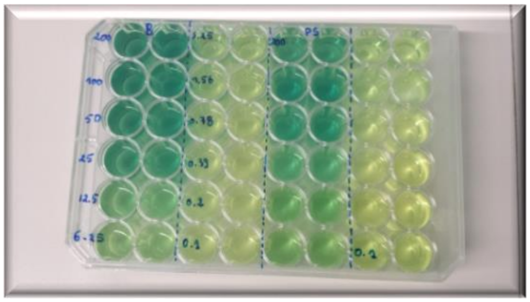

**Fig 10:** Microplates for the reducing power assay.

## 4. Incorporation of the tocopherols rich extract in natural yogurt

The natural yogurts were purchased from a local supermarket.

Four yogurt formulations were prepared (50 g of natural yogurt): i) control (yogurt without any type fortifying agent); ii) yogurt with potassium sorbate (E202; 20 mg); iii) yogurt with α-tocopherol (E307; 8 mg); and iv) yogurt with *G. lucidum* mycelium tocopherol enriched extract (33 mg, according to the $EC_{50}$ value obtained from the reducing power assay). **(Fig. 11)**. All the yogurts were prepared in duplicate.

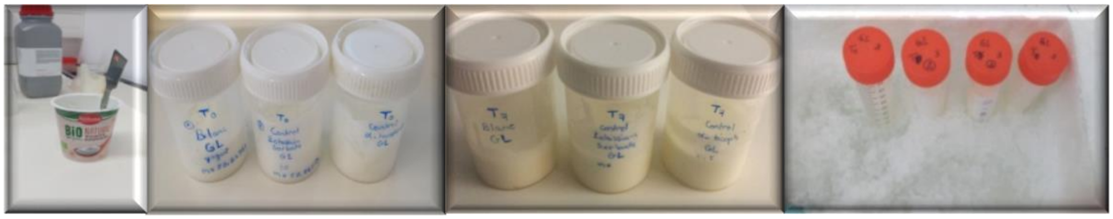

**Fig 11:** Preparation of the three groups of samples.

### 4.1. Nutritional composition and evaluation of the antioxidant activity of the samples along the shelf-life period

The samples were analyzed immediately after preparation and after seven days of storage at 4 ºC. All the analyses were performed in triplicate.

**Nutritional parameters.** The nutritional value of the samples was evaluated (moisture, protein, fat, ash and carbohydrates) using the AOAC (2016) standard procedures (George & Latimer., 2016). The crude protein content (N × 6.38) of the samples was estimated by 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 ± 15 ºC 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 coupled to refraction index (RI) detector.

Briefly, the lyophilized samples (≈ 1 g) were spiked with melezitose (internal standard; IS) and were extracted with 40 ml of 80% aqueous ethanol at 80 °C for 90 min. The resulting suspension was centrifuged at 15,000g for 10 min. The supernatant was concentrated at 40 °C under reduced pressure and defatted three times with 10 ml of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 ml and filtered through 0.22 µm disposable LC nylon disk filters and transferred into an injection vial to be analyzed by HPLC-RI.

The HPLC equipment consisted of an integrated system with a Smartline 1000 pump (Knauer, Berlin, Germany), a Smartline manager 5000 degasser, an AS-2057 auto-sampler (Jasco, Easton, MD) and a Smartline 2300 refraction index (RI) detector (Knauer). Data were analysed 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 30 °C. 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 sample peaks with standards. Quantification was made by the internal standard method based on the RI signal response of each standard, using the internal standard (IS, melezitose) method and by using calibration curves obtained from commercial standards of each compound. The results are expressed in g/100 g of yogurt, calculated by internal normalization of the chromatographic peak area (Caleja et al., 2016; Heleno et al., 2015; Heleno et al., 2009; Reis et al., 2011).

Fatty acids were analyzed by gas chromatography (GC) coupled to a flame ionization detector (FID) detector.

Briefly, the fatty acids obtained after Soxhlet extraction, were subjected to a trans-esterification procedure (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 50 °C and 160 rpm). Afterwards, 3 ml of deionised water were added in order to obtain phase separation; the fatty acids methyl esters (FAME) were recovered by shaking in a vortex with 3 ml of diethyl ether, and the upper phase was passed through a micro-column of anhydrous sodium sulfate to eliminate the water. The sample was recovered in a vial with Teflon and filtered through a 0.2 µm Whatman nylon filter.

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Heleno et al., 2009). The fatty acid profile was analyzed with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID) and a Macherey–Nagel column (30 m × 0.32 mm ID × 0.25 µm df). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 10 °C/min ramp to 240 °C and held for 11 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. For each analysis 1 µl of the sample was injected in GC. Fatty acid identification was made by comparing the relative retention times from samples with FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid (Heleno et al., 2009; Reis et al., 2011).

**Antioxidant activity.** The lyophilized samples (≈ 1 g) were extracted with methanol at room temperature during 1 h under stirring. The extract was filtered with Whatman paper filter No 4, and the remaining solid residue subjected to an additional extraction at the same conditions. The resulted combined extracts were evaporated at 40 ºC under reduced pressure (rotary evaporator Büchi R-210, Büchi, Flawil, Switzerland)) until complete removal of methanol **(Fig. 12)**. Finally, the evaporated extracts were dissolved in methanol at a concentration of 200 mg/ml.

DPPH radical-scavenging activity and reducing power were evaluated at 515 and 690 nm, respectively, using the ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, Vermont, USA), as previously described.

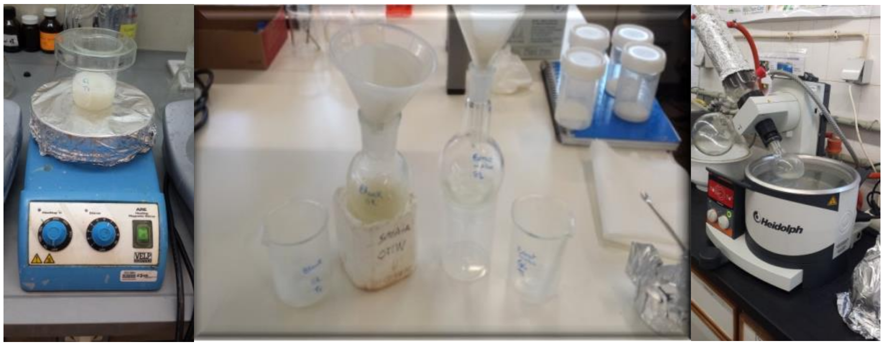

**Fig 12:** Antioxidant activity extracts preparation from modified yogurt

## 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 were compared through an analysis of variance (ANOVA) with type III sums of squares using the general linear model (GLM) procedure. The dependent variables were analyzed using 2-way ANOVA with the factors “yogurt formulation” (YF) and “storage time” (ST). When a statistically significant interaction among these two factors was detected, their effects 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’ $\Lambda$ 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). The main objective was estimating correlations between single categorical dependent variables (yogurt formulations) and quantitative independent variables (results obtained in the laboratorial assays). The LDA outputs identified the independent variables with highest contribution to the differences in the average score profiles of different yogurt formulations. To verify the significance of the canonical discriminating functions, Wilk’s $\Lambda$ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

# RESULTS AND DISCUSSION

Nowadays, natural antioxidants are among the most searched compounds due to the several issues related to the oxidative stress and the increasing awareness of the possible side effects of the synthetic alternatives. Indeed, it has been reported that mushrooms constitute a valuable source of several antioxidants including phenolic compounds, vitamin C and E and carotenoids. Moreover, it has been established a direct relationship between the compounds present in mushrooms extracts and their verified bioactivity, making them potentially bioactive ingredients for use in the nutraceutical formulations (Reis et al., 2017).

Hence, in a continuous way of mushrooms valorization, and for the first time, this study aims to evaluate the antioxidant capacity of a tocopherols rich extract obtained from the mycelium of *Ganoderma lucidum* produced by *in vitro* culture, as well as evaluate its antioxidant effects when incorporated in natural yogurt.

As abovementioned, although several studies have reported that the antioxidant activity of *G. lucidum* is correlated mainly with the phenolic compounds, polysaccharides, peptides and polysaccharides-peptides complexes present in their chemical constitution (Ćilerdžić et al., 2014; Heleno et al., 2011; Stojković et al., 2013), there are no information regarding the tocopherols content and the antioxidant potential of tocopherol extracts from this species. Moreover, the inclusion of such extract, as an antioxidant additive, in foodstuff has not been tested yet.

In general, fruiting bodies seem to have highest antioxidant properties than the *in vitro* produced mycelia. However, some studies proved that mycelia produced by *in vitro* culture may have higher tocopherols content than the fruiting bodies (Reis et al., 2011).

## 1. Mycelium production

In the present work, the *in vitro* culture was exploited for the production of *G. lucidum* mycelium, in order to obtain enough biomass for the incorporation and the subsequent analysis. Therefore, this study started with the evaluation of the mycelium growth in different culture media (MMN, modified MMN and PDA).

After 7 days of the inoculation time, it was possible to measure the mycelium growth on both MMN and PDA media. Mycelium revealed a better growth on MMN medium, comparing with PDA (**Fig 14**), however, these differences were not statistically significant. Besides, Heleno et al. (2012) reported, for this species, better results for mycelium growth, on PDA medium. Some slight differences on both studies may be explained by the fact that the culture was carried out after a long period without mycelia sub-culturing, therefore, the starting conditions of the mycelia were different.

The mycelium was sub-cultured several times and over a period of six months (**Fig 13A**). During the first months, it was noticed that, on both media, the mycelium was covering the surface of the plates in a thinly layer and seemed to had a more dehydrated aspect mainly on the center of the Petri dish (where mycelium cells are older) (**Fig 13B**). During the last two months, deep changes were noticed. Mycelium became colonizing the plate area in more dense layers with a clear cottony aspect, and without a dehydrated appearance (**Fig 13C** and **D**). After covering all the surface of the petri dishes, the growth remains constant. Hence, the repeated sub-cultures of the mycelium seem “reactivate” and “refresh” *G. lucidum* mycelium cells.

It was also performed an experiment to test the mycelium growth in modified MMN medium (mMMN), but it showed a very poor and insufficient mycelium development due to the stress caused by the absence of some nutrients in the culture medium. These results are in agreement with the results published by Heleno et al. (2012) (**Fig 13E**).

PDB liquid medium was inoculated using the Petri dishes with the best growth rates. After four days of the inoculation time, it was possible to see the starting growth in the majority of the flasks. In five weeks, the entire medium surface became covered by the mycelium. Whereas, in many flasks the mycelia did not cover the entire surface medium and stopped growing at the end of the five weeks (**Fig 13F**). Overall, ≈ 250 flasks with 30 ml of PDB liquid medium were used.

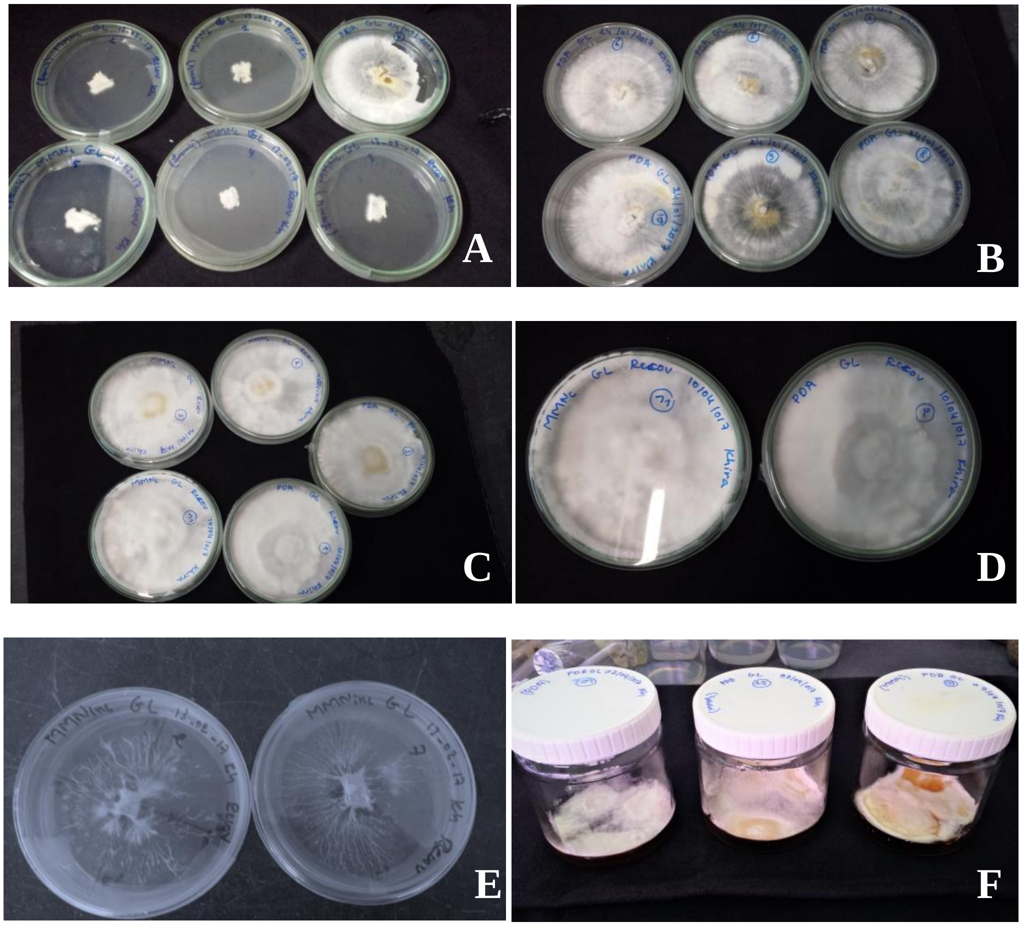

**Fig 13:** *Ganoderma lucidum* mycelium growth in solid and liquid media. A- Sub-culture of the mycelium; B- Mycelium growth aspect in the solid medium during the first months of the growth; C- Mycelium growth aspect during the last two months; D- Mycelium growth aspect in the mMMN; E- mycelium growth aspect in the PDB liquid medium

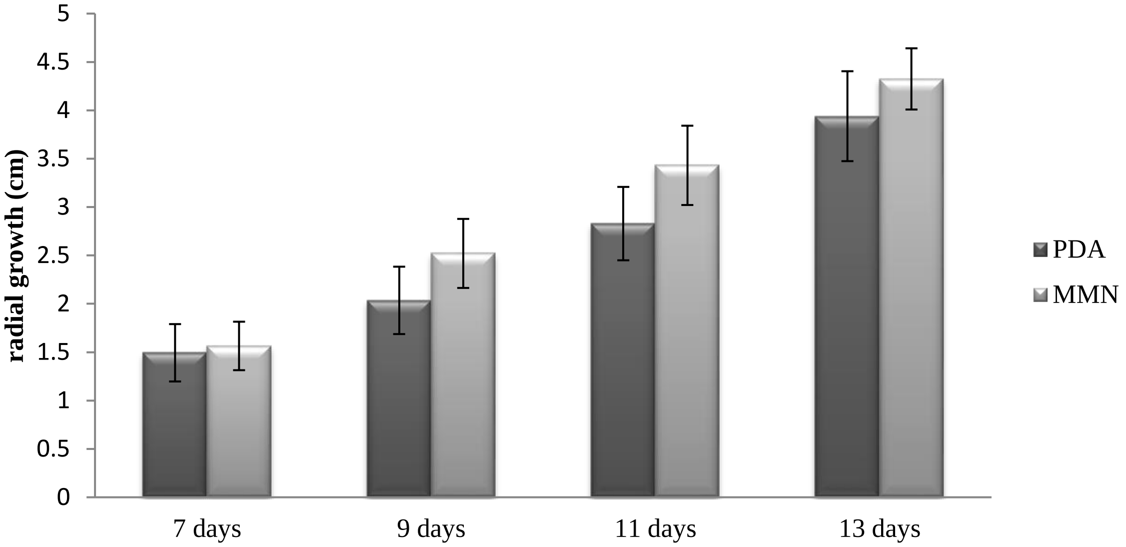

**Fig 14:** Means of radial growth of *Ganoderma lucidum* in PDA and MMN complete media during the growth period.

## 2. Incorporation of tocopherols in yogurt

Mushrooms are widely recognized for their organoleptic properties, nutritional composition and bioactivity, which is mainly provided by substances such as polysaccharides, lipids (*e.g.*, sterols), peptides or fiber (Cheung, 2010; Ferreira et al., 2009; Kozarski et al., 2015; Manzi et al., 2001; Öztürk et al., 2015; Patel & Goyal, 2012; Reis et al., 2014; Soković et al., 2016; Xu et al., 2011; Zhang et al., 2016). The *Ganoderma* genus, particular *G. lucidum* species, has been the focus of intense scientific research (Cao & Lin, 2004; Ćilerdžić et al., 2014; El Zawawy & Ali, 2016; Heleno et al., 2013; Kamra & Bhatt, 2012; Kohno et al., 2017; Shi et al., 2013; Xiao et al., 2017; Zhao et al., 2010). Nevertheless, most of the conducted studies were based in the fruiting body, while only few studies report the activity of the mycelium (Kamble et al., 2011). However, the advantageous characteristics found in mushroom fruiting bodies are often exacerbated in the corresponding mycelia (Kamble et al., 2011; Kuo et al., 2006; Li et al., 2013; Li et al., 2013; Mohamad Ansor et al., 2013; Oka et al., 2010; Shen et al., 2014).

In either case, several different natural species are being considered as potential sources of bioactive extracts to be incorporated in functionalized/fortified food products. Among the most commonly tested food products, yogurt has been the subject of intensive research (Ghorbanzade et al., 2017; Karaaslan et al., 2011; Karam et al., 2013; Santillán-Urquiza et al., 2017; Singh & Muthukumarappan, 2008).

After obtaining good results with the incorporation of hydrophilic extracts (decoctions of *Matricaria recutita* and *Foeniculum vulgare* Mill.) in several yogurt parameters (Caleja et al., 2016), we are now interested in evaluating the effects of lipophilic extracts, particularly obtained from *G. lucidum* mycelium.

The study was initiated by characterizing the antioxidant activity and tocopherol composition of the mycelium itself. According to the obtained results, the mycelium of *G. lucidum* achieved good results in DPPH scavenging activity ($EC_{50}$ = 10.4±0.2 mg/mL), but especially good in the reducing power assay ($EC_{50}$ = 0.32±0.01 mg/mL). In terms of tocopherol profile, *G. lucidum* mycelium was particularly rich in δ-tocopherol (362±7 µg/g extract) and β-tocopherol (272±8 µg/g extract), followed by γ-tocopherol (68±3 µg/g extract) and α-tocopherol (15±1 µg/g extract), showing relevant differences in comparison to the corresponding fruiting body from the Northeastern region of Portugal (α-, β- and δ-tocopherol 1.93±0.03, 267±4 and 15.4±0.4 µg/g extract, respectively), as also with *G. lucidum* fruiting body from Serbia and China (Stojković et al., 2014). Owing to the detected contents, the mycelium of *G. lucidum* (717±12 µg tocopherols/g extract) might certainly be considered as a potential source of these lipophilic antioxidants. In order to verify its effectiveness, other yogurt formulations, namely including typical commercial antioxidants (potassium sorbate and α-tocopherol), were also prepared and compared with yogurt incorporating *G. lucidum* mycelium.

### 2.1. Characterization of different fortified yogurts

Considering the increasing interest of consumers in food products prepared with natural additives instead of synthetic compounds, which are frequently associated with adverse effects (Carocho et al., 2014), this type of studies has high usefulness, also because functionalized products are expected to present better rheological and technological properties (Caleja et al., 2016; Santillán-Urquiza et al., 2017).

Accordingly, four yogurt formulations (YF) were prepared: i) control (yogurt without any type fortifying agent); ii) yogurt with potassium sorbate; iii) yogurt with α-tocopherol; and iv) yogurt with *G. lucidum* mycelium extract. Besides evaluating the effects of the incorporated agent in the same day yogurts were prepared, a further comparison was performed after 7 days of storage, in order to assess the possible influence of storage time (ST).

In order to understand the true effect of each factor (YF and ST), their interaction (YF×ST) was also evaluated to assess possible cooperative effects (*i.e.*, if the effect of ST over a determined parameter varied with the functionalizing agent). In all cases where a significant interaction was found ($p<0.050$), the multiple comparisons could not be performed. In those cases, some overall trends were tentatively obtained from the corresponding estimated marginal means (EMM) plots.

Regarding nutritional composition (**Table 2**), the interaction among factors was only significant for fat, protein and lactose, indicating that ST influenced these parameters differently for each YF. Furthermore, the effect of YF was only significant for fat, protein, carbohydrates and lactose, resulting that the statistical classification could only be indicated for carbohydrates, which reached maximum values in yogurts prepared with potassium sorbate and lowest values in control yogurts. Regarding fat, protein and lactose, the EMM did not show unequivocal tendencies.

Similarly, ST caused significant changes in the contents of fat, protein and lactose. In this case, fat and lactose tended to higher values in yogurts stored during 7 days, but the protein content did not showed any identifiable tendency. The proximate composition of yogurts prepared in this work is in general agreement with that reported previously (Caleja et al., 2016).

Fatty acids profiles were also assayed, since these molecules are considered as reliable indicators of adequate conservation processes (Barreira et al., 2010; Pereira et al., 2016). In addition to the tabled fatty acids (**Table 3**), 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 3**, the changes induced by ST vary according to YF in most cases, since the interaction was not significant only for C10:0, C12:0, C14:0, C15:0, C16:1, C17:0 and SFA. Furthermore, the different yogurt formulations showed significant changes only in C4:0, C6:0, C8:0, C18:0 C18:2n6, C18:3n3 and PUFA. From the EMM plots, some overall tendencies could be verified for C8:0 (higher percentages prepared with *G. lucidum* mycelium extract), C18:0 (higher percentages in yogurts incorporating α-tocopherol), C18:2n6, C18:3n3 and PUFA (all with lower percentages in yogurts including α-tocopherol). Likewise, ST did not induce significant changes in most fatty acids, except for C4:0, C8:0 and C18:1n9 (this last presenting lower percentages in stored samples).

Considering the *p*-values of the interaction among factors and of each factor *per se*, it was not possible to indicate the statistical classification in any case.

Additionally, the antioxidant activity of each YF was evaluated by performing DPPH scavenging activity and reducing power assays. As it might be concluded from **Figure 15** and **Figure 16**, yogurts prepared with α-tocopherol or *G. lucidum* mycelium extract, were clearly the ones with highest antioxidant activity. On the contrary, yogurts prepared with potassium sorbate did not produce great improvements in the antioxidant activity of the control yogurt itself. Moreover, no particularly significant differences were observed among 0 and 7 days, particularly in yogurts incorporated with *G. lucidum* mycelium extract, which were maintained throughout storage.

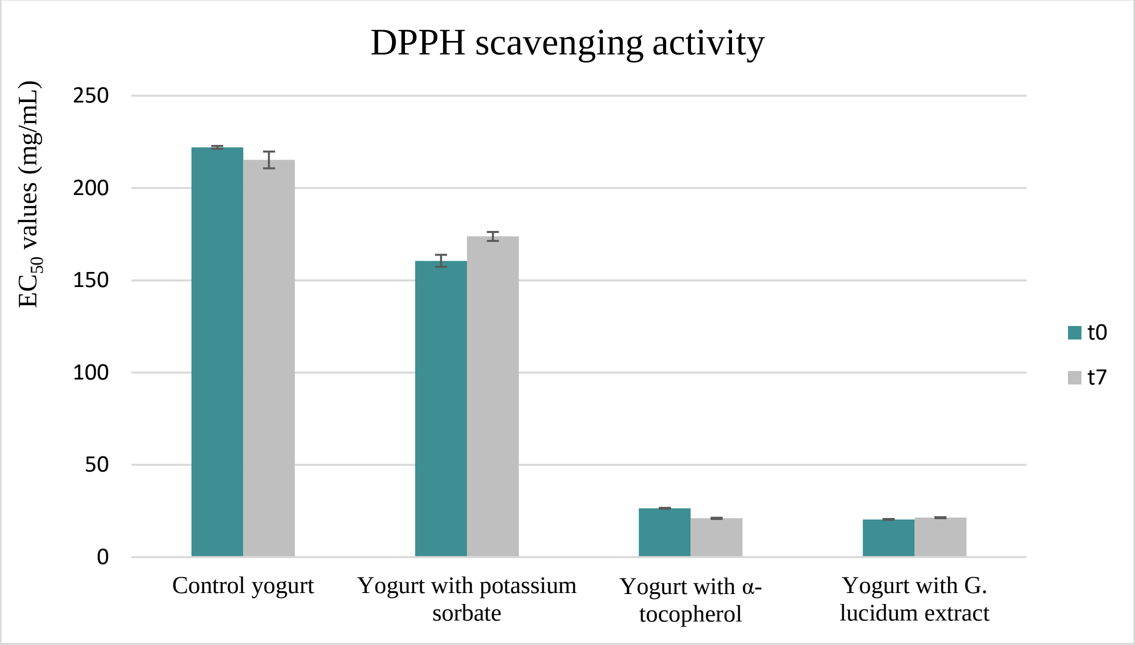

**Figure 15.** DPPH scavenging activity of different yogurt formulations assayed at preparation day and after 7 days of storage.

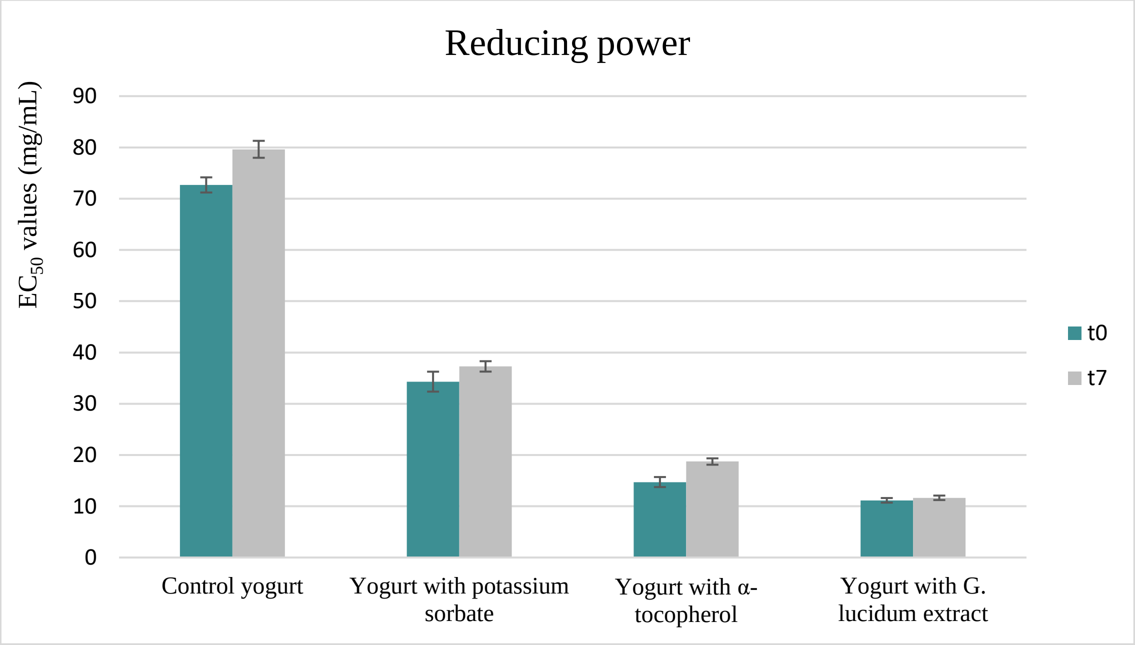

**Figure 16.** Reducing power of different yogurt formulations assayed at preparation day and after 7 days of storage.

**Table 2.** Nutritional composition and energy values for different yogurt formulations (YF) and storage times (ST). Results are presented as mean±standard deviation.[1]

| | | Water | Fat | Protein | Ash | Carbohydrates | Lactose | Energy |
|---|---|---|---|---|---|---|---|---|
| YF | Control | 85±1 | 3.7±0.3 | 4.7±0.2 | 0.88±0.04 | 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 | 5.9±0.2 a | 3.8±0.2 | 74±2 |
| | α-Tocopherol | 85±1 | 3.5±0.2 | 4.9±0.2 | 0.86±0.05 | 5.8±0.2 ab | 3.6±0.5 | 75±1 |
| | *G. lucidum* | 85±1 | 3.7±0.2 | 4.7±0.2 | 0.88±0.04 | 5.8±0.2 ab | 4.1±0.2 | 75±2 |
| | ANOVA *p*-value (n = 18)[2] | 0.951 | 0.012 | 0.001 | 0.318 | 0.026 | <0.001 | 0.243 |
| ST | 0 days | 85±1 | 3.5±0.2 | 4.7±0.2 | 0.86±0.04 | 5.8±0.2 | 3.7±0.4 | 74±2 |
| | 7 days | 85±1 | 3.7±0.2 | 4.7±0.2 | 0.89±0.05 | 5.8±0.2 | 4.1±0.2 | 76±2 |
| | ANOVA *p*-value (n = 36)[3] | 0.253 | <0.001 | 0.812 | 0.007 | 0.846 | <0.001 | <0.001 |
| YF×ST | *p*-value (n = 72)[4] | 0.587 | 0.002 | <0.001 | 0.665 | 0.951 | <0.001 | 0.168 |

[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 3.** 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 | C17:0 | 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 | 11.2±0.3 | 0.8±0.1 | 1.4±0.1 | 31±1 | 1.4±0.1 | 0.9±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 | 11.1±0.2 | 0.8±0.1 | 1.4±0.1 | 30±1 | 1.3±0.1 | 0.9±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 | 11.2±0.2 | 0.8±0.1 | 1.4±0.1 | 31±1 | 1.4±0.1 | 0.9±0.1 | 11.2±0.4 | 24±1 | 2.1±0.2 | 1.2±0.2 | 71±1 | 26±1 | 3.5±0.4 |
| | *G. lucidum* | 3.6±0.5 | 2.6±0.2 | 1.4±0.1 | 3.0±0.1 | 3.4±0.2 | 11.2±0.3 | 0.8±0.1 | 1.4±0.1 | 31±1 | 1.3±0.1 | 0.9±0.1 | 10.9±0.4 | 24±1 | 2.5±0.2 | 1.6±0.1 | 70±1 | 26±1 | 4.4±0.2 |
| | ANOVA *p*-value (n = 18)[2] | 0.002 | 0.001 | <0.001 | 0.685 | 0.907 | 0.496 | 0.692 | 0.512 | 0.328 | 0.091 | 0.141 | 0.002 | 0.066 | <0.001 | <0.001 | 0.068 | 0.093 | <0.001 |
| ST | 0 days | 3.6±0.5 | 2.7±0.4 | 1.4±0.1 | 3.0±0.1 | 3.4±0.2 | 11.2±0.1 | 0.8±0.1 | 1.4±0.1 | 31±1 | 1.4±0.1 | 0.9±0.1 | 11.0±0.3 | 24±1 | 2.4±0.3 | 1.4±0.3 | 70±1 | 26±1 | 4.1±0.5 |
| | 7 days | 3.9±0.3 | 2.8±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 | 0.9±0.1 | 10.9±0.4 | 23±1 | 2.3±0.2 | 1.5±0.1 | 70±1 | 26±1 | 4.2±0.3 |
| | ANOVA *p*-value (n = 36)[3] | <0.001 | 0.112 | 0.011 | 0.061 | 0.411 | 0.460 | 0.432 | 0.415 | 0.925 | 0.114 | 0.561 | 0.181 | <0.001 | 0.402 | 0.430 | 0.342 | 0.152 | 0.177 |
| YF×ST | *p*-value (n = 72)[4] | <0.001 | <0.001 | <0.001 | 0.119 | 0.804 | 0.480 | 0.028 | 0.152 | 0.001 | 0.343 | 0.327 | 0.007 | 0.016 | <0.001 | <0.001 | 0.250 | 0.030 | <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.

## 2.2. Linear Discriminant Analysis

In the previous section, it was possible to identify statistically significant changes in each individual parameter (mostly in result of YF). Complementarily, besides identifying individual changes, it would be interesting to find the parameters that typify each YF, namely by evaluating changes in all parameters simultaneously. Therefore, a linear discriminant analysis (LDA) was performed to evaluate the correlations among YF (categorical dependent variables) and all obtained results (quantitative independent variables). The significant independent variables were selected following the stepwise method of LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p<0.050$) were maintained by the statistical model.

The three defined discriminant functions included 100.0% (first function: 95.3%; second function: 4.1%; third function: 0.6%) of the observed variance (**Figure 17**). From the 37 variables included in the analysis, the model selected protein, C20:0, C20:3n6, C20:4n6, C20:3n3+C21:0, C20:5n3, C22:0, C23:0, DPPH scavenging activity and reducing power as those having discriminant ability, indicating that the most relevant changes occurred mainly in polyunsaturated fatty acids and antioxidant activity.

Considering the correlations among functions and variables, function 1 was highly correlated with DPPH scavenging activity, clearly separating markers corresponding to yogurts added with α-tocopherol or *G. lucidum* mycelium extract (both with low $EC_{50}$ values in this assay) from the remaining formulations. Function 2, which was more highly correlated with reducing power, allowed the separation of markers corresponding to yogurts incorporated with potassium sorbate, while function 3, mainly correlated with C20:5n3, C20:3n6 (both presenting higher percentages in yogurts added with *G. lucidum* mycelium extract) and lactose (higher content in yogurts added with α-tocopherol) was effective in separating yogurts added with α-tocopherol and *G. lucidum* mycelium extract from each other.

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

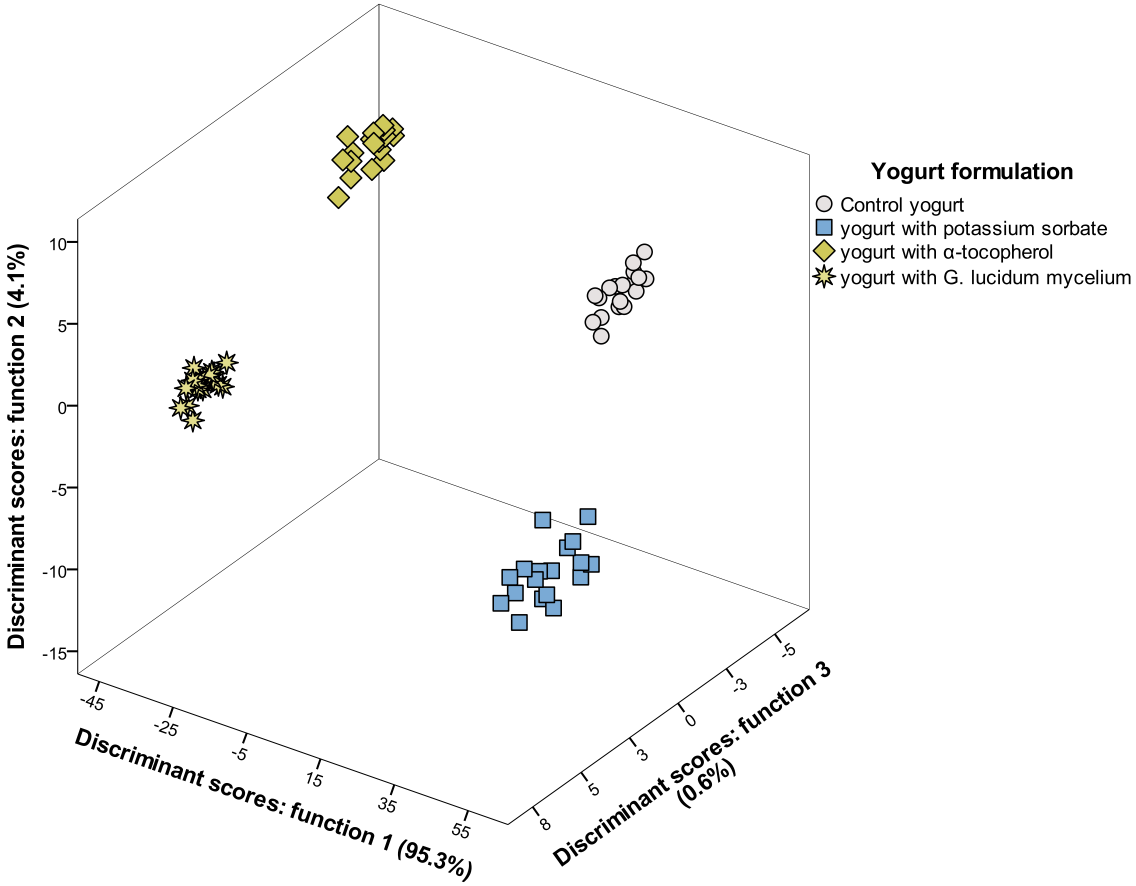

**Figure 17.** Canonical discriminant functions coefficients defined from the evaluated parameters to assess the overall effects of yogurt formulation.

In conclusion, the extract of *G. lucidum* mycelium has good potential to be employed as a lipophilic antioxidant, showing preservative properties similar to those provided by α-tocopherol. This similarity might probably be explained by the composition of the mycelium extract, which was nearly a mixture of δ-tocopherol and β-tocopherol. Another interesting outcome was the maintenance of nutritional properties (except for protein content, which was classified as a discriminant variable) among all tested YF.

# CONCLUSIONS AND FUTURE PERSPECTIVES

The main objective of the present work was to evaluate the antioxidant capacity of a tocopherols enriched extract obtained from the well-known medicinal mushroom *Ganoderma lucidum*, obtained by *in vitro* culture, and study its effectiveness as an antioxidant preservative additive in yogurts.

During the *in vitro* culture period, *G. lucidum* mycelium, revealed similar radial growth in both MMN and PDA media.

According to the chemical analysis, the tocopherols profile shows that the *in vitro* produced mycelium was particularly rich in δ-tocopherol (362±7 μg/g extract), and β-tocopherol (272±8 μg/g extract) and followed by γ-tocopherol (68±3 μg/g extract) and α-tocopherol (15±1 μg/g extract), with relevant differences in comparison to the corresponding fruiting body. Indeed, owing to the detected contents (718±12 μg tocopherols/g extract), the mycelium of *G. lucidum* might certainly be considered as a potential source of these lipophilic antioxidants.

In terms of antioxidant activity, the mycelium of *G. lucidum* showed good results in the DPPH radical scavenging assay ($EC_{50}$ = 10.4±0.2), and the reducing power assay ($EC_{50}$ = 0.32±0.01), although, different results were registered for the yogurt formulations (YF). Indeed, yogurts prepared with α-tocopherol or *G. lucidum* mycelium extract, were clearly the ones with highest antioxidant activity; however, yogurts prepared with potassium sorbate did not produce great improvements in the antioxidant activity. Moreover, no particularly significant differences were observed among 0 and 7 days, especially in yogurts incorporated with the *G. lucidum* mycelium extract.

Regarding the nutritional composition, the interaction between the factors yogurt formulations (YF) and storage time (ST) were only significant for fat, protein and lactose, indicating that ST influenced these parameters differently for each YF. Furthermore, the effect of YF was only significant for fat, protein, carbohydrates and lactose, resulting that the statistical classification could only be indicated for carbohydrates, which reached maximum values in yogurts prepared with potassium sorbate (5.9±0.2 g/100 g) and lowest values in control yogurts (5.7±0.2 g/100 g). Regarding fat, protein and lactose, the estimated marginal means (EMM) did not show unequivocal tendencies. Also, the ST caused significant changes in the contents of fat, protein and lactose. Regarding the fatty acids profile, it was noticed that the changes induced by the ST vary according to YF in most cases, since the interaction was not significant only for C10:0, C12:0, C14:0, C15:0, C16:1, C17:0 and SFA. Additionally, the different YF showed significant changes only in C4:0, C6:0, C8:0, C18:0 C18:2n6, C18:3n3 and PUFA. Furthermore, the EMM plots shows some overall tendencies for C8:0 (higher percentages prepared with *G. lucidum* mycelium extract), C18:0 (higher percentages in yogurts incorporating α-tocopherol), C18:2n6, C18:3n3 and PUFA (all with lower percentages in yogurts including α-tocopherol). Likewise, the ST did not induce significant changes in most fatty acids, except for C4:0, C8:0 and C18:1n9 (this last presenting lower percentages in stored samples).

According to the Linear Discriminant Analysis (LDA), it was possible to identify the most distinctive parameters of each YF, allowing to choose the functionalizing agent (potassium sorbate, α-tocopherol or the enriched extract in tocopherols obtained from *G. lucidum* mycelium), according to a specific objective (in this case preservative effect). Considering the values of the antioxidant activity, it can be concluded that the yoghurt incorporated with α-tocopherol or extract enriched in tocopherols obtained from the mycelium of *G. lucidum*, presented a greater preservation potential.

In conclusion, the extract of *G. lucidum* mycelium has good potential to be employed as a lipophilic antioxidant, showing preservative properties similar to those provided by α-tocopherol. This similarity might probably be explained by the composition of the mycelium extract, which revealed high contents of β- and δ-tocopherol. Another interesting outcome was the maintenance of nutritional properties (except for the protein content, which was classified as a discriminant variable) among all tested YF.

The obtained results could be the base for new studies regarding the use of tocopherols in other food matrices and the production of new natural compounds to be used as food additives. Besides, it is also interesting to think about the incorporation of the mycelia itself in food as a source of several compounds with nutritional and bioactive properties. Actually, the *in vitro* culture may be explored and more optimized, in order to obtain high quantities of interesting compounds (e.g., by inducing stress to the cultures) that can be scaled at the industrial level.

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