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PhD SCHOOL IN FOOD SYSTEMS
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DEVELOPMENT OF FUNCTIONAL INGREDIENTS FROM *PLEUROTUS OSTREATUS* OBTAINABLE FROM AGRI-FOOD WASTE RECYCLING

[Scientific field – AGR/15]

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“La professione del ricercatore deve tornare alla sua tradizione di ricerca per l'amore di scoprire nuove verità. Poiché in tutte le direzioni siamo circondati dall'ignoto, e la vocazione dell'uomo di scienza è di spostare in avanti le frontiere della nostra conoscenza in tutte le direzioni, non solo in quelle che promettono più immediati compensi o applausi.”

Enrico Fermi

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ABSTRACT

The *Pleurotus* genus can use by-products of agri-food industry as substrates to develop its edible fruiting body. *Pleurotus ostreatus*, which is the most cultivated specie of *Pleurotus*, has high nutritional and nutraceutical properties; in particular, it is a source of vitamin D_2 and fiber named β-glucans. However, food uses of this mushroom are limited because of the lack of value-added applications.

The overall aim of this PhD thesis was to develop functional ingredients from *P. ostreatus* biomass.

Specific goals were:

a) development of a food application and production of a powdered ingredient with a target amount of vitamin D_2;

b) encapsulation of an oxidizable target using *P. ostreatus* β-glucans as antioxidant and emulsifying agent.

The results showed that using an adequate concentration of dehydrated and milled *P. ostreatus* for the development of new food applications could help against the increasing vitamin D_2 deficiency among specific groups of populations. To enhance the level of vitamin D_2 in *P. ostreatus*, a combined process of UV irradiation and air-drying was applied, and a new powdered ingredient with a target amount of vitamin D_2 was obtained. The kinetics of vitamin D_2 degradation in *P. ostreatus* powder was studied as a function of water activity and temperature to predict its stability during storage. The kinetics data obtained can be used as a starting point to project the best formulation strategy to deliver vitamin D_2 with functional foods. Since there is a gap of knowledge about the effect of UV irradiation on mushroom bioactive compounds, the antioxidant and antiglycation activities of *Agaricus bisporus* and *P. ostreatus* were investigated and the results pointed out that UV irradiation reduced the antioxidant activity, but not the antiglycation properties.

Two different β-glucan-rich extracts were obtained from *P. ostreatus*; after characterization, they were used to stabilized oil-in-water emulsions. Their stability was checked through variation of oil droplets size distribution over time, in order to find the best formulations suitable for further encapsulation, which was carried out using spray drying technique. After production, the powders were analyzed for the particle morphology and in terms of oil protection against oxidation. Results showed that *P. ostreatus* extracts provided good emulsifying properties and it allowed excellent protection of α-tocopherol and polyunsaturated fatty acids against oxidation. The effects of the *P. ostreatus* extracts was tested during *in vitro* digestion; samples underwent partial aggregation, but the oil droplets were still fairly stable, and α-tocopherol degradation was slower in presence of β-glucans up to the beginning of intestinal phase. The protection against oxidation was also studied during 15 days of accelerated storage; β-glucans improved the oxidative stability of the powders, compared to common emulsifier with no inherent bioactivity.

In conclusion, it is evident that *P. ostreatus* and its compounds can be exploited as sustainable food ingredients to deal with the requirements of populations with endemic nutritional deficiencies and to stabilize oxidizable target during encapsulation process, *in vitro* digestion and storage.

RIASSUNTO

Il genere *Pleurotus* è in grado di utilizzare sottoprodotti dell'industria agroalimentare come substrati di crescita per il suo corpo fruttifero. *Pleurotus ostreatus*, la specie di *Pleurotus* più coltivata, ha elevate proprietà nutritive e nutraceutiche; in particolare, è fonte di vitamina D_2 e di fibre dette β-glucani. Tuttavia, gli usi alimentari di questo fungo sono limitati a causa della mancanza di applicazioni ad alto valore aggiunto.

L'obiettivo generale di questa tesi di dottorato è stato quello di produrre ingredienti funzionali partendo dalla biomassa di *P. ostreatus*.
Gli obiettivi specifici erano:
a) sviluppo di un'applicazione alimentare e produzione di un ingrediente in polvere con una quantità target di vitamina D_2;
b) incapsulamento di un composto ossidabile utilizzando β-glucani da *P. ostreatus* come agente antiossidante ed emulsionante.
I risultati hanno dimostrato che l'utilizzo di un'adeguata quantità di *P. ostreatus* in polvere per lo sviluppo di nuove applicazioni alimentari, potrebbe aiutare contro la crescente carenza di vitamina D_2 tra diversi gruppi di soggetti. Per aumentare il livello di vitamina D_2 in *P. ostreatus* è stato applicato un processo combinato di irraggiamento UV ed essicamento con risultati positivi, ed è stato così ottenuto un nuovo ingrediente in polvere con una quantità target di vitamina D_2. La cinetica di degradazione della vitamina D_2 presente nella polvere di *P. ostreatus* è stata studiata in funzione dell'attività dell'acqua e della temperatura, al fine di prevederne la stabilità durante la conservazione. I dati cinetici ottenuti possono essere utilizzati come base per la progettazione della migliore strategia di formulazione atta a fornire vitamina D_2 attraverso degli alimenti funzionali. Dal momento che mancano dati relativi all'effetto dell'irraggiamento UV sui composti bioattivi dei funghi, sono state studiate le attività antiossidanti e antiglicanti di *Agaricus bisporus* e *P. ostreatus*, e i

risultati hanno evidenziato che l'irraggiamento UV riduce l'attività antiossidante, ma non le proprietà antiglicanti.

Due differenti estratti ricchi di β-glucani sono stati ottenuti da *P. ostreatus*; dopo la caratterizzazione, sono stati utilizzati per stabilizzare le emulsioni olio-in-acqua. La stabilità è stata verificata attraverso la variazione, nel tempo, della distribuzione dimensionale delle goccioline d'olio, al fine di trovare le migliori formulazioni adatte ad un ulteriore processo di incapsulamento, effettuato mediante spray drying. Dopo la produzione, le polveri sono state analizzate dal punto di vista morfologico e in termini di protezione dell'olio dall'ossidazione. I risultati hanno dimostrato che gli estratti da *P. ostreatus* possedevano buone proprietà emulsionanti e consentivano un'eccellente protezione dell'α-tocoferolo e degli acidi grassi polinsaturi contro l'ossidazione. Gli effetti degli estratti da *P. ostreatus* sono stati testati durante la digestione *in vitro*; i campioni hanno mostrato fenomeni di aggregazione, ma le goccioline d'olio erano ancora abbastanza stabili e la degradazione dell'α-tocoferolo è stata più lenta in presenza di β-glucani, fino all'inizio della fase intestinale. La protezione contro l'ossidazione è stata studiata anche durante 15 giorni di stoccaggio accelerato; i β-glucani hanno migliorato la stabilità ossidativa delle polveri rispetto ai comuni emulsionanti privi di bioattività intrinseca.

In conclusione, *P. ostreatus* e i suoi composti possono essere utilizzati come ingredienti alimentari sostenibili per far fronte alle esigenze di popolazioni con carenze nutrizionali endemiche e per stabilizzare target ossidabili durante il processo di incapsulamento, digestione *in vitro* e conservazione.

CHAPTER 1

INTRODUCTION and LITERATURE REVIEW

Towards sustainability in functional food design: the role of *Pleurotus* spp.

Nowadays, worldwide food production is dealing with many difficult challenges, including the noticeable impact of climatic change on agricultural production, the effects of agro-food system on the environment, growing populations and an imbalanced economic situation (Foley et al., 2011; Godfray et al., 2010). Moreover, with the rise in the cardiovascular disease, type-2 diabetes and cancer occurrence, the development of new dietary strategies is required, together with the design of foods that can possibly help in disease prevention (Martirosyan and Singh, 2015). In this context, the genus *Pleurotus* can have an important role since it can grow with a slight utilization of bioresources and, moreover, it can support the production of value-added food products. Indeed, *Pleurotus* spp. can grow fast, both in tropical and in temperate regions, and with low technical skill and capital investment.

The bio-transformation of agri-food by-products into high quality products materials is one of the different approaches of food waste reduction strategies (FAO, 2017). Over the years, the use of agri-food wastes as substrate for producing specific products has gained importance due to their economic and environmental advantages. The production of *Pleurotus* spp. biomass by conversion of lignocellulosic substrates, due to their enzymatic complexes (e.g., phenol oxidases and peroxidases), is a representative example (Sánchez, 2010). Thanks to this conversion, the fungal biomass of *Pleurotus* spp. represents a sustainable source of protein, dietary fiber, vitamins, minerals and mycochemicals (i.e., low-molecular weight bioactive compounds present in mushrooms) (Corrêa et al., 2016). These basidiomycetes have been long known for their nutritional value; however, since their nutritional composition is affected by the species and strain, harvest location, part of the mushroom body, stage of maturation and most of all the composition of the substrate, results regarding the identification and quantification of some of their components are contradictory (Lavelli et al., 2018). Nevertheless, there

are an increasing number of studies on the use of this mushroom for the development of new drugs and functional foods, due to their potential anticancer, immunomodulatory, anti-inflammatory, antihypertensive, antinociceptive, hypoglycemic, hypolipidemic, antioxidant, antimicrobial, antiviral and antiproliferative properties among others (Carrasco et al., 2017; Corrêa et al., 2016; Manzi et al., 1999; Manzi et al., 2001; Mattila et al., 2002). Among food applications, *Pleurotus* spp. powder or β-glucan-rich fractions extracted from *Pleurotus* spp. have been employed as fortifying agents, high-cost protein replacers and prebiotic ingredients. However, to take advantage of the great potential of *Pleurotus* genus, more studies are needed on its micronutrients and bioactive compounds. Moreover, since the addition of *Pleurotus* spp. to functional foods elicit their sensory properties and, therefore, play a key role in food acceptability, sensory evaluation with a proper number of judges can have an important influence on product optimization (Lavelli et al., 2018).

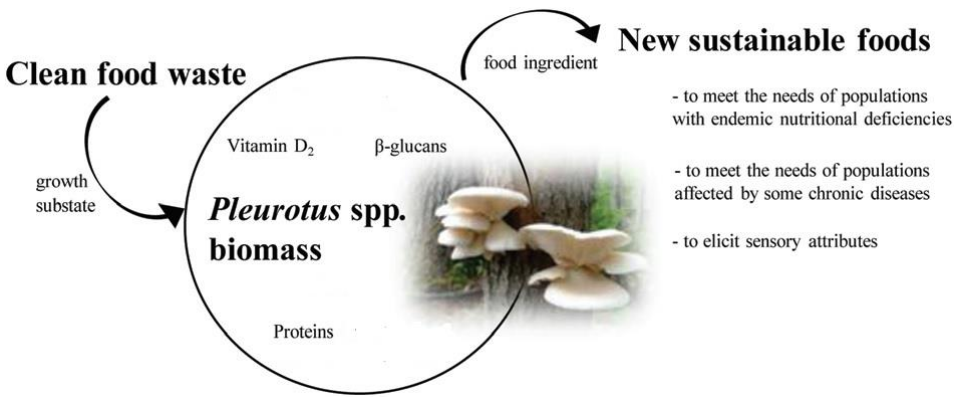


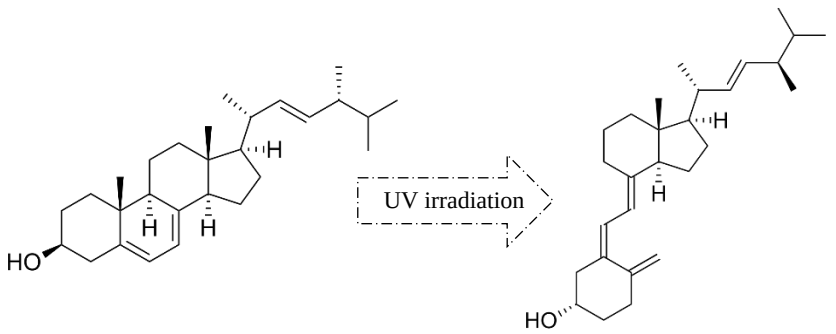
Figure 1. Modified graphical abstract from Lavelli et al., 2018.

Among the around 200 species of *Pleurotus* identified, only a few have been used for food applications and, in particular, *Pleurotus ostreatus* (Jacq.). P. Kumm. 1871, which is the second-largest commercially cultivated edible mushroom (Naim et al., 2020).

Hence, is this PhD thesis *P. ostreatus* biomass was used in order to develop a new functional food accepted by consumers and new functional ingredients, one with a target amount of vitamin D_2 and the other one obtained using *P. ostreatus* β-glucans as antioxidant and emulsifying agent for the encapsulation of an oxidizable target.

Pleurotus ostreatus as a source of vitamin D_2 for functional foods

Among malnutrition concerns, vitamin D deficiency is now recognized as a pandemic. In children it causes rickets, while it aggravates osteopenia, osteoporosis and fractures in adults. Vitamin D deficiency has also been related with increased risk of autoimmune diseases, common cancers, hypertension and infectious diseases (Holick and Chen, 2008). *P. ostreatus* has variable natural levels of vitamin D_2. However, vitamin D_2 in mushrooms is converted from its precursor ergosterol through irradiation (Fig. 2), particularly using UV-B, which have been found to be more efficient than UV-A and UV-C (Jasinghe and Perera, 2006).



Ergosterol Ergocalciferol (vitamin D_2)

Figure 2. Conversion of ergosterol to vitamin D_2 through UV irradiation.

Irradiation protocols of mushroom have been proposed at laboratory scale. On the contrary, the information necessary for scaling up of this technology is still scarce (Lavelli et al., 2019). Studies so far performed on *P. ostreatus* irradiation are summarized in Table 1.

Table 1. Irradiation conditions applied to increment the vitamin D_2 level in *P. ostreatus*.

P. ostreatus **sample and batch size**	**UV-B Intensity (W/m^2)**	**Time (h) Temp (°C)**	**Vitamin D_2 before – after treatment (µg/g d.w.)**	**Ref.**
80% moisture whole, n.s.	4.9	2 35	n.d. – 184 ± 6	Jasinghe and Perera, 2006
fresh sliced, 5 mm, 2 kg	11.5	1 20	0 – 141 ± 0.93	Wittig et al., 2013
fresh whole, n.s.	1.2	2 25	< 2.0 – 239 ± 35	Wu and Ahn, 2014
lyophilized, 60 mesh, 1 g	1.14	0.17 28	< 2.0 – 498	
fresh whole, 0.5 kg	3.6	2 25	0.83 ± 0.06 – 69 ± 2	Huang et al., 2015
fresh whole, 0.3 kg	2.3	0.5 20	n.d. – 56.60 ± 0.82	Sławinska et al., 2016
air-dried, 20 g	2.3	0.5 20	n.d. – 31.85 ± 1.86	
freeze-dried, 20 g	2.3	0.5 20	n.d. – 34.63 ± 0.91	
sliced, 4 mm, during drying, 10 kg	0.4	24 37	3.5± 0.4 – 44 ± 1	Lavelli et al., 2019

n.s. = not specified (regarding the batch size); n.d. = not detected.

Usually, irradiation of fresh mushroom is done with the lamellae facing the UV source, because this part of the fruit body is rich in ergosterol (Wu and Ahn, 2014; Huang et al., 2015; Sławinska et al., 2016). Otherwise, fresh mushrooms are irradiated after slicing (Wittig et al., 2013). The rate of ergosterol conversion is affected by moisture content; in partially dehydrated mushroom (i.e., when moisture is the range 60-85%), vitamin D_2 generation is maximum at the moisture level of 80% (Jasinghe and Perera, 2006). Irradiation of freeze-dried mushroom powder (60 mesh) was found to produce more vitamin D_2 than irradiation of fresh sliced mushroom, probably due to a much higher

surface exposed to UV light (Wu and Ahn, 2014). In fact, increase in conversion rate was not observed in air-dried and sliced freeze-dried mushroom in comparison with fresh sliced mushroom (Sławinska et al., 2016). All these treatments confirm that *P. ostreatus* irradiation can serve for the production of vitamin D_2 to be used for the treatment of endemic deficiency and malabsorption. Nevertheless, the conversion rate depends on a number of factors including UV-B intensity, duration and temperature of the treatment, product shape and surface exposure, moisture content, strain and cultivation parameters. Thus, process monitoring should be implemented to accomplish the target value of vitamin D_2 in the final product and to avoid excessive and toxic levels. In fact, in all the treatments presented in Table 1, the amount of vitamin D_2 of irradiated mushroom is high with respect to the recommended nutrient intakes (RNIs), which is between 5 and 15 μg/day (WHO/FAO, 2004).

Pleurotus ostreatus as a source of β-glucans for functional foods

P. ostreatus fiber fraction is mainly constituted by β-glucans; these polysaccharides have a backbone of D-glucose-linked β-(1→3) with no branches or variable amounts of β-(1→6) branches (Lavelli et al., 2018). The content of total dietary fiber has been evaluated by several researchers and is in the range 12.5–47.3 g per 100 g of fruit body dry weight (d.w.) (Dundar et al., 2008, 2009; Koutrotsios et al., 2014; Manzi et al., 2001; Mattila et al., 2002). The effect of the growth substrate and the method used to determine dietary fiber contents could be the reasons for the different values found by various authors. Concerning the evaluation of β-glucans quantity, it has been recommended to determine it as the difference between the total glucans and the α-glucans. Total glucan measure corresponds to the glucose obtained through a controlled acid hydrolysis with H_2SO_4 and an enzymatic hydrolysis with β-glucosidase and *exo*-1,3-β-glucanase, while the amount of α-glucans is obtained by measurement of the glucose released from α-glucans through enzymatic hydrolysis with amyloglucosidase and α-amylase (McCleary and

Draga, 2016). Using this approach, a study on 16 strains of *P. ostreatus* discovered that the total glucan content varied in the range 14–25 g per 100 g d.w., with β-glucans in the range of 10.9–22.9 g per 100 g d.w. (Koutrotsios et al., 2017). A higher β-glucan content, *i.e.* 35 g per 100 g d.w. with 36 g per 100 g d.w. corresponding to total glucans, was also observed in the strain of *P. ostreatus* used in this PhD thesis (Gallotti et al., 2020), very similar to the one studied by McCleary and Draga (2016), where the total glucans content was found to be 32.7 g per 100 g d.w., while the β-glucan content was 32.3 g per 100 g d.w. The β-glucan content is affected, along with the genetic factors, by the growing conditions; however, knowledge on this matter is still scarce. Since *P. ostreatus* health effects are mostly due to its β-glucans (Dalonso et al., 2015), procedures to recover concentrated β-glucan fractions from *P. ostreatus* have been proposed (Fig. 3).

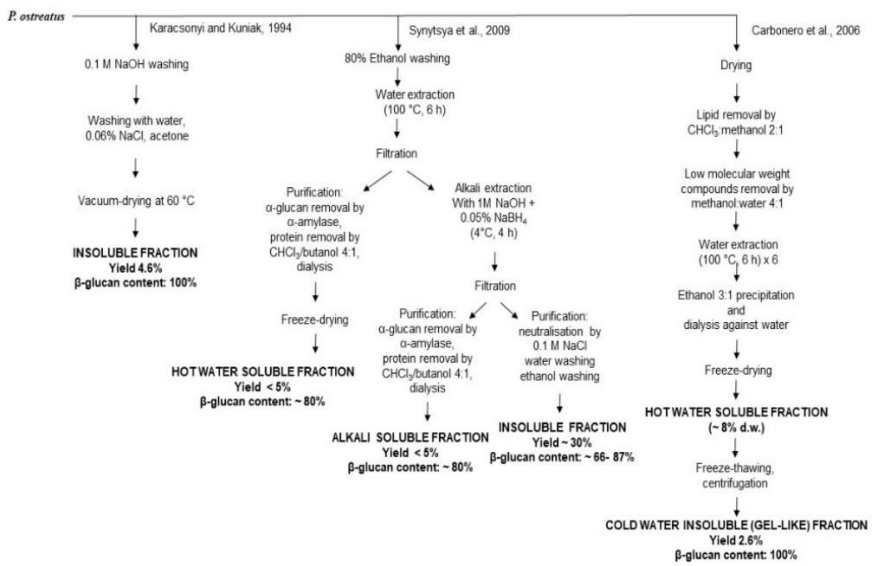
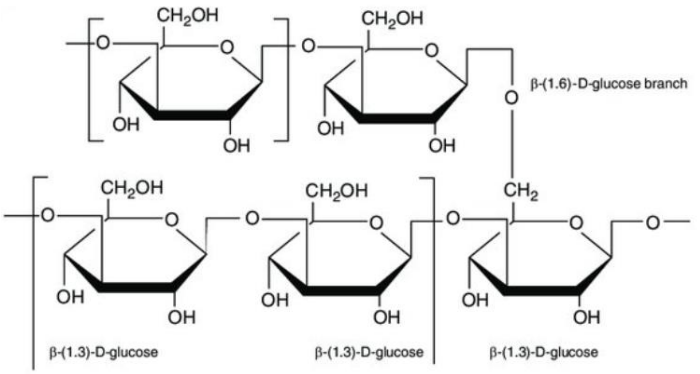


Figure 3. Examples of processes to obtain concentrated and purified β-glucan-rich fractions from *P. ostreatus*. Modified from Lavelli et al., 2018.

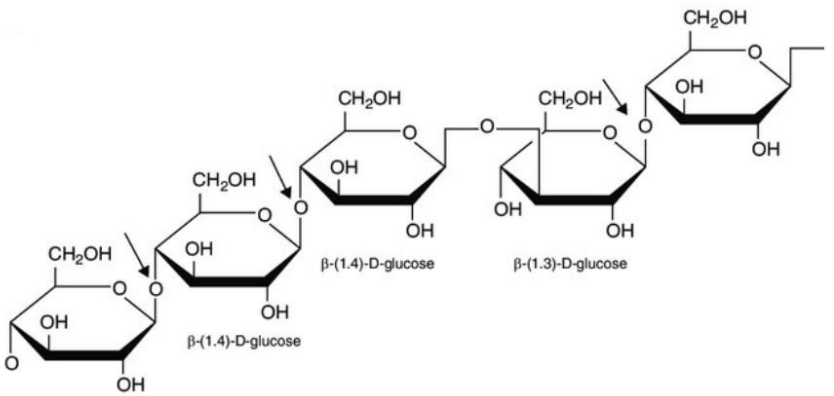
There is a growing number of studies on the use of insoluble β-glucans of *P. ostreatus* (named pleuran) due to their numerous biological activities such as antitumor, antioxidant, antiallergic, prebiotic, immunomodulatory,

hepatoprotection and gastroprotection properties (Huang and Nie, 2015; Lavelli et al., 2018). Moreover, they have enormous potential in a wide variety of fields due to their gel-forming capacity.

In cereals, these polysaccharides have a different molecular structure, with a backbone of D-glucose-linked β-(1→3) with β-(1→4) branches (Fig. 4). In the recent years, many authors have investigated their properties as encapsulation carriers for delivery-controlled release of probiotics and nutraceuticals (Lazaridou et al., 2015; Salgado et al., 2015). However, there is a lack of information about the same properties of mushrooms' β-glucans.



Structure of (1→3)β-glucans with ramifications β(1→6)



Structure of (1→3)β-glucans with ramifications β(1→4)

Figure 4. Structure of mushrooms and cereals' β-glucans (Abuajah, 2017).

The usage of carriers fabricated by polymeric constituents with inherent bioactivity, such as *P. ostreatus* β-glucans, can enhance the nutritional value of the pharmaceutical/nutraceutical preparations or the food matrix in which these materials would be incorporated.

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CHAPTER 2

AIMS and OBJECTIVES

The overall aim of this PhD thesis was to develop functional ingredients from *P. ostreatus* biomass. Specific objectives were:

a) development of a food application and production of a powdered ingredient with a target amount of vitamin D_2. The dehydrated fungal biomass was used as ingredient for a model functional food (breadsticks), designed for children as target population with vitamin D_2 deficiency. To select which formulation had the highest potential liking by consumers, the breadsticks were assessed by consumers' preference studies. The level of vitamin D_2 in *P. ostreatus* powder was enhanced through UV irradiation from its precursor ergosterol. The kinetics of vitamin D_2 degradation was studied as a function of water activity and temperature to predict its stability during storage. The effect of UV irradiation on antioxidant and antiglycation activities was investigated. This research has been conducted at University of Milan (DeFENS).

b) encapsulation of an oxidisable target using *P. ostreatus* β-glucans as antioxidant and emulsifier agent. Extraction of β-glucans with a "food-grade" procedure was optimized. The different extracts obtained were used in the oil-in-water emulsion preparation; emulsion stability was checked over time. The encapsulation technique was carried out using a pilot scale spray dryer and the powders so obtained were characterized. The oxidizable target content (α-tocopherol) and the oxidation primary product (conjugated dienes) were measured after powder production and during accelerated storage. The stability of encapsulated α-tocopherol was also tested during *in vitro* digestion. This research has been conducted both at University of Milan (DeFENS) and at Université Paris-Saclay (UMR SayFood).

CHAPTER 3

RESULTS and DISCUSSION

Chapter 3a

Development of a food application and production of a powdered ingredient with a target amount of vitamin D_2

nutrients

MDPI

Article

Effect of Vitamin D_2 Fortification Using *Pleurotus ostreatus* in a Whole-Grain Cereal Product on Child Acceptability

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ABSTRACT

Vitamin D_2 deficiency is one of the most common micronutrient insufficiencies among children. Few foods, mainly from animal sources, naturally contain this vitamin. The basidiomycete mushroom *Pleurotus ostreatus* could be used as an innovative and sustainable ingredient for food fortification with vitamin D_2. This study was aimed at exploring children's acceptance of a whole cereal-based product (breadsticks) added with increasing concentrations of *P. ostreatus* powder rich in vitamin D_2. The food neophobic traits (fear to try unfamiliar and new food) on samples acceptability was also investigated. One hundred and three children (47 girls and 56 boys, aged 9-11 years) were recruited and breadsticks liking was studied in relation to gender and neophobic traits. Results showed that the samples enriched in vitamin D_2 were well accepted by the children even if liking decreased with increasing concentration of mushroom powder. Generally, neophilic subjects gave higher liking scores compared with the neophobic ones, especially for the modified samples. New well accepted fortified products could be developed using an adequate concentration of mushroom powder to deal with the increasing vitamin D_2 deficiency among children.

1. Introduction

Vitamin D deficiency is recognized to be a worldwide problem [1], which has raised the need to develop new dietary strategies to increase its intake. Vitamin D has a well-known role related to calcium absorption and homeostasis, bone mineralization and bone health. Additionally, a nuclear receptor for the bioactive form of vitamin D (i.e., $1,25(OH)_2D$) is present in at least 38 human tissues and organs [2] and it is probably related to the risk-reduction potential of vitamin D towards other diseases, including psoriasis, multiple sclerosis, inflammatory bowel disease, type 1 and 2 diabetes, hypertension, cardiovascular disease, metabolic syndrome and various cancers [3].

Based on these considerations, it is crucial to maintain, both by sunlight exposure and by dietary intake [4], an adequate vitamin D status. Indeed, sunlight exposure that leads to the endogenous production of this vitamin is not sufficient to reach the recommended vitamin D daily intake, especially in specific populations, such as African Americans with dark skin pigmentation that decreases UVB exposure, as well as elderly people with a low level of vitamin D_3 precursor [1]. In both adults and children more than one year old, the recommended vitamin D intake is 15 µg/day [5]. Since few foods are a source of this vitamin, food enrichment is considered a useful strategy to achieve this recommendation. In most studies, the prevalent form of vitamin D used for food enrichment was vitamin D_3, which is found only in animal sources such as fish oil and liver. However, vitamin D_2, which mainly comes from mushrooms, can provide a more sustainable alternative, considering the low environmental impact of mushroom production. Among mushrooms belonging to basidiomycete phyla, the *Pleurotus* genus is recognized as having an interesting nutritional profile due to its valuable essential amino acid scoring pattern and high β-glucan content, as well as important micronutrients concentrations. This mushroom presents a high level of some vitamins of the B group and vitamin D_2—the latter up to 5.93 µg/g in the *Pleurotus ostreatus*

species [6]. Thus, *P. ostreatus* appears to be an interesting natural source of vitamin D_2 for specific consumer groups, including vegetarians, vegans, and people intolerant to lactose, since most of the fortified products with vitamin D_2 available on the market are dairy-based products. Moreover, *P. ostreatus* is able to grow efficiently on low-cost substrates such as lignocellulosic agri-food byproducts, leading to the possible production of sustainable food ingredients [7–9].

However, mushroom powder addition to foods elicits sensory attributes that could potentially affect product acceptability [7]. It has been highlighted that liking of vegetable soups where *P. ostreatus* powder was added was affected in adult subjects. In particular, consumers' liking decreased as the concentration of mushroom powder increased [8]. Other findings revealed that unfortified wheat pasta obtained the lowest liking scores, while acceptability increased with the addition of a low amount of the insoluble β-glucan fraction from *P. ostreatus* powder [10]. It has recently been shown that a cereal-based product (flatbread) enriched with *P. ostreatus* powder obtained higher liking scores compared with the control sample without mushroom addition in a group of adolescents [9]. Thus, it is clear that developing new food formulations is challenging considering that food perception and food-liking—one of the main drivers of food consumption—differs greatly among individuals. Moreover, developing food products with optimized nutritional and sensory characteristics is of crucial importance, especially for specific target populations such as children, who show well-known neophobic reactions [11]. Food neophobia, defined as the reluctance to eat unfamiliar foods [12], is considered a negative eating behavior because it can decrease diet variety and reduce daily fruit and vegetable intake [13]. This behavior also seems to be related to the nutritional status of subjects, considering neophobic individuals may be less willing to try healthy alternative versions of familiar products, leading to weight gain [14].

The aim of the present study was to investigate children's acceptance of a product based on whole-wheat cereal supplemented with increasing

concentrations of *P. ostreatus* powder rich in vitamin D_2. The influence of food neophobic traits on sample acceptability was also investigated.

Breadsticks were chosen as the enriched food prototype, since they could be easily consumed as snacks by children during the day, and because cereal-based products are generally well accepted by this specific target population [15,16]. Moreover, since breadsticks were made with whole-wheat flour, they could also be used to meet the recommended intake of whole-grain foods, considering the reported low consumption in children [17].

2. Materials and Methods

2.1. Participants

One hundred and three children (47 girls and 56 boys, aged 9–11 years) were recruited in the Milan area (Italy) via primary schools. Parents received full information about the research study and provided written informed consent for their children's participation. Children without a signed informed consent were not involved in the study. Children who suffered from food allergies or did not like the tested samples, which could negatively affect hedonic responses, performed alternative activities so that they did not feel excluded from the test activities. The study was performed in adherence with the principles established by the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the University of Milan (protocol n°19/18).

2.2. Stimuli

A commercial strain of *P. ostreatus* deposited at the mycotheca of the Società Agricola IoBoscoVivo (Vergiate, Varese, Italy) was used. This strain was cultivated on wood of *Populus tremula* in a greenhouse at room temperature. After one year of incubation, about 10 kg of body fruits were collected, cut into 4-mm slices, spread on an oven rack in a single layer, and air-dried at 37 °C for 48 h. At the end of the process, about 1 kg of dried mushroom was obtained and all the product was ground to a fine powder using a Thermomix TM 31 (Vorwerk Contempora S.r.l., Italy). The production of breadsticks added with

P. ostreatus was performed at the Società Agricola IoBoscoVivo (Vergiate, Varese, Italy), according to the addition levels decided on by the sensory group at the University of Milan, based on preliminary trials. The breadstick ingredients were whole-wheat flour, sunflower oil, yeast, and salt. The experimental samples were prepared by adding different increasing concentrations of *P. ostreatus* powder (B_2 = 2%, B_4 = 4%, and B_6 = 6%) to the standard formulation (B_0). A hidden control of the unmodified sample (B_{hc} = 0%) was also included to verify that children were able to assign a comparable score to the two samples without mushroom powder addition (B_0 and B_{hc}).

The nutritional composition and the energy content of the developed samples, which were provided by Società Agricola IoBoscoVivo (Vergiate, Varese, Italy), are reported in Table 1.

Table 1. Nutritional values (g per 100 g dry weight) and energy content (kcal per 100 g) of the developed breadsticks.

Nutritional values	**Samples**			
	B_0	**B_2**	**B_4**	**B_6**
Energy	390 kcal	384 kcal	372 kcal	372 kcal
Fat	11.7 g	11.7 g	11.6 g	11.6 g
Saturated fat	1.3 g	1.3 g	1.2 g	1.25 g
Carbohydrates	64.6 g	64.5 g	64.2 g	64.2 g
Proteins	12.2 g	12.6 g	13.3 g	13.3 g
Fiber	11 g	11 g	11.5 g	11.5 g
Salt	0.4 g	0.4 g	0.4 g	0.4 g

Approximately 30 g of each sample were presented at room temperature to the children in plastic bags labelled with three-digit codes. Water was available for rinsing the palate.

2.3. Determination of Vitamin D_2 Content

Vitamin D_2 was extracted according to the procedure of Sławinska and collaborators [18] and identified by the HPLC procedure by Huang and colleagues [6]. Analysis was performed on breadsticks enriched with 2%, 4%, and 6% of *P. ostreatus* powder and on the control breadstick after processing

and on the breadstick enriched with 6% of *P. ostreatus* after storage at room temperature for six months.

2.4. Experimental Procedure

The liking evaluation was performed at school in the classroom in the presence of a teacher and an experimenter in a single day. During the evaluation, each child was seated at their own table and received the questionnaire, which was extensively explained by the experimenters. The questionnaire consisted of an evaluation of food neophobia followed by the evaluation of breadstick liking. The questionnaire was self-completed by the children. To increase ecological validity, the breadsticks were offered as a snack and liking was assessed during the mid-morning break. Each of the five formulations, coded with three-digit numbers, were randomly presented to each child in blind condition. Children were instructed to drink a sip of water between each sample. The experimenters monitored the children to ensure that they did not influence each other during the evaluation. The session took approximately 30 min and children received a small award (e.g., pencil case) for their participation.

2.5. Food Neophobia Assessment

The Italian Children Food Neophobia Scale (ICFNS), previously validated by Laureati and collaborators [11] with a large sample of school-aged children, was used to investigate children's neophobic traits. The ICFNS consists of eight items, and it is a simplification of the original Food Neophobia Scale (FNS) of Pliner and Hobden [12] composed of 10 items. Each item offers five graded responses by a facial expression, in order to help the child better understand the level of agreement or disagreement for each item alternatives, from "very false for me" (score 1) to "very true for me" (score 5). Half of the items represent neophobic whereas the other half represent neophilic food situations. Thus, responses to neophilic statements were reversed when calculating the score. The ICFNS score was calculated as a sum of the responses, yielding a range of 8–40.

2.6. Liking Assessment

Children were asked to taste the products and to express their liking scores using a seven-point facial hedonic scale from “super good” (score 7) to “super bad” (score 1) [19] which has been proven to be a reliable tool to be used with young consumers [20]. The experimenters provided instructions for the use of the scale prior to tasting and during the session.

2.7. Data Analysis

Data on vitamin D_2 content were analyzed by one-way ANOVA using the least significant difference (LSD) as a multiple range test. Results are reported as average ± standard error (SE).

A preliminary linear mixed model procedure was carried out on overall liking data considering samples (B0, B2, B4, B6, and Bhc), gender (female and male), and their interactions as fixed factors. Age was added to the model as a covariate. Considering that this first analysis showed that the liking scores of samples B0 and the control (Bhc) were not statistically different, Bhc was not considered in the subsequent analysis. Another model was constructed following the previous approach on food neophobia data.

To determine the influence of food neophobia on liking scores, a linear mixed model was carried out with liking data as the dependent factor, and food neophobia levels (low, medium, high), sample, gender, and their interactions as fixed factors. Subjects were considered as random factors in all the analyses. When a significant difference ($p < 0.05$) was found, the LSD post hoc test was performed as a multiple comparison test. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Vitamin D_2 Content and Product Design

The vitamin D_2 contents of the developed breadsticks are reported in Table 2.

As shown in Table 2, the addition of *P. ostreatus* from 2% to 6% significantly increased the level of vitamin D_2 from not detectable to 28.4 µg in 100 g. Breadsticks enriched with 2%, 4%, and 6% can provide 32%, 54%, and 95% of the recommended daily amount of vitamin D_2 per single dose (50 g). The 6% enriched breadsticks were also analyzed after being stored for six months at room temperature and found to have the same vitamin D_2 content as just after production. According to the USDA database, milk and orange juice fortified with vitamin D provide 30% of the Recommended Dietary Allowance (RDA), while 20% and 10% of RDA is provided by fortified yogurt and cereal breakfast, respectively (https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/#h3). Hence, all the enriched breadsticks could be of interest to improve the dietary intake of vitamin D.

Table 2. Vitamin D_2 content of breadsticks enriched with *Pleurotus ostreatus*.

Samples	µg in 100 g	µg per dose (50 g)	% daily dose
B_0	N.d.		
B_2	$9.5^a \pm 0.3$	4.8	32
B_4	$16.3^b \pm 0.2$	8.2	54
B_6	$28.4^c \pm 1.6$	14.2	95
B_6, six months stored	$27.1^c \pm 0.9$	14.2	95

N.d. = not detectable. Values with different superscripts in column are significantly different (LSD, $p < 0.05$).

3.2. Liking Assessment

A significant sample effect was found on liking scores ($F_{(4; 404)} = 12.38$, $p < 0.0001$). As reported in Figure 1, B0 and Bhc samples obtained comparable scores (MB0 = 5.5 ± 0.2; MBhc = 5.3 ± 0.2). Children equally liked these samples, which were significantly preferred to samples with increasing added concentrations of *P. ostreatus* powder. Samples enriched with mushroom powder at 2% and 4% obtained comparable liking scores to each other (MB2 = 4.9 ± 0.2; MB4 = 4.9 ± 0.1) while the sample with the highest concentration of *P. ostreatus* powder received the lowest liking scores (MB6 4.3 ± 0.2). However, all the vitamin D_2-enriched samples were well accepted by the children (liking scores were still up the middle of the scale).

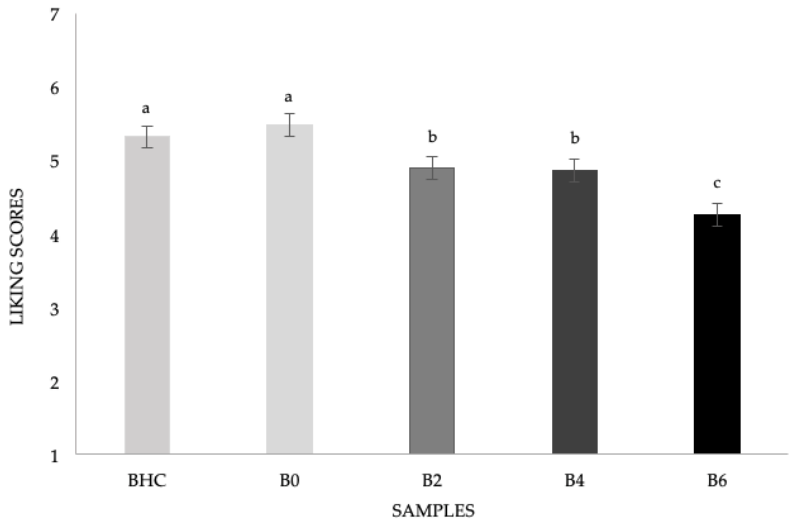

Figure 1. Mean liking scores ± SEM by samples. Different letters indicate significant differences according to post hoc test. Bhc and B0 = 0%; B2 = 2%; B4 = 4%; and B6 = 6% of mushroom powder.

The main factor "gender" and the interaction "sample*gender" were not significant ($F_{(1;141)}$ = 0.01, p = 0.9; $F_{(4;410)}$ = 0.9, p = 0.5, respectively).

Satisfactory internal consistency, as calculated through Cronbach's alpha test (alpha = 0.75), was observed among the ICFNS items. The mean food neophobia value of the children was 19.5 ± 0.3. No significant differences in neophobic traits were found between girls and boys ($F_{(1;101)}$ = 0.42, p = 0.52). To investigate the relationship between food neophobic traits and breadstick formulation liking, children were categorized according to their neophobia scores into the following three groups, after verifying that data were normally distributed [11]: children with scores in the lower 25th percentile of ICFNS scores, score < 16, n = 23 (LOW_FN); children with scores between the 25th and 75th percentiles, 16 ≤ ICFNS score ≤ 23, n = 59 (MEDIUM_FN); and children with scores in the upper 25th percentile, ICFNS score > 23, n = 21 (HIGH_FN). A significant effect of food neophobia on liking scores was found ($F_{(2;98)}$ = 3.32, p = 0.04). As reported in Figure 2, children categorized by LOW_FN gave generally significantly higher liking scores (M = 5.3 ± 0.2) compared with MEDIUM_FN (M = 4.7 ± 0.1) and HIGH_FN (M = 4.9 ± 0.2).

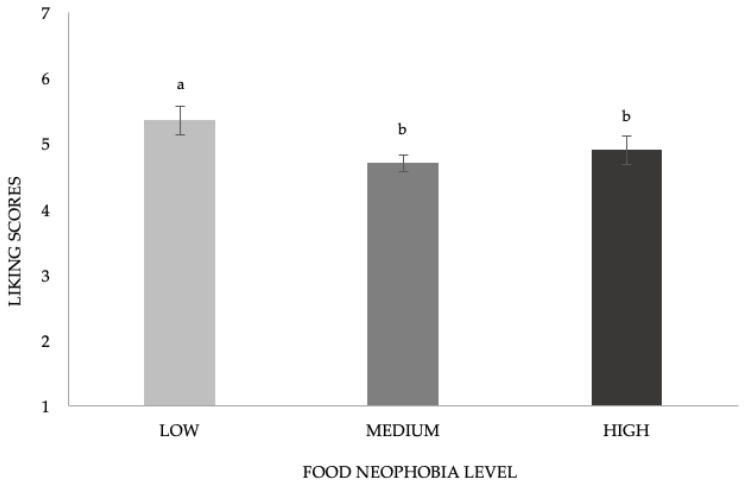

Figure 2. Mean liking scores ± SEM according to food neophobia levels. Different letters indicate significant differences according to post hoc test.

In Figure 3 the results of the interaction “sample*food neophobia levels”, which was also significant ($F_{(6;291)}$ = 2.33, p = 0.03), are reported.

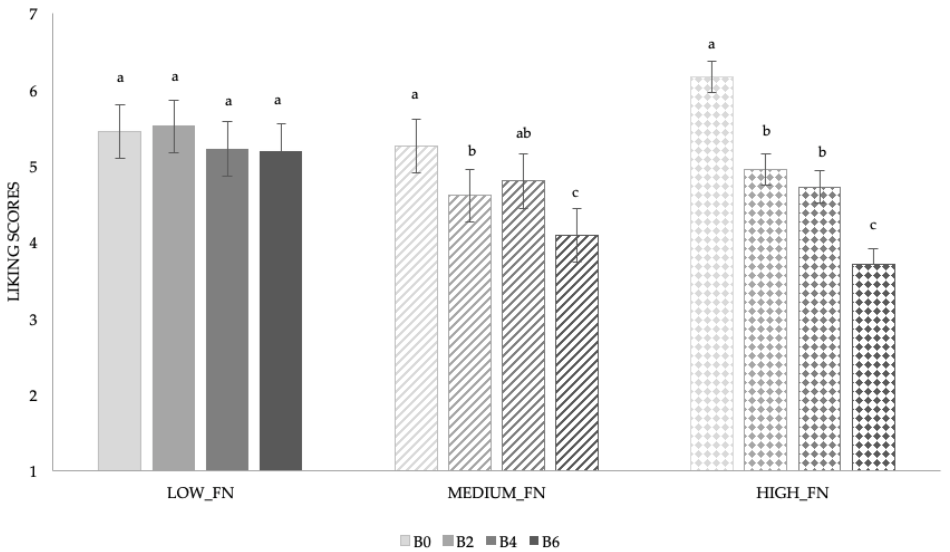

Figure 3. Mean liking scores _ SEM by samples according to food neophobia levels. Different letters indicate significant differences according to post hoc test for each sample. B0 = 0%; B2 = 2%; B4 = 4%; and B6 = 6% of mushroom powder.

Looking at liking scores obtained by the children categorized according to their food neophobia level, no differences were found in sample liking scores in the LOW_FN group. Interestingly, results obtained by the HIGH_FN children showed that they gave significantly lower liking scores to sample B2 (M = 4.9 ± 0.3) and B4 (M = 4.7 ± 0.4), which were comparable to each other, than the unmodified sample (MB0 = 6.2 ± 0.2). Neophobic children gave the lowest liking rating (M = 3.7 ± 0.3) to the sample with the highest concentration of mushroom sample. A similar trend was highlighted for the MEDIUM_FN children.

4. Discussion

The recommended intake of vitamin D has been defined as 15 µg/day [5]. There is no general consensus as to whether the mushroom source D_2 is equally as effective as the animal source D_3.

Indeed, some studies have demonstrated that vitamin D_3 is more efficient than vitamin D_2 [21], while other studies have shown that vitamin D_2 and vitamin D_3 treatment yield equivalent outcomes in the treatment of hypovitaminosis D among young children [22]. What can be stated is that plant sources of vitamin D_2 are more sustainable than D_3 sources of animal origin [23].

Aside from the different sources of vitamin D_2 and D_3, issues regarding compliance with the dietary regimen have also been highlighted. For instance, in a study by Johnson and colleagues [24], cheese was fortified with 17.6 µg of vitamin D_3/100 g showing an effect in increasing vitamin D status. However, there were five dropouts due to gastrointestinal problems, a dislike for the saltiness of the cheese, and medical advice due to the relatively high levels of salt and saturated fat provided by the portion of fortified cheese (85 g) to be ingested. In a study by Daly and collaborators [25], milk fortified by 5.0 µg of vitamin D_3/100 g caused some dropouts due to the large volume of milk (400 mL/day) required to be consumed.

In the present study, whole-grain breadsticks were used as a model food to be enriched with vitamin D_2 from *P. ostreatus*. This represents a sustainable

vitamin source because it can grow efficiently on various clean by-products of food processing and has low production costs. Therefore, this mushroom represents a value-added ingredient for food fortification. The breadsticks developed in this study could also be conveniently sized in the right amount and provide a significant percentage of the recommended daily level of both vitamin D and dietary fiber, since these products could be easily consumed as snacks during the day.

The liking results of the present study demonstrated that even if the acceptability decreased with increasing concentration of mushroom powder, all the samples were clearly well accepted by the children. Thus, the developed food could be useful to integrate the vitamin D_2 daily intake, which depends on clinical and environmental factors such as latitude of residence, level of exposure to the sun, and dietary practices [26]. In this context, consuming fortified food could be a complementary strategy to sunlight exposure and the ingestion of some food that naturally contains vitamin D_2 to meet recommended vitamin daily intake. Different types of fortified food have already been developed, such as fortified milk, cheese, soy drink, and fish [27]. However, these mentioned products might not be suitable for subjects who are intolerant to lactose or for who consume low amounts of fish, such as children [28]. The low fish consumption reported among children could be associated with behavioral factors such as food neophobia, which shape the development of fish disliking [29]. Thus, it is important to develop new foods enriched in vitamin D_2 considering children's food preferences, habits, food intolerance, and neophobic traits.

The present results demonstrate that children characterized by a high food neophobia score were less prone to accept the enriched samples, especially with regard to the breadsticks with the highest amount of mushroom powder. Although low liking ratings were highlighted for this product, acceptability could be increased by enhancing its familiarity through repeated exposure. Indeed, it is widely recognized that repeated exposure to a specific food increases the liking and consumption of that food due to a "learned safety" behavior [30,31].

Thus, the repeated consumption of an unfamiliar food without undesirable consequences leads to increased acceptance of that food. Neophilic children, and partially children with medium levels of neophobia, showed acceptance of all the developed breadstick samples, even the one with the higher amount of mushroom powder.

Some limitations of the study should be mentioned. The present results provide evidence of product acceptability, but no suggestions about which sensory characteristics are related to hedonic perceptions. This information could be useful during product development in order to optimize the sensory quality. Thus, a future perspective of study could be to consider a sensory description of fortified model foods enriched with *P. ostreatus* powder in order to identify drivers of liking and disliking of these products. Moreover, it could be interesting to add the mushroom powder in other food products and investigate consumers' responses in other specific target populations, such as elderly people. Indeed, it has been widely reported that elderly subjects show a direct relation between vitamin D levels and frailty syndrome as well as an inverse association between vitamin D levels and the risk of oral, gastrointestinal, urinary, ocular, and respiratory infections [32]. Finally, even if samples were provided to the children in blind condition and in a randomized order, there could be other factors such as social determinants as well as the children's natural preference toward the snack (breadsticks) that also might influence their responses.

In conclusion, the findings of the present study suggest that it is feasible to develop new food formulations using an adequate concentration of mushroom powder rich in vitamin D_2 that could also satisfy children's preferences. The developed fortified foods could be considered more suitable to prevent vitamin D_2 deficiency in children than the fortified products already available on the market. Indeed, the developed breadsticks could potentially be launch on the market as a healthy snack since they received a positive hedonic response by the young consumers involved.

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Kinetic study of vitamin D_2 degradation in mushroom powder to improve its applications in fortified foods

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ABSTRACT

The aim of this study was to develop a value chain approach to address the need for sustainable vitamin D_2 food sources. A combined process of UV-irradiation and air-drying was used to produce a *Pleurotus ostreatus* powder with a target amount of vitamin D_2 as an ingredient for functional foods. The kinetics of vitamin D_2 degradation was studied as a function of water activity (a_w) in the range 0.11–0.75 at 30 °C, and temperature in the range 20–40 °C, at aw 0.32. The Guggenheim–Anderson–de Boer equation was applied to model experimental data of moisture content as a function of aw at 30 °C, and the resulting monolayer water activity was found to correspond to the a_w level of 0.45. During storage under different environmental conditions, vitamin D_2 degradation followed first-order kinetics. The rate constants ranged from 0.00308 to 0.0121 d−1 at aw 0.11 and 0.75 at 30 °C, respectively. The Arrhenius equation was applied to calculate the activation energy for vitamin D_2 degradation at a_w 0.32, which resulted 37.6 kJ/mol. The kinetics data obtained can be used as a basis to design the best formulation strategy to deliver this vitamin with functional foods.

1. Industrial relevance

The production of vitamin D_2 through mushroom irradiation is a sustainable strategy to address vitamin D deficiency, which is recognized as a worldwide problem. *Pleurotus ostreatus* is a basidiomycete mushroom that can grow efficiently in different climates on low-cost substrates, including waste from the agri-food system. In this study, a combined UV-B irradiation and air-drying process was applied on *P. ostreatus* to produce vitamin D_2. There is no information regarding vitamin D_2 stability in irradiated mushroom powder, as a function of temperature and environmental humidity. Nevertheless, high environmental humidity combined with high temperature is the main cause for loss of food commodities, especially in the developing countries. This study provides the kinetic parameters necessary to predict vitamin D_2 stability during storage as a function of water activity and temperature conditions.

Main chemical compounds studied in this article:

- Vitamin D_2, PubChem CID: 6449838
- Ergosterol, PubChem CID: 5280793

2. Introduction

Among malnutrition concerns, vitamin D deficiency is recognized as worldwide problem. The major sources of vitamin D are sunlight exposure and intake by foods. Upon sunlight exposure, the vitamin D_3 precursor in the skin is converted in previtamin D_3, which is then metabolized in the liver and in the kidney to give the biologically active form of vitamin D, i.e., 1,25-dihydroxy vitamin D. Alternatively, a few foods of animal origin, especially fish oils, are sources of vitamin D_3 while yeasts and mushrooms are sources of vitamin D_2 (Misra, Pacaud, Petryk, Ferrez Collett-Solberg, & Kappy, 2008). Populations at risk of vitamin D deficiency are: African Americans, who live in a temperate climate due to dark skin pigmentation that decreases UV-B exposure; elderly people because of a diminished level of vitamin D_3 precursor and hence decreased endogenous formation; people affected by some diseases due to

malabsorption and decrease efficiency of conversion from vitamin D precursors; obese people due to the sequestration of vitamin D by the large body fat pool. Vitamin D deficiency causes the well-known skeletal consequences such as rickets in children and osteoporosis, osteopenia and fractures in adults. Moreover, it has been associated with increased risk of common cancers, autoimmune diseases, hypertension, and infectious diseases (Holick & Chen, 2008).

Throughout Europe, surveys on vitamin D intake have shown that for 77-100% of adults (19–64 years old) and for 55–100% of elderly adults (> 64 y) the intake of vitamin D is inadequate (Roman Viñas et al., 2011). Vitamin D intakes from food are higher in the USA than in Europe (Moore, Radcliffe, & Liu, 2013), which could depend on higher availability of foods fortified with vitamin D in the USA than in Europe (Milešević, Samaniego, Kiely, Glibetić, Roe, & Finglas, 2018).

Indeed, food fortification with vitamin D is considered a fundamental strategy to combat vitamin D malnutrition and its associated diseases (Holick & Chen, 2008; O'Mahony, Stepien, Gibney, Nugent, & Brennan, 2011; Milešević et al., 2018). Some in vivo studies have shown that vitamin D_2 and D_3 can be considered equally bioavailable, while from other studies vitamin D_3 resulted to be more efficient than vitamin D_2 (Shieh et al., 2016). However, the comparison is complicated by the multiplicity of variables that affect the outcome of in vivo studies, such as the variance in age groups studied, the doses administered, the duration of supplementation (O'Mahony et al., 2011). Indeed, food fortification strategies designed to address the pandemic vitamin D deficiency have considered the use of both vitamin D_2 and vitamin D_3 (Milešević et al., 2018; O'Mahony et al., 2011). A sustainable solution for vitamin D production can arise from the recovery of food waste, thus closing the cycle of a worthy food supply chain. Indeed, accumulation of food waste in the environment is a practice that could not be continued for a long time within the sustainability and bioeconomy framework of the modern food industry (Galanakis, 2015). In this context, the basidiomycetes *Pleurotus* spp. can play

a positive role as they are able to grow on various agri-food waste and their cultivation requires low capital investment and low technical skills (Lavelli, Proserpio, Gallotti, Laureati, & Pagliarini, 2018). In general, mushrooms have low natural levels of vitamin D_2, but high levels of its precursor ergosterol, from which this vitamin can be generated through irradiation of either the fresh or the dried fruit body, particularly using UV-B (Nolle, Argyropoulos, Ambacher, Müller, & Biesalski, 2017; Taofiq, Fernandes, Barrosa, Barreiro, & Ferreira, 2017).

A detailed information is available on vitamin D_3 degradation, which occurs via light-, oxygen- and acid-induced isomerization and oxidation (Mahmoodani, Perera, Fedrizzi, Abernethy, & Chen, 2017). These latter reactions do not involve the side chain that differs between vitamin D_2 and D_3 and hence they most likely occur in vitamin D_2 as well. Soybean oil was used as a model matrix to study the influence of both light- and oxygen-exposure on vitamin D_3 degradation. In this approach, soybean oils with different oxidative status and vitamin E contents were stored in the dark, semi-dark, or exposed to natural light; the determining factors of vitamin D_3 losses were (in decreasing order) the storage time, the exposure to light and the oxidative status of the oil, whereas vitamin E content had a protective role (Hemery et al., 2015). A bread model provided evidence of the effect of acid compounds on vitamin D_2 degradation. In fact, in rye and wheat bread fortified with vitamin D_2 from yeast, the retention after baking was 73% and 89%, respectively. Rye bread has a lower pH than wheat bread, which was the explanation for the lower retention due to the known acidic isomerization of vitamin D to isotachysterol (Jakobsen & Knuthsen, 2014).

Air drying of irradiated *P. ostreatus* has no effect on vitamin D_2 content, while after 1.5 year storage of the powder (moisture content not specified) at 20 °C the level of vitamin D_2 was found to decrease to 66.90%, but final moisture was not specified (Slawinska et al., 2016). However, the final level of moisture achieved after heat-drying depends on ambient humidity and has a fundamental effect on product stability. Indeed, the high ambient humidity

prevalent in the humid climates of developing countries is recognized as the main cause of loss of dried commodities during storage (Bradford et al., 2018), since products in contact with an atmosphere of a given relative humidity will reach the equilibrium water activity (a_w), which could correspond to values above 0.6, were the growth of microorganism is possible. Moreover, even at a_w levels below 0.6 were microbial growth is arrested, various chemical reactions can occur (Bell, 2007). To implement vitamin D_2 enriched foods in the perspective of a value chain approach, long-storage stability is crucial.
Hence, in this study, an ingredient based on *P. ostreatus* powder enriched in vitamin D_2 was obtained and studied during storage in the dark, at various a_w levels and temperatures with the ultimate aim to develop a predictive model for vitamin D_2 stability.

3. Material and methods

3.1. Chemicals

Standards and chemicals were purchased from Sigma Aldrich (Milan, Italy).

3.2. Mushrooms

For the irradiation study, a commercial strain of *P. ostreatus* deposited at the mycotheca of the Società Agricola IoBoscoVivo (Vergiate, Varese, Italy) was used. This strain was cultivated on wood of *Populus tremula* in a green-house at room temperature. After 1 year of incubation, mushrooms were collected for the irradiation process carried out as described previously (Lavelli et al., 2019). In brief, about 10 kg of body fruits were collected, cut into 4 mm slices, spread on an oven rack in a single layer and air-dried at 37 °C for 48 h. During the first 24 h of air-drying, the mushrooms were irradiated using two fluorescent lamps installed at 30 cm above the mushroom layer. The lamps delivered an UV-B (280–315 nm) irradiance of 0.4 W/m2. At the end of the process, about 1 kg of dried mushroom was obtained and all the product was grinded to a fine powder using a Thermomix TM 31 (Vorwerk Contempora S.r.l., Italy).

3.3. Storage study

Mushroom powders were weighed into Petri dishes (6 cm diameter, 5.5 g of product in each dish). The dishes were placed inside airtight plastic chambers on wire-mesh racks situated above saturated salt solutions. To create different relative humidity environments, the following saturated salt solutions were used: LiCl (a_w = 0.113 ± 0.002), CH_3COOK (a_w=0.216 ± 0.005), $MgCl_2$ (a_w=0.324 ± 0.002), NaBr (a_w = 0.560 ± 0.004), and NaCl (a_w = 0.7509 ± 0.0011). The chambers at a_w levels 0.11–0.75 were stored for 150 d at 30 °C. Storage at 0.75 was stopped at 36 d because mold growth was observed. The powders stored at the a_w level of 0.32 were also stored at 20 and 40 °C. Samples were extracted in triplicate at time zero and in duplicate at following time intervals as described in Section 3.5 and analysed for vitamin D_2 content. At each aw level and temperature, vitamin D_2 decrease was analysed according to a first-order kinetics, as follows:

$$\ln(A) = \ln(A_0) - \int_0^t (dt * k) \quad (1)$$

where A_0 is the initial content, A is the content at time t, t is the storage time (d) and k is the rate constant (d^{-1}).

The effect of temperature on vitamin D_2 degradation was investigated by applying the Arrhenius equation:

$$\ln(k_t) - \ln(k_0) = -Ea * R^{-1} * T^{-1} \quad (2)$$

where T is the storage temperature (K), k_T is the rate constant at the temperature T (d^{-1}), Ea is the activation energy ($J*mol^{-1}$), R is the universal gas constant (8.314 $J*mol^{-1}*K^{-1}$).

3.4. Moisture content and a_w

Moisture content of mushroom powders after equilibration at the various aw levels was determined using a vacuum oven at 70 °C and 50 Torr for 18 h (AOAC, 1990). The a_w of samples and saturated salt solutions was checked using a dew point hygrometer (Aqualab, Decagon Devices, Pullman, WA, USA). Duplicate determinations were made for each sample. Moisture isotherms were developed by plotting the equilibrium moisture content (M)

versus the storage a_w. The Guggenheim- Anderson-de Boer (GAB) equation was used to fit the experimental data:

$$M = \frac{M_0 C K a_w}{(1-Ka_w)(1-Ka_w+CKa_w)} \quad (3)$$

where M is the equilibrium moisture content on a dry basis (g of water/ g of dry solids); M_0 is the monolayer moisture content on a dry basis; C and K are constants (Nurhadi, Roos, & Maidannyk, 2016).

3.5. Determination of vitamin D_2

Free and bound vitamin D_2 and ergosterol were extracted from not irradiated and irradiated powders without saponification (Huang, Lin, & Tsai, 2015) and after saponification (Slawinska et al., 2016), respectively. For the storage study, only the procedure with saponification was performed (Fig. 1). In brief, for evaluation of free vitamin D_2 and ergosterol, mushroom powder (5 g) was mixed with 10 mL of dimethyl sulfoxide and ultrasound-oscillated at 45 °C for 30 min. Then 10 mL of methanol and water (1:1, v/v) and 20 mL of n-hexane were added and the mixture was ultrasound-oscillated at 45 °C for 30 min and centrifuged at 3000×g for 10 min. The residue was extracted twice with 20 mL of n-hexane and centrifuged. The combined extracts were rotary evaporated at 40 °C to dryness, redissolved in 1 mL of methanol: water (95:5, v/v) and filtered using a 0.45-mm polyvinylidene difluouride (PVDF) membrane prior to HPLC analysis. For the evaluation of bound vitamin D_2 and ergosterol, mushroom powder (2 g) was weighed into a 250 mL flask and mixed with 1 g of L-ascorbic acid, 50 mL of 96% ethanol and 25 mL of 50% potassium hydroxide. The mixture was heated at 85 °C for 30 min and then cooled to ambient temperature and extracted twice with 10 mL of deionized water and 30 mL of n-hexane in a separating funnel. The pooled organic phase was washed three times with deionized water until it was neutralized. Next, the organic phase with added 2 mL of butylated hydroxytoluene (BHT) solution (1 mg/mL in n-hexane) was poured in a flask and rotary evaporated to dryness at 40 °C. The dried extract was recovered by washing the flask with 5 mL of

methanol: water (95:5, v/v) twice and filtered using a 0.45-μm PVDF membrane prior to HPLC analysis.

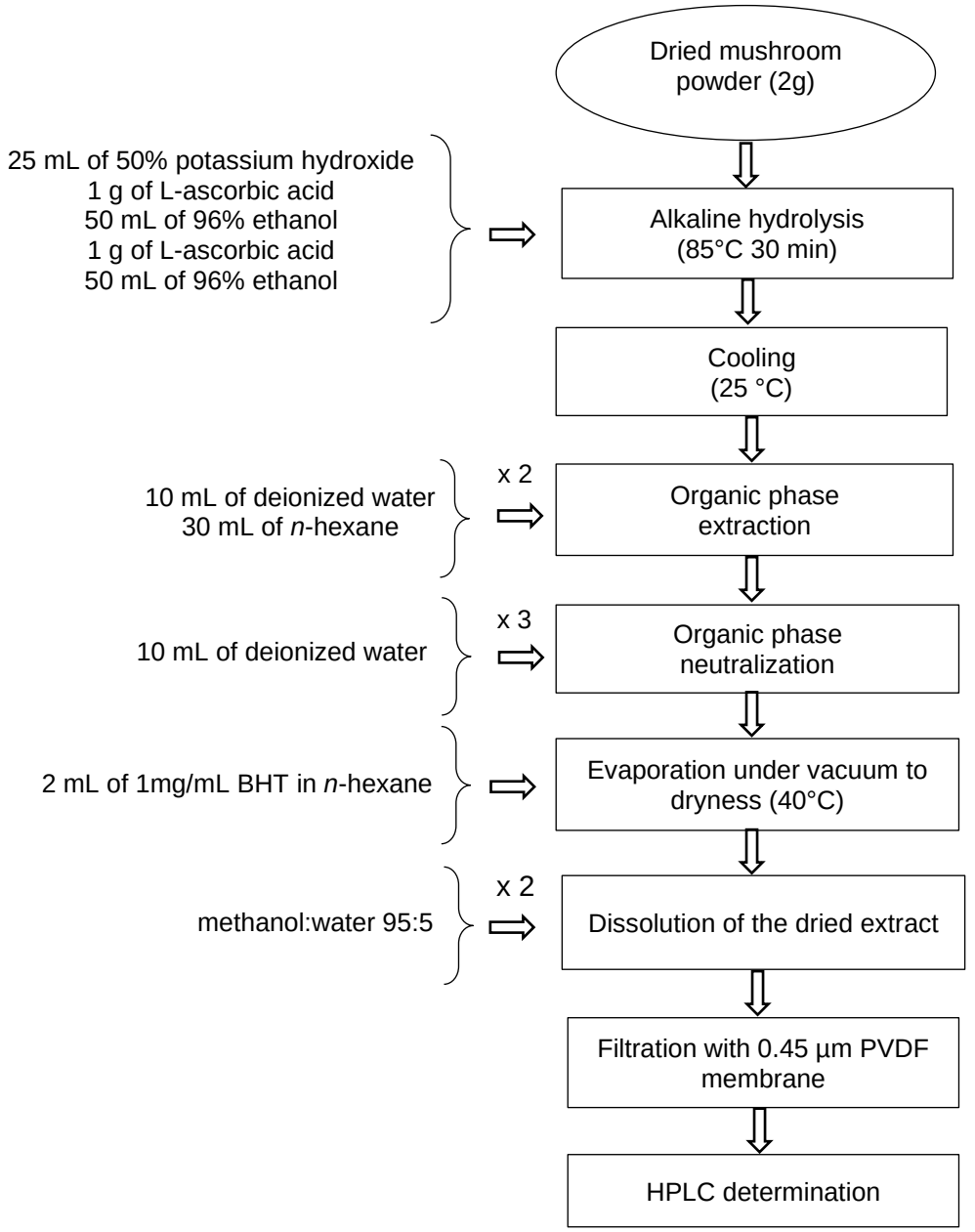

Fig. 1. Scheme for *P. ostreatus* treatment for extraction of bound vitamin D_2 and ergosterol.

To check the recovery, 0.1 mL of vitamin D_3 (400 mg/L in methanol) was added to the mushroom powder. The observed recovery was always higher than 90%. Vitamin D_2 and ergosterol were identified by the HPLC procedure by Huang et al. (2015). The HPLC system consisted of a model Shimadzu LC-20 AD pump coupled to a model Shimadzu SPDM20A photodiode array detector operated by Labsolution Software Shimadzu, Kyoto, Japan. A C18 Sunfire column (4.6 mm× 250 mm x 5 mm, Waters) was used for the separation. The mobile phase was methanol: water (95:5, v/v) at a flow rate of 1.0 mL/min and UV detection was performed at 254 nm. The content of vitamin D_2 was calculated on the basis of the calibration curve of authentic vitamin D_2.

3.6. Statistical analysis of data

Analytical data were analysed using Statgraphics 5.1 (STCC Inc.; Rockville, MD). Simple regression was performed to analyse vitamin D_2 decrease as a function of time at fixed temperature for every aw (equation (1)) and activation energy of this degradation (equation (2)). Nonlinear regression was performed to analyse the relationship between M and aw by fitting to the GAB equation (equation (3)). The models were considered significant when the P-value was less than 0.05 (95% confidence level). Vitamin D_2 and ergosterol contents were analysed using one-way ANOVA with the least significant difference (LSD) as a multiple range test. Results are reported as the average ± standard deviation (SD).

4. Results and discussion

4.1. Moisture properties of mushroom powder

The a_w level of mushroom powder after air-drying was 0.32. The moisture sorption isotherm was built at 30 °C and modelled according to the GAB equation (Fig. 2). Fitting to the GAB model gave parameters of C = 1.4 ± 0.3, K = 1.02 ± 0.02 and M_0 = 0.052 ± 0.008 g moisture/g dry solids, corresponding to an aw level of 0.45 ± 0.05 (R^2=99.8). Similar GAB parameters have been reported for *Auricolaria auricola* at 35 °C, with C=1.59, K=0.94 and M_0=0.045

g moisture/ g dry solids (Fan, Chen, Wei, He, & Yan, 2015). However, the moisture sorption properties of mushrooms depend on the drying process. In fact, the GAB monolayer is lower for air-dried mushroom than vacuum-dried and freeze-dried mushrooms due to a decrease in the hydrophilicity upon heat treatment (Ho & Lee, 2008). In general, foods are very stable at a_w levels close to the monomolecular moisture content, where the rate for lipid peroxidation is minimum (Bell, 2007).

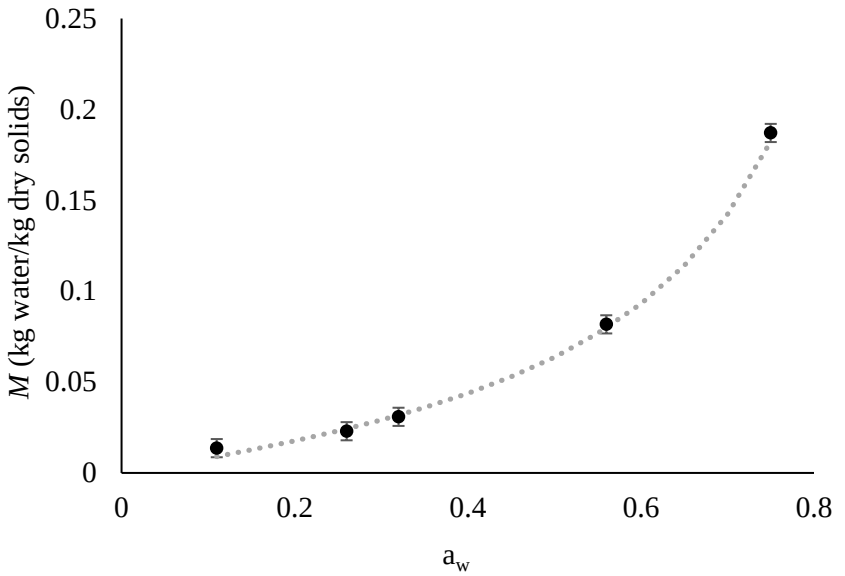

Fig. 2. Water sorption properties of the mushroom powder equilibrated at various a_w levels at 30 °C. Symbols indicate the experimental data;
error bars indicate SD; the dotted line indicates the absorption isotherm obtained by fitting experimental data with the GAB model.

Besides a_w, the phenomenon of glass transition could be applied as an integrated approach to determine food stability. Maximum food stability occurs when storage temperature is below the glass transition temperature, i.e., food is in the glassy state (Nurhadi et al., 2016). In mushroom, no glass transition is observed at very low moisture content up to 0.11, and hence no information can be derived from this parameter on their physical structure. At moisture contents above 0.11, glass transition in mushrooms occurs at temperatures

below zero and hence at the room temperature mushrooms are in the most unstable amorphous state (Guizani, Rahman, Klibi, Al-Rawahi, & Bornaz, 2013).

4.2. Initial vitamin D_2 content of mushroom powders

In *P. ostreatus* powder, vitamin D_2 and its precursor ergosterol were mostly present in esterified forms, since their contents greatly increased by alkaline hydrolysis (Table 1).

Table 1 Free and bound vitamin D_2 and ergosterol contents (mg/kg d.w.) of *P. ostreatus* mushroom before and after irradiation treatment.

		Vitamin D_2	**Ergosterol**
Not irradiated			
	free	$0.35^{a} \pm 0.12$	$1254^{a} \pm 225$
	bound	$3.5^{b} \pm 0.2$	$3451^{b} \pm 671$
UVB-irradiated			
	free	$17.5^{c} \pm 0.0$	$1254^{a} \pm 149$
	bound	$44.2^{b} \pm 1.3$	$3090^{b} \pm 706$

Different letters in the same column indicate significant differences among average values (n = 3; LSD, $p < 0.05$).

Previous irradiation trials were carried out on *Pleurotus* spp. used batch sizes of 0.3–2 kg of fresh mushroom or 1–20 g of dried mushroom; the delivered UV-B irradiance ranged between 1.14 and 11.5 W/m^2 for 0.17–2 h at 25–35 °C (Taofiq et al., 2017). In this study, the batch size was increased to 10 kg and the UV-B irradiance was decreased to 0.4 W/m^2 to ensure greater protection for workers in case of accidental UV radiation exposure. Consequently, UVB irradiance was applied for a longer time, i.e., 24 h. However, irradiation was combined with drying and process duration was not increased. As expected, irradiation increased the vitamin D_2 level, which raised from 3.5 to 44.2 mg/kg d.w. (Table 1). The content of vitamin D_2 obtained was in the range of those obtained by Slawinska et al. (2016) and Huang et al. (2015), while levels up to 498 mg/kg d.w. were attained by Wu and Ahn (2014). It is worth noting that there is not a general consensus on the optimal daily

intake for vitamin D. The FAO/WHO suggested a recommended daily intake of vitamin D of 5 μg for children and up to15 μg for adolescents, adults and elderly (WHO/FAO, 2004). According to the Institute of Medicine, the daily intake for vitamin D should be 15 μg for children and adults, but a higher amount, equal to 20 μg, is recommended for the elderly (O'Mahony et al., 2011). Moreover, the Endocrine Society recommended a daily intake 2 or 3 times higher for the obese people with respect to the normal weight subjects, which was confirmed by a large-scale observational study (Ekwaru, Zwicker, Holick, Giovannucci, & Veugelers, 2014). Considering a recommended daily intake in the range 5–45 μg, a dose in the range 0.1–0.9 g of the mushroom powder produced in this study could be formulated in a food matrix and provide the necessary daily intake without compromising consumers' liking. In fact, functional foods such as bakery products and pasta were formulated with the addition of 2–8% of *P. ostreatus* powder in order to increase protein and β-glucan content, obtaining good overall liking rates from consumers (Kim, Lee, Heo & Moon, 2016; Ng, Robert, Ahmad, & Ishak, 2017; Proserpio, Pagliarini, Laureati, Frigerio, & Lavelli, 2019).

It is also important to consider that the formation of vitamin D_2 by irradiation depends on a number of factors, including: mushroom strain and cultivation parameters, that affect the concentration of its precursor, product moisture content and shape, which influence the surface exposure to light source (Taofiq et al., 2017). There is still a knowledge gap to allow the identification of operating conditions to obtain a target vitamin D_2 content in the final product. However, the content of vitamin D_2 in a mushroom powder can be standardized by mixing the irradiated powder with not irradiated powder in order to accomplish the standardize vitamin D_2 in the final product and to avoid excessive and toxic levels (Fig. 3).

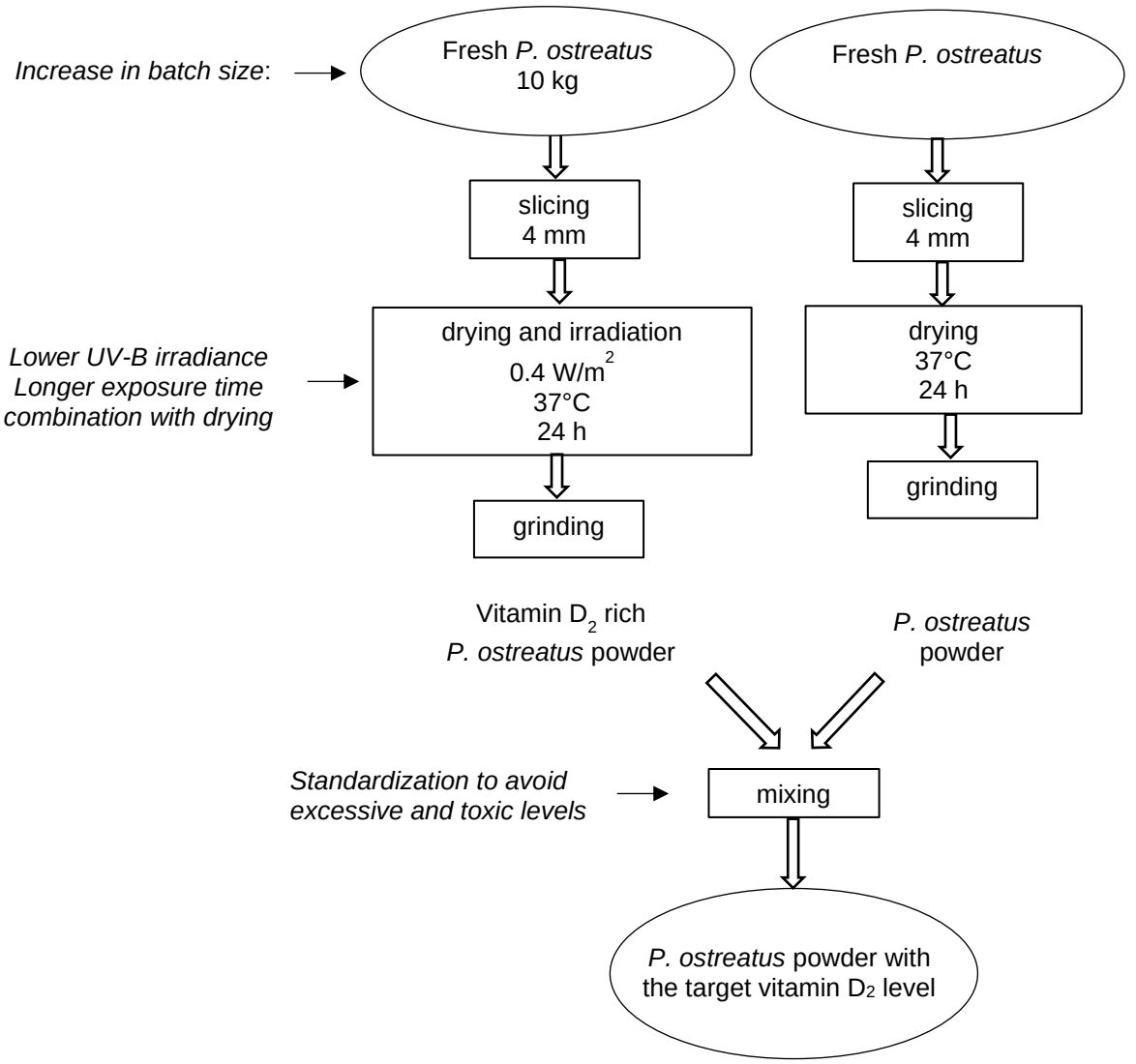

Fig. 3. Process to produce irradiated *P. ostreatus* powder with a target value of vitamin D_2. Information in *Italics* highlights the main differences of the proposed process with respect to the previously proposed approaches.

4.3. Kinetics of vitamin D_2 degradation in the powder

Beside standardization of the initial level of vitamin D_2, another issue is to prevent vitamin D_2 degradation in the mushroom powder. During storage of mushroom powder at different aw levels in the temperature range 5–40 °C, degradation of vitamin D_2 followed a first-order kinetics (Fig. 4A and B).

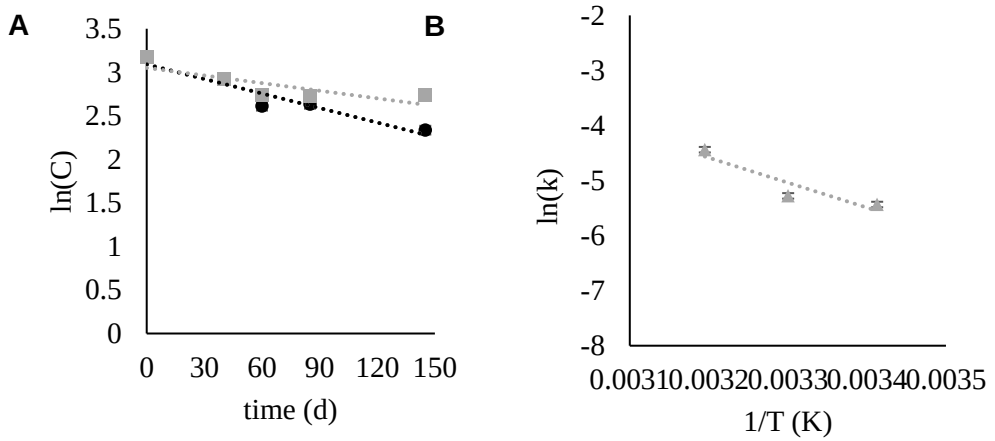

Fig. 4. Time course of vitamin D_2 degradation during storage at a_w 0.11 (■) and a_w 0.56 (●) at 30 °C fitted to a first-order kinetics (A) and Arrhenius plot for vitamin D_2 degradation at a_w 0.32 (B).

In a previous study the degradation of vitamin D_2 in fresh irradiated mushrooms was found to follow a first-order kinetics (Roberts, Teichert, & McHugh, 2008). In mushroom powder, the rate constants for vitamin D_2 degradation ranged from 0.0121 to 0.00308 d^{-1} (Table 2). In general, the rate of degradation of vitamin D_2 in mushroom powder observed in the current study was much lower than that observed in fresh mushrooms. In fresh *Agaricus bisporus* mushroom stored at 2 °C, a decrease of about 70% occurs in 4 days (Roberts et al., 2008). Likewise, vitamin D_2 content in the caps of white and brown *A. bisporus* was found to decrease markedly from day 1 to day 7, and then kept stable until day 14 (Guan et al., 2016). Longer storage times were not considered because the shelf life of fresh mushroom is only a few days (Marshall & Nair, 2009). These results point that irradiated fresh mushroom is hardly useful as a functional ingredient rich in vitamin D_2. There is no information on vitamin D_2 stability in solution, but free vitamin D_3 in water–ethanol mixture degraded rapidly and reduced to 4% of the initial value after 7 days. Considering the dry state, encapsulation of vitamin D_2 in spray dried casein micelles resulted in complete recovery after 4-months of storage at room temperature, while the a_w was not specified (Moeller, Martin, Schrader,

Hoffmann, & Lorenzen, 2018). For the whole mushroom powders considered in the present study, the half-life of vitamin D_2 calculated from the degradation rate constants is in the range between 57 and 225 d (Table 2).

Table 2 First-order rate constants (k) correlation coefficient (R) and predicted half-lives (t ½) for the degradation of vitamin D_2 during storage in the dark as a function of a_w and temperature.

a_w	T (°C)	$k*10^3$ (d^{-1})	R	t ½ (d)
0.11	30	3.08 ± 0.7	-0.779	225
0.26	30	5.07 ± 0.8	-0.915	137
0.32	30	5.1 ± 1	-0.867	136
0.56	30	6.04 ± 1.1	-0.862	115
0.75	30	12.1 ± 1.8	-0.968	57
0.32	20	4.33 ± 0.7	-0.971	160
0.32	40	11.8 ± 0.8	-0.978	59

The degradation rate of vitamin D_2 decreased progressively with decreasing the a_w, as expected for diffusion-controlled reactions. In fact, as water is removed the viscosity of the medium increases and molecular mobility decreases (Rahman, 1995). A similar moisture-dependent degradation was observed in various polar oxygen-sensitive molecules, such as for instance ascorbic acid and phenolic compounds (Corey, Kerr, Mulligan, & Lavelli, 2011; Lavelli, Sri Harsha, Laureati, & Pagliarini, 2017). Conversely, oxidation of lipid soluble oxygen sensitive molecules, such as for instance β-carotene, generally displays a U-shaped curve with respect to a_w level, with minimum oxidation rate occurring when sample moisture is near the monolayer moisture content and a rapid degradation below the monolayer moisture content (Sri Harsha & Lavelli, 2019). The lipid soluble vitamin D_2 did not show a rapid degradation at very low a_w, conversely it showed maximum stability. It is known that vitamin D_3 is stable in air at ambient temperature; however, its acid-catalyzed isomerization product, i.e., isotachysterol, is very labile in air even in the dark (Jin, Yang, Yang, Liu, Z. & Zhang, 2004). Vitamin D_2 also forms isotachysterol

through acid-catalyzed isomerization (Kobayashi, 1965). One possible explanation of the results observed in the present study is that an increase in a_w favored the acid-catalyzed isomerization of vitamin D_2 and then the isotachysterol formed underwent degradation through autoxidation. Indeed, the HPLC profile of the mushroom powders during storage evidenced the appearance of a peak that was not present at the beginning of incubation (Fig. 5), which according to its UV-VIS spectrum could be assigned to isotachysterol (Mahmoodani et al., 2017).

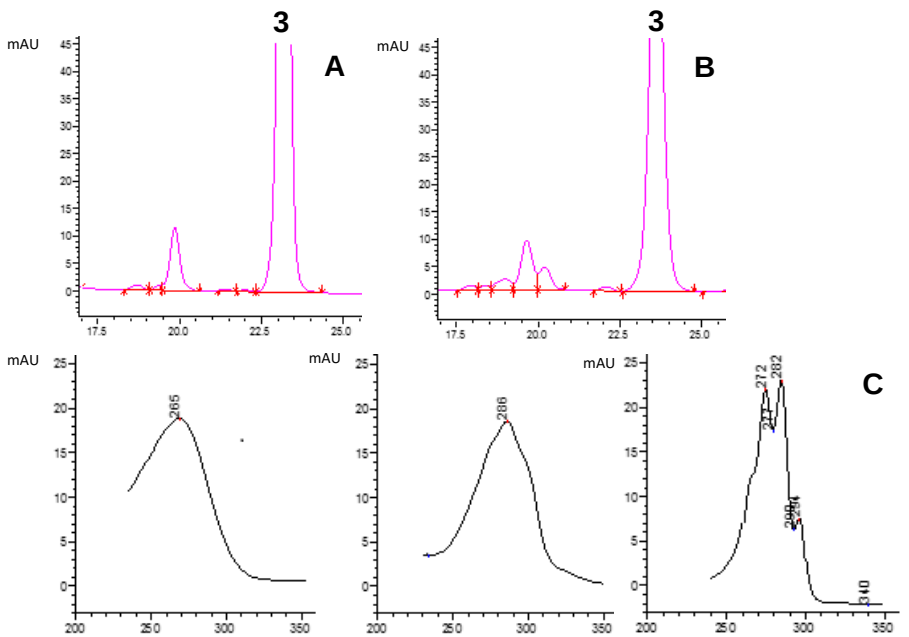

Fig. 5. HPLC profile of the n-hexane extract of *P. ostreatus* after irradiation (A) and after storage at 30 °C for 60 d at a_w 0.32 (B) and UV spectra of compounds 1, 2, 3 (C). 1, vitamin D_2, 2, tentatively identified as isotachysterol, 3, ergosterol.

However, this latter peak did not show a clear trend of variation during storage. The activation energy of vitamin D_2 degradation was calculated at the a_w level of 0.32 and found to be 37.6 kJ/mol (Fig. 3). Interesting, this value is close to the activation energy for oxygen diffusion in unsaturated lipids, i.e., 24 kJ/mol (Zhu & Sevilla, 1990). The activation energy observed also agrees with activation energies found for the free radical chain reactions in triglycerides.

For instance, the activation energy for carbon radical decay by radical-radical recombination in triolein, trilinolein, and trilinolenin, is in the range 34.0–37.3 kJ/mol, while that for peroxyl radicals decay is in the range 42–56 kJ/mol (Zhu & Sevilla, 1990). Hence, autoxidation could be the mechanism by which vitamin D_2 is lost. Whatever the degradation mechanism, the low activation energy observed for vitamin D_2 points the need to dehydrate the product at very low aw levels to ensure long stability.

5. Conclusion

In conclusion, UV-B irradiation of *P. ostreatus* during air-drying can be used to produce a powdered ingredient with a target amount of vitamin D_2. In the perspective of a value-chain approach, drying of mushroom, which is notably an energy-consuming operation, should be followed by long-time storage at ambient temperature, i.e., with no further energy input. This condition can be achieved by storage at room temperature at a_w levels around 0.11. Moreover, kinetics data for vitamin D_2 degradation in mushroom powder provided in this study can be used as a basis to design the best formulation strategy to deliver this vitamin with functional foods.

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The Effect of UV Irradiation on Vitamin D_2 Content and Antioxidant and Antiglycation Activities of Mushrooms

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ABSTRACT

Mushroom irradiation has been considered a sustainable process to generate high amounts of vitamin D_2 due to the role of this vitamin for human health and of the global concerns regarding its deficient or inadequate intake. Mushrooms are also receiving increasing interest due to their nutritional and medicinal properties. However, there is still a knowledge gap regarding the effect of UV irradiation on mushroom bioactive compounds. In this study, two of the most cultivated mushroom species worldwide, *Agaricus bisporus* and *Pleurotus ostreatus*, were irradiated with UV-B, and the effect of processing was investigated on the contents of vitamin D_2 as well as on antioxidant and antiglycation activities. UV irradiation increased vitamin D_2 up to 57 µg/g d.w, which is an adequate level for the fortification of a number of target foods. UV irradiation decreased the antioxidant activity when measured by the Folin–Ciocalteu reagent, the 2,2-diphenyl-1-(2,4,6 trinitrophenyl) hydrazyl radical assay and the ferric ion-reducing antioxidant power assay, but did not decrease the mushroom's ability to inhibit glycation of a target protein. These results open up a new area of investigation aimed at selecting mushroom species with high nutraceutical benefits for irradiation in order to maintain their potential properties to inhibit oxidative and glycation processes responsible for human diseases.

1. Introduction

Deficient and inadequate vitamin D intake (vitamin D represents both D_2 and D_3 forms) is a worldwide public health issue causing both skeletal diseases and increased risk of various chronic diseases [1]. Sources of vitamin D for humans are sunlight exposure, yielding vitamin D_3; a few foods of animal origin providing vitamin D_3; and yeasts and mushrooms, providing vitamin D_2. Both vitamin D_2 and D_3 are metabolized in the liver to 25-hydroxyvitamin D (25(OH)D) and then in the kidneys to 1,25-dihydroxyvitamin D (1,25(OH)2D). According to the serum concentration of 25(OH)D, vitamin D status is defined as deficient when serum 25(OH)D is lower than 25 nmol/L (10 ng/mL) and insufficient when serum 25(OH)D is between 25 and 50 nmol/L (10–20 ng/mL). Vitamin D status can also be assessed by validated food questionnaires [2]. Populations at risk of vitamin D deficiency are young children, the elderly, pregnant women and non-western immigrants in Europe [1]. The occurrence of vitamin D deficiency was found to be 48.1% in a Chinese population of preschool-aged children [3]. A large survey throughout Europe has shown that the intake of vitamin D is inadequate for 77–100% of adults (19–64 years old) and for 55–100% of elderly adults (>64 years old) [4]. From the 2005–2016 National Health and Nutrition Examination Surveys (NHANES), the prevalence of inadequacy for vitamin D intake among US population was found to be 95% [5].

Despite the fact that vitamin D_3 can be formed by the conversion of its precursors in the skin upon sunlight exposure, the above-reported studies document that this process is not sufficient to ensure appropriate levels of this vitamin. Moreover, the intake of vitamin D_3 from animal-based foods is not a proper solution because the current food systems, which are highly dependent on animal-based food sources, are not sustainable from an environmental point of view but also from a health and food security perspective [6]. Hence, one strategy to overcome the global demand of vitamin D in a sustainable way is to produce this vitamin from mushroom irradiation. This way, vitamin D can

be delivered through fortified foods [7]. The amount of vitamin D_2 in mushrooms is generally low. However, mushrooms have high amounts of the vitamin D_2 precursor, i.e., ergosterol. Processes that generate vitamin D_2 from ergosterol have been developed by UV irradiation on either the fresh or dried fruit body [8]. Recent trends have tested the efficiency of vitamin D_2 generation by combining supercritical CO_2 extraction with dissolution of the extract in ethanol or methanol followed by UV irradiation [9]. *Pleurotus* spp. are among the basidiomycetes that can be used for vitamin D_2 generation because their cultivation can be performed on various agri-food waste at a low cost [10,11]. Another basidiomycete of interest is *Agaricus bisporus*, which is the most cultivated mushroom worldwide [12].

Mushrooms are known to have medicinal properties and interest into this food source is increasingly expanding due to many studies that demonstrated their potential rules on human health due to antioxidant, antitumor, antimicrobial, anti-inflammatory, immunomodulator, antiatherogenic and hypoglycemic activities [10-11].

There is still a knowledge gap regarding the effect of UV-irradiation on mushroom bioactive properties. Nevertheless, it is known that the UV radiation can promote photo-oxidation via two major routes. The first of these involves direct photo-oxidation arising from the absorption of UV radiation by chromophores, thereby generating excited state species (singlet or triplets) or radicals as a result of photo-ionization. The second major process involves indirect oxidation of targets via the formation and subsequent reactions of singlet oxygen ($^1O_2$). Antioxidant enzymes that eliminate $^1O_2$ have not evolved. Instead, the highly reactive $^1O_2$ can interact with potential targets by either physical quenching and chemical reaction. The former results in energy transfer and de-excitation of the singlet state but no chemical change in the energy acceptor. The latter causes modification of the target and in the initiation of radical type reactions [13]. Hence, it may be expected that UV irradiation affects the potential ability of mushrooms to prevent cell damage.

Oxidative stress has been found to mediate cell damage thus triggering a number of human diseases [14]. The term is used to describe the condition of oxidative damage resulting when the critical balance between reactive oxygen species (ROS) enzymatic and non-enzymatic generation and antioxidant defenses (low molecular weight antioxidants, antioxidant enzymes and repair enzymes) is unfavorable.

Moreover, the carbonyl stress is another route leading to cell damage that is associated to a number of human diseases [15]. It involves highly reactive dicarbonyl compounds that are formed mainly through non-enzymatic protein glycation and in turn are involved in the formation of various harmful cross-linked adducts, which are collectively called advanced glycation end-products (AGEs).

The bioactive properties of mushrooms are due to their polysaccharides, proteins, lipids and molecules from mushroom secondary metabolism, such as terpenoids, eritadenine, ergothioneine and phenolic compounds [10-11, 16-18]. The antioxidant activity of edible mushrooms was studied in mice and has been associated mainly with the polysaccharide fraction, ergothioneine, and phenolic compounds [9, 10]. There is little information on the antiglycation activity of mushrooms. In a previous study, polysaccharides isolated from *Ganoderma lucidum* were supplemented in high-fat-diet/streptozotocin diabetic rats and found to decrease the level of AGEs and augment the activities of antioxidant enzymes [19].

In this study, *P. ostreatus* and *A. bisporus* were submitted to UV-irradiation and the effects of the process on vitamin D_2 content, as well as antioxidant and antiglycation properties were investigated to get an overall knowledge on the impact of this process on the potential health benefit of mushrooms.

2. Materials and Methods

2.1 Chemicals

Chemicals were purchased from Sigma Aldrich (Milan, Italy).

2.2 Mushrooms

P. ostreatus and *A. bisporus* were purchased from the market. The irradiation treatment was performed as described previously [20]. In brief, about 10 kg of body fruits were used for each mushroom type, cut into 4 mm slices, spread on an oven rack in a single layer and air-dried at 37 °C for 48 h. During the first 24 h of air-drying, the mushrooms were irradiated using two fluorescent lamps installed at 30 cm above the mushroom layer. In accordance to most of the previous studies so far performed [8, 12], an UV-B source was chosen for the irradiation. The lamps delivered an UV-B (280–315 nm) irradiance of 0.4 W/m^2. At the end of the process, about 1 kg of dried mushroom was obtained from each mushroom type and the product was grinded to a fine powder using a Thermomix TM 31 (Vorwerk Contempora S.r.l., Italy). Control *P. ostreatus* and *A. bisporus* powders were obtained by processing mushrooms with the same drying and grinding procedure without UV-irradiation.

2.3 Determination of vitamin D_2

Vitamin D_2 and ergosterol were extracted in triplicate from not irradiated and irradiated mushroom powders after saponification as described previously [21]. To check the recovery, preliminary samples of mushroom powder were added with 0.1 mL of vitamin D_3 (400 mg/L in methanol). The observed recovery was always higher than 90%. Then, samples were analyzed without vitamin D_3 in order to check the possible presence of vitamin D_2 photoproducts with elution times close to that of vitamin D_3 [22]. Vitamin D_2 and ergosterol were identified by a previously published procedure [23]. A Shimadzu HPLC system (Kyoto, Japan) including a LC-20 AD pump and a SPD-M20A photodiode array detector operated by Labsolution Software was used.. The column was a C18 Sunfire (4.6 mm x 250 mm x 5 mm, Waters) and the mobile phase was methanol:water (95:5, v/v), at a flow rate of 1.0 mL/min. UV detection was performed at 254 nm. The retention times for vitamin D_2, vitamin D_3 and ergosterol were 19.5, 20.5 and 23 min, respectively. The content of vitamin D_2 and ergosterol were calculated on the basis of the calibration curve of pure compounds.

2.4 Recovery of bioactive fractions

Mushroom powders of *P. ostreatus* and *A. bisporus* were extracted in triplicate with cold water (0.25 g into 5 mL) for 24 h at 4 °C under magnetic stirring. Then, the mixtures were centrifuged (Centrikon T-42K) at 12000 rpm for 30 min at 20 °C and the supernatant was used for further characterization [24]. The ethanol-insoluble fractions were obtained by adding 8.3 mL of 96% ethanol to 2 mL of the water extracts. The precipitate was recovered by centrifugation (Centrikon T-42K) at 12000 rpm for 30 min at 20 °C and redissolved in water for further characterization [25]. The yield of water-soluble extracts and ethanol-insoluble precipitates was determined by measuring the weight of dry solids after drying in a vacuum oven at 70 °C and 50 Torr for 18 h.

2.5 Antioxidant activity

The Folin–Ciocalteu (FC) assay was performed on the water-soluble fractions and the ethanol-insoluble fractions redissolved in water, as described previously [26]. A calibration curve was built using gallic acid. FC reducing compounds were expressed as milligram of gallic acid equivalents (GAE) per gram of dry fraction.

The free radical scavenging capacity of the water-soluble fractions and the ethanol-insoluble fractions redissolved in water was evaluated using the stable 2,2-diphenyl-1-(2,4,6 trinitrophenyl)hydrazyl radical (DPPH) as described previously [27]. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a reference antioxidant and results were expressed as micromoles of Trolox equivalents (TE) per gram of dry fractions.

The ferric ion reducing antioxidant power (FRAP) assay was performed on of the water-soluble fractions and the ethanol-insoluble fractions redissolved in water as described previously [26]. $FeSO_4$ was used for calibration and results were expressed as micromoles of Fe II equivalents per gram of dry fraction.

2.6 Antiglycation activity using bovine serum albumin (BSA)/fructose model systems

The antiglycation activity was determined on the water-soluble fractions and the ethanol-insoluble fractions redissolved in water, as described previously [28]. The reaction mixture was prepared by adding 100 µL of sample extracts or standard diluted in water, 900 µL of 200 mM potassium phosphate buffer, pH 7.4 with 0.02% sodium azide, 300 µL of bovine serum albumin *(*BSA) solution (50 mg/mL of BSA in phosphate buffer) and 300 µL of fructose solution (1.25M fructose in phosphate buffer). A BSA solution (blank sample) and control reaction without sample addition were prepared in parallel. The mixtures were incubated at 37 °C for 3 days in the dark and then analyzed for fluorescence on a Perkin-Elmer LS 55 Luminescence Spectrometer (Perkin-Elmer, United Kingdom) with an excitation/emission wavelength pair of 350/420 nm and 5 nm slit width, read against phosphate buffer. Aminoguanidine was used as a positive control. For each sample extract, 3–4 dilutions were assessed in duplicate. Dose–response curves were built reporting % inhibition of BSA glycation as a function of sample or standard concentration. Results were expressed as milligrams of aminoguanidine equivalents (AG) per gram of dry fraction.

2.7 Statistical analysis of data

Experimental data were analysed using one-way ANOVA with the least significant difference (LSD) as a multiple range test using Statgraphics 5.1 (STCC Inc.; Rockville, MD). These results are reported as the average ± standard error (SE).

3. Results and Discussion

3.1 Effect of mushroom irradiation on Vitamin D_2 content

Initial content of vitamin D_2 was low in both commercial mushrooms considered. This result is consistent with previous studies and could be related with the conditions used for industrial cultivation of mushrooms that generally

occurs with low exposure to sunlight [8, 12]. UV-irradiation led to an increase of vitamin D_2 content from 3.1 to 37 µg/g d.w. and from 0.3 to 57 µg/g d.w. in *P. ostreatus* and *A. bisporus,* respectively (Table 1). The initial contents of ergosterol were 3600 and 5700 µg/g d.w. in *P. ostreatus* and *A. bisporus,* respectively (Table 1). These values fall in the range previously reported [29] and remained high after the generation of significant amounts of vitamin D_2. This result may suggest that upon UV irradiation the residual amount of ergosterol is still relevant for drug development and could contribute to the health-promoting effects of mushrooms [30].

Table 1. Vitamin D_2 and ergosterol content in non-irradiated and UV-irradiated mushrooms[a].

Mushroom	Vitamin D_2 µg/g d.w	Ergosterol µg/g d.w.
P. ostreatus	$3.1^{c} \pm 0.1$	$3600^{c} \pm 400$
P. ostreatus $_{UV}$	$37^{b} \pm 0.1$	$3200^{c} \pm 300$
A. bisporus	$0.31^{d} \pm 0.1$	$5700^{a} \pm 300$
A. bisporus $_{UV}$	$57^{a} \pm 0.1$	$4800^{b} \pm 300$

[a]Values are average ± SE. Different letters in the same column indicate significant differences among samples (LSD, $p < 0.05$).

The contents of vitamin D_2 obtained were in the recovery range described by previous studies, i.e., from 3.9 to 741 µg/g d.w. for *A. bisporus* and from 27.89 to 239.67 µg/g d.w. for *P. ostreatus,* respectively [8]. In the present study, no vitamin D_2 photoproducts were detected (Figure 1).

According to [22], irradiation of *P. ostreatus* led to significant formation of vitamin D_2, tachysterol$_2$ and lumysterol$_2$, but the conditions applied were different from those used in the present study and the amount of vitamin D_2 produced was 141.2 mg/kg, i.e. higher than those observed in the present study. The yield of vitamin D_2 formation upon irradiation was previously found to depend on conditions that can be controlled during processing, such as light source, distance between the lamp and the mushroom, temperature and duration of the irradiation. However, the yield also depends on factors that

cannot be controlled such as the shape of mushroom, which conditions the surface exposed to light and the moisture levels, that varies during drying [8].

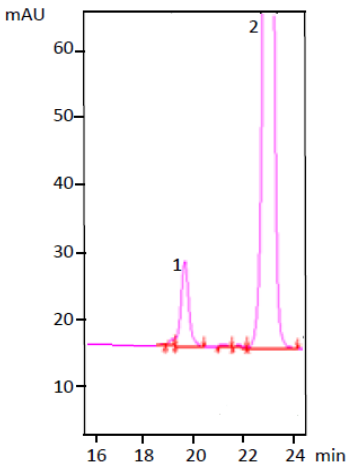

Figure 1. HPLC profile of the *n*-hexane extract of irradiated *P. ostreatus* powder. 1. Vitamin D_2; 2. ergosterol.

The final powder obtained after irradiation can be standardized for vitamin D_2 content by mixing the treated powder with untreated powder. Hence, considering a target final value for vitamin D_2 of 40 µg/g d.w., a simulation was conducted to calculate the amount of irradiated powder that could be necessary to fortify vehicle foods in order to achieve health benefit (Table 2). To perform this simulation, three studies were considered to define the most appropriate target foods and fortification levels, namely, a study in line with the regulation dealing with vitamin D fortification in the United States and Canada [31], a study considering the European regulation scenario on vitamin D fortification [32] and a study aimed at exploring food matrices for vitamin D fortification in low/lower-middle income countries [33]. These latter studies defined somewhat different fortification strategies, due to different dietary habits and needs of the populations involved and to different regulations of the countries of interest. In any case, it can be observed that very low fortification levels of mushroom powder, in the range 0.03 to 0.38 g/100 g of food, could lead to the target fortification level, thus confirming the potential rule of irradiation technology in increasing the intake of vitamin D (Table 2).

Table 2. Modelled formulations of foods with irradiated mushroom powder to achieve a target level of vitamin D_2[a]

Food category	Target level for vitamin D µg/100 g food	Ref.	Amount of UV- irradiated mushroom powder g/100 food
Ready-to-eat breakfast cereals	8.75	[31]	0.22
Milk	1.05	[31]	0.03
Yogurt	2.225	[31]	0.06
Margarine	8.275	[31]	0.21
Edible oil	15	[32]	0.38
Milk	2	[32]	0.05
Wheat flour	2.8	[32]	0.07
Milk	1	[33]	0.03
Orange juice	10.5	[33]	0.26

[a]The mushroom powder considered was obtained from *P. ostreatus* and *A. bisporus* through UV-irradiation as described under Material and Methods and contained 40 µg/g of vitamin D_2.

3.2 Effect of mushroom irradiation on antioxidant activity

Considering the variety of mushroom compounds that can act as antioxidants, various extraction procedures have been proposed to study the antioxidant activity of mushrooms. Comparing the yields of ethanol and hot water extractions for various mushroom species, it was observed that ethanol only accounted for 5.89 – 18.89 % of solids, while hot water fraction accounted for 38.3 – 57.2 % of total solids [34]. Moreover, information obtained by using hot water is considered more valuable because it corresponds to the procedure used to recover bioactive compounds from medicinal mushrooms by Chinese traditional medicine [34]. However, studies conducted on *P. citrinopileatus* have shown a greater bioactivity *in vitro* of the cold aqueous extract compared to the hot water extract, due to protein denaturation and degradation of phenolic compounds at high temperatures [24]. Moreover, the ethanol precipitation of the aqueous extract has been proposed for the isolation of proteoglycans with anticancer properties [25]. Hence, in the current study both the cold water soluble and ethanol precipitate fractions of *P. ostreatus* and *A. bisporus* were studied to assess the effects of UV-irradiation. The water-

soluble fraction of *P. ostreatus* accounted for 54 % of solids, while the ethanol insoluble fraction was 7.2 % of solids (Table 3).

Table 3. Fraction yields and antioxidant activity evaluated by the FC, DPPH and FRAP assays of the water extracts and ethanol-insoluble fractions of *P. ostreatus* and *A. bisporus* before and after UV-irradiation.[a]

Mushroom and treatment	Fraction yield g/100 g	Antioxidant activity		
		FC mg GAE/g fraction	DPPH µmol TE/g fraction	FRAP µmol FeII/g fraction
P. ostreatus WE	54^{b} ± 3	24.6^{c} ± 1.3	56^{c} ± 4	82^{d} ± 5
P. ostreatus $_{UV}$ WE	54^{b} ± 3	19.6^{d} ± 1.5 (20%)	48^{d} ± 1 (14%)	58^{e} ± 14 (29%)
A. bisporus WE	67^{a} ± 1	16.2^{e} ± 0.5	48^{d} ± 6	208^{b} ± 7
A. bisporus $_{UV}$ WE	70^{a} ± 1	15.0^{f} ± 0.2 (7%)	37^{e} ± 7 (23%)	131^{c} ± 28 (37%)
P. ostreatus EP	7.2^{c} ± 0.1	32.1^{b} ± 0.6	71^{b} ± 1	222^{b} ± 1
P. ostreatus $_{UV}$ EP	7.3^{c} ± 0.1	25.8^{c} ± 0.7 (20%)	49^{d} ± 3 (31%)	127^{c} ± 14 (43%)
A. bisporus EP	3.4^{d} ± 0.1	53.4^{a} ± 1.6	119^{a} ± 14	473^{a} ± 43
A. bisporus $_{UV}$ EP	3.2^{d} ± 0.1	53.4^{a} ± 0.8	103^{a} ± 17	505^{a} ± 14

[a]Values are average ± SE; values in brackets are the percent decrease for the UV irradiated samples with respect to non-irradiated samples. WE = water extract, EP = ethanol precipitate. Different letters in the same column indicate significant differences among samples (LSD, $p < 0.05$).

Higher percentage of the water extracts was observed for *A. bisporus*, i.e., 69 %, but the percentage of ethanol insoluble fraction was lower, i.e., 3.3 % (Table 3). Due to the high extraction yield, the cold-water extracts can be considered representative of the mushroom matrix to investigate the effect of irradiation on potential bioactive components.

The antioxidant activity in both non-irradiated and UV-irradiated mushrooms was evaluated by the FC, FRAP and DPPH assays (Table 3).

The levels of FC reducing compounds for the aqueous extracts of *P. ostreatus* and *A. bisporus* before irradiation were 24.6 and 16.2 mg GAE/g of fraction, respectively. These values can be considered to be high according to the ranking defined previously in a screening study on 23 species of mushrooms, which reported levels of FC reducing compounds of the aqueous extract in the range from 2 to 37 mg GAE/g [35]. The ethanol-insoluble fractions of both

mushrooms showed a higher specific content of FC reducing compounds than those of the respective water-soluble fraction for both mushrooms. This result may suggest that bioactive compounds partitioned mostly into the ethanol insoluble fraction. UV-irradiation decreased the FC reducing compounds of *P. ostreatus* by 20%, while the decrease was lower for *A. bisporus* (7%).

The DPPH values for the water extracts of *P. ostreatus* and *A. bisporus* before irradiation were 56 and 48 µmol TE/g fraction. As observed for FC reducing compounds, the specific DPPH values for the ethanol-insoluble fractions of both mushrooms were higher than those of the corresponding water-soluble fractions. The genus *Agaricus* has been previously found to have the highest DPPH radical scavenging properties among the most widely appreciated cultivated mushrooms [36]. UV-irradiation decreased the DPPH values for all fractions, except for the ethanol-insoluble fraction of *A. bisporus*.

The FRAP values for the water extracts of *P. ostreatus* and *A. bisporus* before irradiation were 82 and 208 µmol FeII/g fraction, respectively. As observed for FC and DPPH, higher specific FRAP values were present in the ethanol-insoluble fractions than in the water-soluble fractions for both mushroom species, with values of 222 and 473 µmol FeII/g fraction. This result may be related to the preferential partition of the bioactive compounds in the ethanol-insoluble fractions. A screening of 1943 plant-based foods revealed that the average FRAP value is 115.7 µmol FeII/g [37]. Hence mushrooms can be considered important as a dietary source of reducing compounds. On the other hand, irradiation caused a marked decrease in the FRAP values, except for the ethanol-insoluble fraction of *A. bisporus*.

3.3 Effect of mushroom irradiation on antiglycation activity

The antiglycation activity in both non-irradiated and UV-irradiated mushrooms was evaluated by the BSA/fructose assay (Table 4).

The water extracts of *P. ostreatus* and *A. bisporus* were able to inhibit protein glycation, exhibiting 113 and 83 mg AG/g fraction.

Table 4. Antiglycation activity of the water extracts and ethanol-insoluble fractions of *P. ostreatus* and *A, bisporus* before and after UV irradiation.

Mushroom and treatment	Antiglycation agents mg AG/g fraction
***P. ostreatus* WE**	$113^{c} \pm 22$
***P. ostreatus* $_{UV}$ WE**	$131^{c} \pm 25$
***A. bisporus* WE**	$83^{d} \pm 9$
***A. bisporus* $_{UV}$ WE**	$89^{d} \pm 10$
***P. ostreatus* EP**	$177^{b} \pm 10$
***P. ostreatus* $_{UV}$ EP**	$170^{b} \pm 6$
***A. bisporus* EP**	$555^{a} \pm 80$
***A. bisporus* $_{UV}$ EP**	$500^{a} \pm 89$

Values are average ± SE. WE = water extract, EP = ethanol precipitate. Fraction yield is reported in Table 3. Different letters in the same column indicate significant differences among samples (LSD, $p < 0.05$).

As observed for the antioxidant activity, which was higher in the ethanol-insoluble fractions, higher levels of antiglycation activity was observed in the ethanol-insoluble fractions with respect to the water-soluble fractions, corresponding to 177 and 555 mg AG/g fraction for *P. ostreatus* and *A. bisporus*, respectively. There is no information on the antiglycation activity of these mushroom species. In previous studies, the antiglycation activities of a crude water- soluble fraction extracted from the sclerotia of *Inonotus obliquus* [38] and *Lignosus rhinocerus* [39] were found to be 23 and 133 mg AG/g fraction. Compared to these latter studies, the antiglycation activities of the ethanol-insoluble fractions of *P. ostreatus* and *A. bisporus* were found to be much higher. However, even higher antiglycation activity was observed for a polysaccharide purified from the fruiting body of *Boletus snicus*, which was found to have the same antiglycation activity of aminoguanidine in the BSA/glucose model system, i.e., 1000 mg AG/g [40].

The UV-irradiation process did not cause any significant change to the antiglycation activity of *P. ostreatus* and *A. bisporus* (Table 4). Despite redox

active compounds generally possess antiglycation activity, this activity is also dependent on metal chelation and carbonyl-trapping abilities [16].

4. Conclusions

P. ostreatus and *A. bisporus*, which are among the most cultivated mushroom species in the world, were able generate relevant amounts of vitamin D_2 through UV-irradiation. The concept that this latter technology can address the global need of vitamin D_2 was highlighted by providing a scenario of possible future application of the vitamin D_2 enriched mushroom in target foods. *P. ostreatus* and *A. bisporus* were also found to possess high antioxidant and antiglycation activity. Irradiation caused a decrease of antioxidant activity in both mushroom species but did not affect antiglycation activity. The ethanol-insoluble fraction of *A. bisporus* was not affected by UV-irradiation. Results from this research open up a new area of investigation. In fact, while the relevance of UV irradiation of mushrooms to address the global need of vitamin D_2 has attracted a lot of research interest, the identification of the effects of irradiation on the mushroom matrices is still lacking and deserves particular attention.

One limitation of the current study is that it included only two mushroom species. Considering the wide biodiversity among mushrooms, future studies should be directed to extend knowledge on the effects of irradiation to other mushroom species in order to assist a more efficient design of the process for the generation of vitamin D_2 with a major focus on the retention of bioactive properties.

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Chapter 3b

Encapsulation of an oxidisable target using *P. ostreatus* β-glucans as antioxidant and emulsifier agent.

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Production of Stable Emulsions Using β-glucans Extracted from *Pleurotus ostreatus* to Encapsulate Oxidisable Compounds

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ABSTRACT

The basidiomycete *Pleurotus ostreatus* is a sustainable food source known to be rich in β-glucans, functional compounds in mushrooms recognised for their numerous health, nutraceutical and physicochemical properties. The aim of the present study was to extract β-glucans from *P. ostreatus* powder and to study the feasibility of using the extract to prepare oil-in-water emulsions formulated for the encapsulation of lipophilic antioxidants by spray drying e.g. with an oil droplet size about 2 µm, a dry matter content of 40% w/w and with a physical stability of at least 2 h. Hot-water soluble β-glucans fraction was extracted from *P. ostreatus* powder following a method adapted from Synytsya et al (2009). Emulsions were prepared by rotor-stator homogenisation using maltodextrin DE12 as wall material, β-glucans extract as emulsifier and commercial sunflower oil as a model for lipophilic active compounds. The emulsion stability was estimated from the evolution over time of the oil droplet size distribution measured by laser light diffraction. Extracts containing up to 27% w/w water-soluble β-glucans were produced. Physically stable mono-modal emulsions were obtained when MD/β-glucans weight ratio was lower than about 500 to avoid depletion and β-glucans/oil weight ratio was more than 0.014 to stabilize oil droplets. The usage of emulsifiers made-up by polymeric constituents with inherent bioactivity, such as mushroom β-glucans, could be a sustainable and healthy alternative to common emulsifiers.

1. Introduction

Bioactive lipophilic compounds, such as fatty acids, aroma and flavour compounds, vitamins, antioxidants, phytosterols and essential oils are widely used in the food industry due to their multifunctional roles. However, these compounds are also involved in chemical processes linked to degradation; therefore, it is important to protect these active compounds against factors promoting their degradation (oxygen, humidity, light, etc.). To this aim, bioactive lipophilic compounds can be encapsulated by spray drying. The first step of lipophilic compounds encapsulation by spray drying consists in the preparation of an oil-in-water liquid emulsion with formulation, microstructure and properties suitable for further spray drying and use (Hernandez-Sanchez et al, 2015). Moreover, the emulsion needs to be physically stable until and during spray drying in order to ensure correct oil distribution within the matrix of encapsulation: this means that no evolution of the size distribution should occur due to emulsion destabilization or shear stress during atomisation (Turchiuli et al, 2017; Munoz-Ibanez et al, 2016). The selection of an efficient combination of wall materials and emulsifier agent with the suitable ratio of each constituent in the blend has therefore to be carefully studied. Wall materials must be soluble in water, allow forming solutions with a proper viscosity at high solids concentration (e.g. up to 40-60%w/w dry matter) in order to be pumped and sprayed, and bring to a stable powder without sticking during drying. Due to their high solubility in water and low viscosity at high solids concentrations, hydrophilic carbohydrate molecules, such as maltodextrins (MD), are suitable wall materials. However, these components have no interfacial properties and must be used in association with emulsifier or surfactant molecules to provide protection from physical destabilization of emulsions (Turchiuli et al, 2014).

Since the field of encapsulation is expanding, research is required to study the possibility of using alternative and naturally occurring compounds, with health effects and available at low cost (e.g. from local production, by-products, etc.),

as support materials or emulsifiers for a wide variety of active compounds (Falco et al, 2017). In this scenario, the genus *Pleurotus* can make a valuable contribution because these mushrooms are able to grow on by-products with a limited capital investment and technical skills. Besides their nutritional value, it has been demonstrated that *Pleurotus* spp. have health promoting benefits, mainly due to their unique dietary fibre fraction. Among the constituents of this fraction, β-glucans are the major component and among the most studied functional compounds (Lavelli et al, 2018). β-glucans from different sources have different linkage types, branching manners and molecular weight. In mushrooms, these polysaccharides have a backbone of D-glucose-linked β-(1→3) with no branches or variable amounts of β-(1→6) branches; these glucose chains are twisted and create a single or a triple helix stabilized by inter-chain hydrogen bonds. Besides their recognized healthy effects, β-glucans' functionality is also associated with their physicochemical properties, such as thickening, stabilizing, emulsifying, foaming and gelation properties (Zhu, Du and Xu, 2016). These characteristics have been widely studied for β-glucans obtained from different cereals (Burkus and Temelli, 2000; Kontogiorgos et al, 2004) but poorly investigated for β-glucans from yeasts and mushrooms. Thammakiti et al (2004) discovered that β-glucans obtained from brewer's yeast can be used in food products as a thickening, water-holding, or oil-binding agent and emulsifying stabilizer. Since the yeast and fungal glucans share a common structure, it can be assumed that they also share the same physical and chemical properties.

Taking into account this information, the aim of the present work was to propose formulations and protocols to produce stable oil-in-water emulsions with an oil droplet size around 2 μm and a dry matter content suitable for spray drying. Sunflower oil, rich in polyunsaturated fatty acids (PUFA) and vitamin E, was used as a model oil phase containing active lipophilic compounds. Two protocols were tested for the preparation of the β-glucans extract. Emulsion formulation was tested at lab scale in terms of both emulsifier over oil (β-glucans/oil) and wall material over emulsifier (MD/β-glucans) weight ratio in

order to find the suitable β-glucans content allowing to obtain physically stable emulsions with high dry matter content while avoiding depletion phenomena. A protocol for the production of emulsions at pilot scale was also tested.

2. Materials and methods

2.1 Materials

Commercial sunflower oil containing 11% w/w saturated, 29% w/w monounsaturated and 60% w/w polyunsaturated fatty acids and 0.05% w/w α-tocopherol (Cora, France) was used as model oil for lipophilic compounds encapsulation. Maltodextrin DE 12 (MD) (Glucidex®, Roquette, FR) was used wall material for the encapsulation process. The β-glucans-rich extract obtained from *Pleurotus ostreatus* powder (IoBoscoVivo, Vergiate, IT) (see Section 2.2.1) was used to stabilize the emulsions.

2.2 Methods

2.2.1 Extract preparation

Fractions of water-soluble polysaccharides were isolated from *P. ostreatus* powder according to modified Synytsya's method (Synytsya et al, 2009) (Fig. 1a). 3 g of powder were washed with 60 mL of ethanol 80% (w/v) by keeping the suspension under magnetic stirring overnight; then the supernatant was removed by centrifugation (14000 rpm for 1 h at 20°C). The volume of ethanol extract removed was replaced with the same volume of distilled water for another centrifugation under the same conditions in order to wash the powder from the remaining solvent. Again, the aqueous washing solution (WW) was separated and replaced with the same volume of distilled water and the tubes containing the powder in water were placed in boiling water for 6 hours to extract hot water-soluble compounds remaining in the powder (especially β-glucans). The resulting extract (Extract from Washed solids, W) was isolated by centrifugation and used to prepare emulsions E1_lab_W, E2_lab_W, E3_lab_W, E4_lab_W, E4_pilot_W and E5_lab_W (see Section 2.2.3).

This method was further modified by removing the washing step with distilled water, since the results showed that this step of the extraction process caused an important loss of cold water-soluble β-glucans (Fig. 1b). The extract obtained without the washing step (Extract from Unwashed solids, UW) was used to prepare emulsions E4_pilot_UW and E6_lab_UW (see Section 2.2.3).

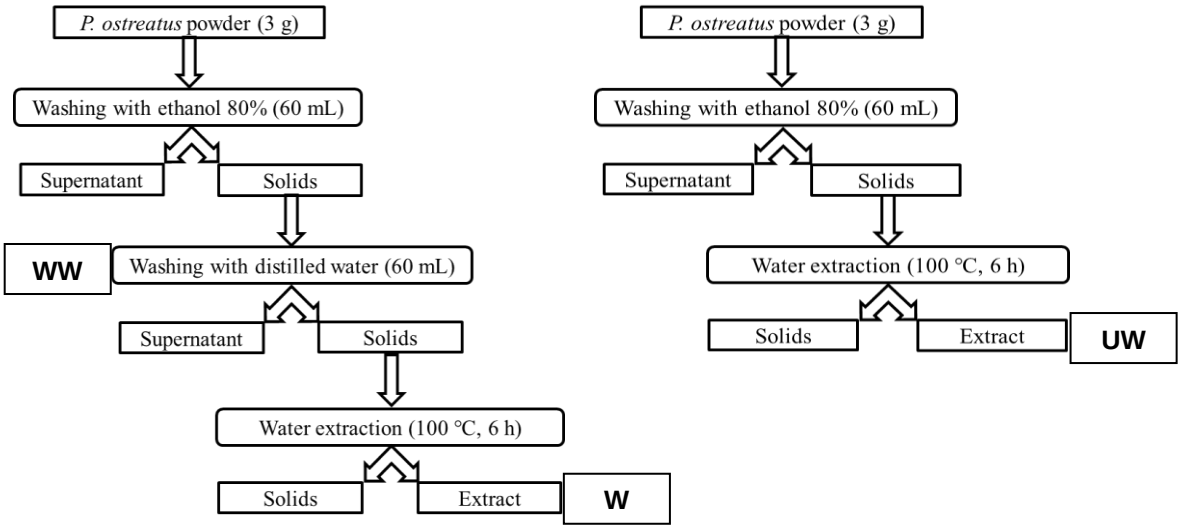

Figure 1: Steps to obtain the W and UW β-glucans-rich extracts from *P. ostreatus.*

2.2.2 Evaluation of β-glucans content

The amount of β-glucans in the mushroom powder, in the extracts and in the spray dried powders was measured with an enzymatic kit purchased from Megazyme (Bray, Co. Wicklow, IRELAND). Briefly, total glucans (plus free glucose and glucose from sucrose) were measured using controlled acid hydrolysis with H_2SO_4 and the glucose released was measured using glucose oxidase/peroxidase reagent. α-glucans (starch/glycogen) plus free glucose and glucose from sucrose were specifically measured after hydrolysis of starch/glycogen to glucose with glucoamylase and sucrose to glucose plus fructose with invertase and the glucose specifically measured with GOPOD reagent (glucose oxidase plus peroxidase and 4-aminoantipyrine dissolved in *p*-hydroxybenzoic acid and sodium azide). β-glucans were determined by the

difference. The same method was used to measure the glucans content of *P. ostreatus* powder, in order to calculate the yield of extraction:

$$\%\ Extraction\ Yield = 100 * \frac{\text{glucans (total or β) in extract (g)}}{\text{glucans (total or β) in powder (g)}}$$

2.2.3 Preparation of emulsions

To prepare emulsion at lab scale (100 mL of volume) and pilot scale (1L of volume) a two-step protocol was used. An aqueous phase was prepared by slow dissolution of wall material (MD) in the extracts containing β-glucans and distilled water at 35°C under mechanical stirring (with a 3-bladed propeller stirrer (Eurostar, IKA, FR) at pilot scale; with magnetic stirrer at lab scale). A glass container instead of a stainless steel one was used to avoid contamination by metal ions, which can start oxidation. Then, in order to obtain an emulsion with the required oil droplet size (e.g. about 2 µm diameter), sunflower oil was added in the aqueous solution under homogenization. At lab scale a Polytron PT 3100 D model homogenizer (KINEMATICA AG, Switzerland) with a PTG 36/4 stator was used at 10000 rpm from 10 to more than 30 min. At pilot scale, a rotor-stator homogenizer (AXR Silverson Machine Ltd, FR) was used at 3900 rpm for 10 min.

2.2.4 Oil droplet size distribution and emulsion stability

The oil droplet size distribution was measured by LASER light diffraction (Mastersizer 2000, Malvern, FR) in wet mode (Hydro 2000) after dispersion in purified water. The refractive index value used for the oil droplets was 1.475. From the volume size distribution obtained, characteristic diameters were deduced: d50 (median diameter), d10 and d90, diameters with respectively 50, 10 and 90% of the particles with a smaller size. The span was calculated as (d90-d10)/d50. The higher is the span value, the wider is the distribution.

To estimate the emulsion stability, the size distribution measurements were repeated after a 2h rest period at room temperature.

3. Results and discussion

3.1 Characterization of β-glucans extracts

P. ostreatus is considered an important source of β-glucans among the most cultivated species of mushrooms worldwide (e.g. *Agaricus bisporus, Lentinula edodes* and *P. ostreatus*) (Correa et al, 2016). Indeed, McCleary and Draga (2016), who have developed the method used in this work for the evaluation of β-glucans, reported that the amounts of β-glucans for *A. bisporus, L. edodes* and *P. ostreatus* were 6.0 g/100g d.w, 23.5 g/100g d.w and 32.3 g/100g d.w., respectively. Using the same approach, a study on 16 different strains of *P. ostreatus* revealed that the total glucans content varied in the range 14-25 g/100 g d.w., with β-glucans in the range of 10.9-22.9 g/100 g d.w. (Koutrotsios et al, 2017). For the strain of *P. ostreatus* selected for this work the total glucans content measured was 36 g/100g d.w. with 35 g/100g d.w. corresponding to β-glucans. These values were used to calculate the yield of extraction (Table 1). The contents of total glucans and β-glucans in extract W were 19.3-20.8 g/100g d.w. and 12.6-14.1 g/100g d.w., respectively corresponding to a low extraction yield of about 10%.

TABLE 1: Characterization of the β-glucans-rich extracts obtained from *P. ostreatus*.

Extract	% dry matter	% yield total glucans	Total glucans (g/100g d.w.)	% yield β-glucans	β-glucans (g/100g d.w.)
W	1.0	10.7-11.6	19.3-20.8	7.2-8.1	12.6-14.1
UW	1.4	19.6-21.1	25.2-27.1	16.6-17.8	20.8-22.3

The analysis of the aqueous washing (WW) showed that it contained a high amount of β-glucans that were lost. Extract UW presented higher contents of total glucans (25.2-27.1 g/100g d.w.) and β-glucans (20.8-22.3 g/100g d.w.) and also higher yields of extraction (19.6-21.1% for total glucans instead of 10.7-11.6% and 16.6-17.8% for β-glucans instead of 7.2-8.1%).

3.2 Emulsions stability

When elevated concentrations of high molecular weight polysaccharides are present in the aqueous phase of an emulsion, the depletion phenomena decrease the number of emulsifying molecules adsorbed at the oil/water interphase, causing flocculation and consequently the coalescence of oil droplets. The polymer concentration must therefore remain below the CFC (Critical Flocculation Concentration) (McClements, 1999). Since this value depends on the emulsifier nature and the oil content, many different formulations of oil-in-water emulsions were tested (Table 2) at lab scale, using both the W and UW extracts, while maintaining the same percentage of dry matter (e.g. 40%w/w) but varying the amount of oil, β-glucans and MD.

TABLE 2: Composition of the different emulsions tested (40%w/w dry matter).

Emulsion	% oil	% β-glucans	% MD	$\frac{\beta-glucans}{oil}$	$\frac{MD}{\beta-glucans}$
E1_lab_W	4.0	0.03	35.8	0.007	1249
E2_lab_W		0.04	35.7	0.009	944
E3_lab_W		0.06	35.5	0.014	620
E4_lab_W		0.08	35.4	0.019	468
E4_pilot_W		0.09	35.4	0.021	418
E4_pilot_UW		0.09	35.6	0.021	415
E5_lab_W	8.0	0.06	31.5	0.007	551
E6_lab_UW		0.13	31.4	0.017	235

The oil droplet size distribution was measured after different homogenization procedures, carried out at lab scale and pilot scale, with the purpose to obtain a median diameter (d50) around 2 μm (Table 3). In some cases (E1_lab_W, E2_lab_W, E3_lab_W and E5_lab_W), even after a homogenization step of more than 30 minutes and at 10000 rpm, the distribution of the emulsion was still bi-modal and with a d50 bigger than 3 μm. These parameters are associated to a low stability and hence the evolution of their size distribution with time was not checked.

TABLE 3: Homogenization conditions and size distributions at the time of preparation (t = 0) and after two hours (t = 2h)

Emulsion	Homogenization		Size distribution (t = 0)				Size distribution (t = 2h)			
	Time (min)	Speed (rpm)	d10 (μm)	d50 (μm)	d90 (μm)	Span	d10 (μm)	d50 (μm)	d90 (μm)	Span
E1_lab_W	>30	10000	1.37	6.11	15.3	2.3	---	---	---	---
E2_lab_W	30	10000	0.99	3.97	11.01	2.5	---	---	---	---
E3_lab_W	>30	10000	1.01	3.14	11.22	3.3	---	---	---	---
E4_lab_W	10	10000	1.02	2.02	3.77	1.4	1.02	2.02	3.83	1.4
E4_pilot_W	10	3900	1.28	2.51	4.55	1.3	1.39	2.56	4.42	1.2
E4_pilot_UW	10	3900	1.44	2.50	4.19	1.1	1.42	2.59	4.51	1.2
E5_lab_W	>30	10000	1.46	7.89	17.57	2.0	---	---	---	---
E6_lab_UW	10	10000	1.30	2.79	5.55	1.5	1.50	2.96	5.58	1.4

---: not measured for non-stable emulsions at time of production.

The other emulsions (E4_lab_W and E6_lab_UW) showed instead a mono-modal distribution and a d50 around 2 μm at the time of preparation. Since these properties were satisfying at the time of preparation, the size distribution of the liquid emulsions was also measured after two hours storage at room temperature. The results obtained show that no significant changes have occurred in the microstructure of the emulsions, confirming that they were physically stable. Consequently, it was possible to establish that an emulsion with 4%w/w of oil was stable when the weight ratio between β-glucans and oil was bigger than 0.014 and the weight ratio between MD and β-glucans was lower than 468. Taking into account that the amount of emulsifier required depends on the oil quantity, it was necessary to increase the percentage of β-glucans up to 0.13%w/w in the formulation of emulsions with 8%w/w of oil to maintain the stability: in this case, a stable emulsion was obtained with a ratio between β-glucans and oil of 0.017 and a ratio between MD and β-glucans of 235 (Fig. 2). E4_lab_w was selected to scale up the protocol at pilot scale (Fig. 3). Changing the homogenization procedure, the diameters d10, d50 and d90 increased but they remained stable after 2h from the preparation.; the span value varied from 1.1 to 1.3 at time 0 and from 1.2 to 1.4 at time 2h, corresponding to relatively narrow distributions. The oil droplet size

distributions of the emulsions homogenized at pilot scale (Fig. 3) were mono-modal with comparable d50 both at time 0 and after two hours.

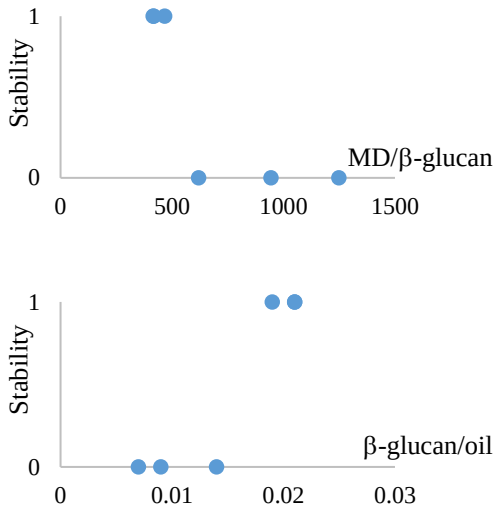

Figure 2: Emulsion physical stability as a function of β-glucans/oil and MD/ β-glucans ratio (0: unstable; 1: stable).

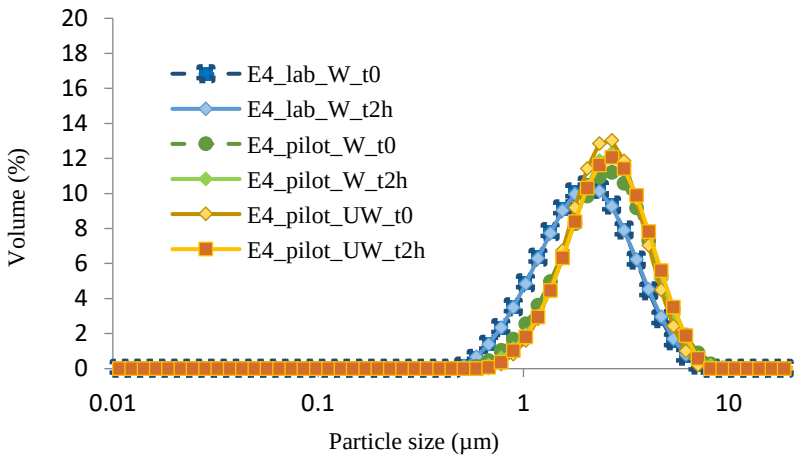

Figure 3: The oil droplet size distribution of the emulsions E4_lab_W, E4_pilot_W and E4_pilot_UW after production (t0) and after 2h storage (t2h)

Thus, the size of these droplets seems to be compatible with a good oil encapsulation in the powder particles produced with a pilot spray dryer that generally have diameters between 20 to 50 µm (Turchiuli et al, 2013). It is

noteworthy that, even if the extract W contained just hot-water-soluble β-glucans and UW contained also β-glucans soluble in water at Tamb, thus with different molecular weight, their emulsifying behavior was the same: however, since the extract UW has a better yield of extraction, it can be chosen over W.

4. Conclusions

The usage of emulsifiers made-up by polymeric constituents with inherent bioactivity, such as mushroom β-glucans, could be a sustainable and healthy alternative to common emulsifiers. Their efficiency regarding spray drying and protection still has to be evaluated.

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Application of *Pleurotus ostreatus* β-glucans for oil–in–water emulsions encapsulation in powder

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ABSTRACT

In this study, sunflower oil containing α-tocopherol (model for oxidizable lipophilic compounds) was encapsulated using a wall material made of maltodextrin and two different extracts rich in β-glucans and proteins, obtained from the basidiomycete *P. ostreatus.* A mixture of maltodextrin and acacia gum was used as control wall material. The aim was to assess *P. ostreatus* extracts as a sustainable and healthy alternative to common emulsifiers. After the evaluation of the emulsion stability, four powders were produced by spray drying and then analysed for the particle morphology and oil droplets distribution within the solid matrix (by scanning electron microscopy), the emulsion microstructure after their dissolution in water (by laser light diffraction) and the encapsulation efficiency (by solvent extraction of surface oil). In addition, powders were analysed in terms of oil protection against oxidation by spectroscopic determination of conjugated dienes and HPLC evaluation of vitamin E. Results showed that *P. ostreatus* extracts provided suitable emulsifying properties both in the liquid and in the spray dried emulsions. Moreover, it allowed excellent protection of vitamin E and polyunsaturated fatty acids against oxidation. Hence, *P. ostreatus* extracts can be exploited as innovative emulsifier to provide physical protection to functional, oxygen sensitive lipophilic ingredients by microencapsulation.

1. Introduction

Polyunsaturated fatty acids (PUFAs) can be a carrier of lipid soluble bioactive compounds and provide health benefits. Due to their highly unsaturated nature, PUFAs are sensitive to oxidation and thermal degradations, leading to the production of hydroperoxides, unpleasant flavours and smells and to the loss of dissolved bioactive compounds. Therefore, it is important to protect these liposoluble oxidizable compounds against factors promoting their degradation, such as oxygen, humidity, light, etc. To this aim, oils containing bioactive compounds have been microencapsulated in polymeric matrices from many years (Lewandowski, Czyżewski, & Zbiciński, 2012) and one of the most widely used technic is spray drying. Microencapsulation by spray drying is a relatively inexpensive, fast and efficient process, which is mostly used for the encapsulation of oils, colorants, vitamins and probiotics (Le Priol et al., 2019).

The first step of lipophilic compounds encapsulation by spray drying consists in the preparation of an oil-in-water (O/W) liquid emulsion with formulation, microstructure and properties suitable for further spray drying and use. This emulsion needs to be physically stable until and during spray drying, in order to ensure correct oil distribution within the matrix of encapsulation: this means that no evolution of the size distribution should occur due to emulsion destabilization or shear stress during atomisation (Munoz-Ibanez et al., 2016). The choice of wall material and emulsifying agent is a vital step in spray drying as they influence the properties of the microparticles produced. Due to their high solubility in water and low viscosity at high solids concentrations (e.g. up to 40-60% w/w dry matter), hydrophilic carbohydrate molecules, such as maltodextrin (MD), are suitable wall materials. However, MD have no interfacial properties and must be used in association with emulsifier or surfactant molecules to produce physically stable emulsions. For the microencapsulation of sunflower oil by spray drying, a mixture of MD and

acacia gum (AG) is frequently used (Gharsallaoui et al., 2007;Munoz-Ibanez et al., 2016; Tolun, Artik & Altintas, 2020).

Since the field of encapsulation is expanding, research is required to study the possibility of using alternative and naturally occurring compounds, with health effects and available at low cost as support materials or emulsifiers for a wide variety of active compounds (Falco, Sotres, Rascón, Risbo, & Cárdenas, 2017). In this context, the basidiomycete *P. ostreatus* can make a valuable contribution as it can be cultivated on different by-products with a limited capital investment and technical skills. Furthermore, this mushroom is considered as a good source of a unique dietary fibre fraction, among which β-glucans with a β-(1→3), (1→6) skeleton are the major component. Antitumor, immunomodulatory, antimicrobial, antinociception, anti-inflammatory, prebiotic, antidiabetic and antioxidant are some of the different properties already described for β-glucans of *P. ostreatus* (Dalonso, Goldman, & Gern, 2015; Lavelli, Proserpio, Gallotti, Laureati, & Pagliarini, 2018). Moreover, β-glucans' functionality is also associated with their physicochemical properties, such as thickening, stabilizing, emulsifying, foaming and gelation properties (Zhu, Du, & Xu, 2016). These latter characteristics have been extensively studied for the β-(1→3), (1→4) β-glucans obtained from different cereals (Burkus & Temelli, 2000; Kontogiorgos, Biliaderis, Kiosseoglou, & Doxastakis, 2004) but poorly investigated for β-glucans from yeasts and mushrooms, which share a common structure. In a previous study, Thammakiti et al. (2004) discovered that β-glucans obtained from brewer's yeast could be used in food products as a thickening, water-holding, or oil-binding agent and emulsion stabilizer. However, the efficacy of fungal β-glucans for encapsulation of O/W emulsions has not been investigated. Moreover, the water soluble β-glucans fractions obtained from *P. ostreatus* are probably bound to proteins, since these components cannot be separated completely (Synytsya et al., 2009). Proteins are natural amphiphilic molecules with good emulsifying and film-forming properties (Encina, Vergara, Giménez, Oyarzún-Ampuero, & Robert, 2016). In

fact, proteins can adsorb at the O/W interface and form viscoelastic film, which provides physical stability to the emulsion during subsequent processing and storage (Dickinson, 2001). In addition to their functional properties, proteins also exhibit antioxidant properties in O/W emulsions. These properties include the chelation of metals, free radical scavenging, binding of secondary lipid oxidation products and formation of a physical barrier protecting the lipid phase (Adjonu, Doran, Torley, & Agboola, 2014; Berton-Carabin, Ropers, & Genot, 2014). It is noteworthy that fungal proteins should be preferred over animal proteins; they are generally less expensive, they may reduce the risk of spreading diseases such as bovine spongiform encephalitis and they are acceptable to both vegetarian and vegan consumers (Nesterenko, Alric, Violleau, Silvestre, & Durrieu, 2013).

Taking into account the gap of knowledge regarding possible use of fungal β-glucans in O/W systems, the main purpose of this study was to evaluate the potential of two β-glucan rich extracts obtained from *P. ostreatus* as emulsifiers with inherent antioxidant activity for the encapsulation of sunflower oil rich in PUFAs and vitamin E by spray drying. Hence, specific objectives were to investigate: a) the emulsion stability by assessing the oil droplet size distribution over time of O/W emulsions obtained at pilot scale; b) the characteristics of the spray dried powders obtained from the emulsions in terms of water activity, moisture content, morphology, as well as size distribution of particles and oil droplets after reconstitution with water; c) the oxidative stability of the oxidizable target vitamin E and the carrier PUFAs during spray drying.

2. Materials and methods

2.1 Materials

Commercial sunflower oil containing 11% w/w saturated, 29% w/w monounsaturated and 60% w/w polyunsaturated fatty acids and 0.05% w/w α-tocopherol (α-toc) (Cora, France) was used as model oil for lipophilic compounds encapsulation. MD DE 12 (Glucidex®, Roquette, France) was

used as main wall material for the encapsulation process. For the control emulsion (Ec), AG (Instantgum AA, Nexira, France) was used for its film forming properties. Other emulsions (E10W, E10UW and E20UW) were stabilized adding β-glucans-rich extracts obtained from *P. ostreatus* powder (IoBoscoVivo, Vergiate, Italy). Standard α-toc (VWR International PBI, Milano, Italy) was used for calibration.

2.2 Methods

2.2.1 Extract preparation

Fractions of water-soluble polysaccharides were isolated from *P. ostreatus* powder according to a modified Synytsya's method (Synytsya et al., 2009) after ethanol extraction and water washing or ethanol extraction only, as previously described (Gallotti, Turchiuli, & Lavelli, 2019) (Fig. 1). The resulting extracts were called W (Extract from Washed solids) and UW (Extract from Unwashed solids, obtained by removing the washing step with distilled water, WW).

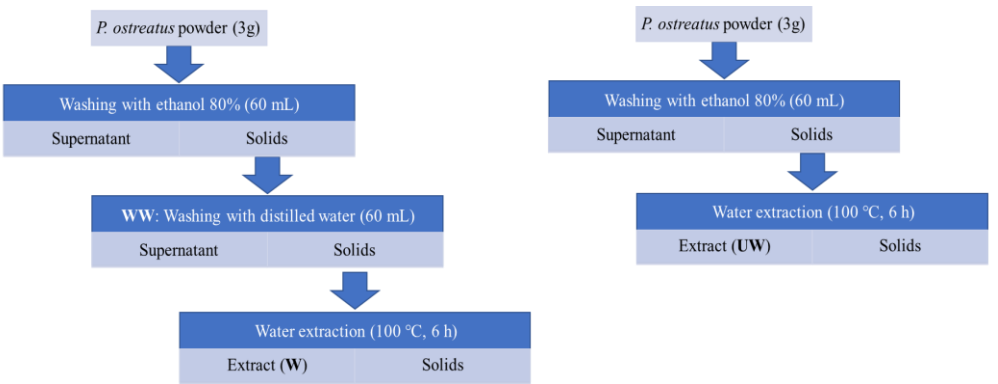


Fig. 1. Steps to obtain the W and UW β-glucans-rich extracts from *P. ostreatus.*

2.2.2 Evaluation of β-glucans content

The amount of β-glucans in the extracts was measured with an enzymatic kit purchased from Megazyme (Bray, Co. Wicklow, Ireland), following the method described by McCleary and Draga (2016). Briefly, total glucans content (plus free glucose and glucose from sucrose) was measured using controlled acid

hydrolysis with H_2SO_4, while α-glucans were specifically measured after hydrolysis with α-amylose to glucose. β-glucans were determined by the difference. The same method was used to measure the glucans content of *P. ostreatus* powder, in order to calculate the yield of extraction according to Eq. (1):

$$\%\ \text{Extraction Yield} = 100 * \frac{\text{glucans (total or β) in extract (g)}}{\text{glucans (total or β) in powder (g)}} \quad (1)$$

2.2.3 Evaluation of protein content

Total nitrogen analysis was performed using the Kjeldahl method (AOAC, 2000). The protein content was calculated as total nitrogen multiplied by the factor 4.38, proposed by Bano et al. (1988), and expressed as grams of protein per 100 g of dry product. In order to calculate the yield of extraction, the protein content of *P. ostreatus* powder was evaluated and used in the following Eq. (2):

$$\%\ \text{Extraction Yield} = 100 * \frac{\text{proteins in d.m. extract (g)}}{\text{proteins in powder (g)}} \quad (2)$$

2.2.4 Evaluation of ash content

Ash content was evaluated by ashing at 500 °C (AOAC, 1990).

2.2.5 Ferric ion reducing antioxidant power (FRAP) assay

The FRAP assay was performed on extracts as described previously (Benzie & Strain, 1996). Briefly, FRAP reagent was prepared by adding 30 mL of 300 mM acetate buffer, pH 3.6; 3 mL of 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl and 3 mL of 20 mM $FeCl_3*6H_2O$. The reaction mixture was prepared mixing 0.4 mL of sample extracts diluted 1:2 with water and 3 mL of FRAP reagent. The absorbance at 593 nm was evaluated using a Jasco UVDEC-610 spectrophotometer (Jasco Europe, Cremella, Italy) after 4 min of incubation at 37°C against a blank with no extract addition. A methanolic solution of $FeSO_4*7H_2O$ was used for calibration. Results were expressed as micromoles of Fe (II) sulfate equivalents per 100 g of dry matter in extract.

2.2.6 Preparation of emulsions

The composition and codes of the initial liquid emulsions produced are reported in Table 1.

TABLE 1: Composition of the different emulsions tested (% w/w), with 40% w/w dry matter including oil, MD, AG and dry matter from the *P. ostreatus* extract.

	Emulsion composition				
Emulsion Code	**Oil (%)**	**AG (%)**	**MD (%)**	**Extract d.m. (%)**	**β-glucan (%)**
Ec		14.40	21.60	---	---
E10W	4	---	35.40	0.60	0.085
E10UW		---	35.59	0.41	0.086
E20UW	8	---	31.19	0.81	0.171

To formulate initial liquid emulsions (1L of volume), aqueous solutions containing wall materials were first prepared by slow dissolution at 35°C, under mechanical stirring with a 3-bladed propeller stirrer (Eurostar, IKA, France). For Ec, the wall materials (MD and AG) were dissolved in distilled water whilst for E10W, E10UW and E20UW MD was dissolved directly in the extract containing β-glucans and distilled water. Glass containers instead of stainless-steel ones were used to avoid contamination by metal ions, which can start oil oxidation especially when in presence of α-toc (McClements, 1999). Then, sunflower oil was added to the aqueous solution under rotor-stator homogenization (AXR Silverson Machine Ltd, France) at 3900 rpm. Homogenization was maintained for 10 min.

In order to be spray dried, the four emulsions had the same total dry matter content fixed to 40% w/w (including oil), and the ratio between β-glucan and oil was kept constant at 0.021. The ratio between MD and β-glucan was therefore 183 and 416 for the emulsions with oil contents of 8 and 4 % w/w, respectively.

2.2.7 Oil droplet size distribution and emulsion stability

The oil droplet size distribution was measured by LASER light diffraction (Mastersizer 2000, Malvern, France) in wet mode (Hydro 2000) after dispersion in purified water. The refractive index value used for the oil droplets was 1.475. From the volume size distribution obtained, characteristic diameters d50 (median diameter), d10 and d90 were deduced. They correspond to diameters with respectively 50, 10 and 90% of the particles with a smaller size. The span was calculated as (d90-d10)/d50. The higher is the span value, the wider is the size distribution.

To estimate the emulsion stability, the size distribution measurements were repeated after a 2h rest period at room temperature.

2.2.8 Preparation of spray dried emulsions

Liquid emulsions were spray dried in a Niro Minor pilot scale spray dryer (Niro, Denmark). It is a one-step co-current spray dryer with an evaporative capacity comprised between 1 and 4 $kg \cdot h^{-1}$. For spraying, a rotary wheel was used with a rotation speed of 22.720 rpm. Emulsions were pumped to the atomization wheel using a peristaltic pump (Masterflex L/S 77201-60, pipe 6429-17) with a feed flow rate of approximately 32 $g \cdot min^{-1}$. It was checked in a previous study that it has no influence on the emulsion oil droplet size distribution (Munoz-Ibanez, Azagoh, Dubey, Dumoulin, & Turchiuli, 2015). Drying air was taken from the ambient by a fan (43 Hz) with a flow rate of 110 $kg \cdot h^{-1}$. The inlet air temperature was fixed at 170 °C and its outlet temperature varied from 85 to 100 °C for the different trials. Four powders were produced: Pc — control powder from emulsion Ec, without β-glucans; P10W — powder from emulsion E10W; P10UW — powder from emulsion E10UW; P20UW — powder from emulsion E20UW. They were stored in hermetical bags at -20°C until analysis.

2.2.9 Particle size distribution, water content and a_w of dry emulsions

The particle size distribution of powders was measured by laser light diffraction (Mastersizer 2000, Malvern, France) in dry mode (Scirocco 2000) with an air

pressure of 4 bar to ensure the dispersion of the powder. Results were analysed in Fraunhofer mode.

The water activity (a_w) was measured with an aw-meter (LabMaster-aw, Novasina AG, Switzerland), while the water content was determined with a moisture analyser at 105°C (Sartorius MA30, Sartorius AG, Germany).

2.2.10 Reconstitution of emulsion

Liquid emulsions were reconstituted by adding 1 g of dry emulsion in 1.5 mL of pure hot water (25°C) under magnetic stirring for 5 minutes, resulting in emulsions having the same dry matter content as the initial liquid emulsions (40 g/100 g emulsion). The oil droplet size distribution was measured in wet mode as described before.

2.2.11 Scanning electron microscopy (SEM)

A Quanta 200 Model Scanning Electron Microscopy (FEI, France) was used to observe the microstructure of the powders (dry emulsions). The samples were mounted on aluminium stubs with double-side sticky carbon tape, placed under low vacuum (0.6 mmHg) at accelerating voltage of 20 kV and analysed with both the Back Scattering Electrons (BSE) and Gas Secondary Electrons (GSE) detectors. The micrographs shown in this work were taken by the instrument's software installed on the PC connected to the SEM.

2.2.12 Surface oil extraction and encapsulation efficiency

The surface oil extraction method used was adapted from the one described by Hernandez Sanchez et al. (2016) for the quantification of non-encapsulated oil. 5 g of powder was weighed on a filter paper (No. 4, Whatman, Maidstone, Kent, United Kingdom), and then washed with 4 × 50 mL of hexane. The recovered organic phase was evaporated in a rotary rotavapor RE 120 (Buchi, Switzerland) and later removed by blowing a stream of nitrogen until constant weight. The non-encapsulated oil (surface oil) was determined by mass difference between the initial clean flask and that containing the extracted oil residue (Jafari, Assadpoor, Bhandari, & He, 2008). The surface oil was

expressed as g surface oil/g powder. Total oil was assumed to be equal to the initial oil, since preliminary tests revealed that 99% of the initial oil was retained in powders obtained by spray drying in similar conditions (Hernandez Sanchez, Cuvelier, Turchiuli, 2016). The encapsulation efficiency (EE) was calculated by the following Eq. (3):

$$\mathrm{EE} = 100 * \frac{(TO - SO)}{TO} \quad (3)$$

where *TO* is the total oil content (calculated) and *SO* is the surface oil content (g/g dry powder).

2.2.13 Oil phase extraction

The oil phase extraction was performed according to the method described by Hernandez Sanchez et al. (2016) with few modifications. In brief, 2 mL of ultra-pure water at 30 °C were added to 0.5 g of powder and agitation was maintained until the emulsion was reconstituted. Then, 50 mL of hexane/iso-propanol (3:1, v/v) were added to the reconstituted emulsion. The mixture was agitated for 15 min and centrifuged at 1000×g for another 15 min at 20°C. Centrifugation allowed to recover around 47 mL of organic phase, where 1 mL was used to measure conjugated dienes and 40 mL was used for α-toc analysis.

2.2.14 Measurement of conjugated dienes

The conjugated dienes (CD) were detected by UV spectrophotometry at 234 nm (standard ISO 3656:2011).

In the case of a measurement in oil, 1 drop of oil were weighed in a 10 mL volumetric flask adding isooctane to get to volume; after, the vial was vortexed for 60 s. Then, the sample absorbance was measured using a UV– visible spectrophotometer (UVIKON 941 Plus, Serlabo Technologies, France) and the Specific Absorbance was calculated according to Eq. (4):

$$\mathrm{SA} = \frac{Abs234nm}{Wg} \quad (4)$$

Where *Abs234 nm* is the absorbance of the sample measured at 234 nm and *Wg* is the oil mass (g) in 100 mL of the solvent solution analysed.

For oil encapsulated in powder, measurements were made directly on the organic phase containing the extracted oil. For that purpose, 1 mL of the oily-organic phase extracted from powder as described above (2.2.13) was diluted 1:6 by adding 5 mL of hexane/iso-propanol (3:1, v/v), and kept under ultrasonic agitation for 30 s. Finally, the specific absorbance (SA) was calculated according to Eq. (5):

$$\mathrm{SA} = 6 * \frac{Abs234nm}{\frac{g\ Calculated\ Oil}{47\ mL} * 100\ mL} \quad (5)$$

Where *Abs 234 nm* is the absorbance of the sample (1 mL) measured at 234 nm, *g Calculated Oil* is the calculated mass of oil contained in 0.5 g of powder and recovered in 47 mL of organic phase.

2.2.15 Measurement of α-toc concentration

To measure the α-toc in the oil phase of powder samples, a precise volume (40 mL) of the oily-organic phase, obtained as described above (2.2.13), was evaporated in a flask by a rotary rotavapor (Heidolph Laborota 4000 efficient, WB eco, Schwabach, Germany). Then, 5 mL of methanol were added and the flask was placed in an ultrasonic bath for 5 minutes. The solution was filtered through a nylon syringe filter with 0.45 µm pore size and 10 µL of the sample were injected and analysed using a model Shimadzu LC-20 AD pump coupled to a model Shimadzu SPD-M20A photodiode array detector and an RF-20 AXS operated by Labsolution Software Shimadzu, Kyoto, Japan. A 5 µm Sunfire C18 column (250 × 4.6 mm; Waters, Milan, Italy) equipped with a C18 precolumn (Waters, Milan, Italy) was used for the separation, performed by isocratic elution at 28°C, at a flow rate of 1 mL*min^{-1} using methanol/water (95:5, v/v) as eluent. Fluorimetric detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 330 nm (Panfili, Fratianni, & Irano 2003). The identification and quantification of α-toc were conducted by using a calibration curve built with a purified standard.

2.2.16 Statistical analysis of data

Experimental data were analysed using one-way ANOVA with the least significant difference (LSD) as a multiple range test, and by linear regression analysis using Statgraphics 5.1 (STCC Inc.; Rockville, MD). These results are reported as the average of duplicate or triplicate values ± standard deviation (SD).

3. Results and discussion

3.1 Characterization of β-glucans extracts

P. ostreatus is considered an important source of β-glucans and proteins among the most cultivated species of mushrooms worldwide (e.g. *Agaricus bisporus, Lentinula edodes* and *P. ostreatus*) (Correa, Brugnari, Bracht, Peralta, & Ferreira 2016; Lavelli et al., 2018).

McCleary et al. (2016), reported that the amounts of β-glucans for *A. bisporus, L. edodes* and *P. ostreatus* were 6.0 g/100g d.m., 23.5 g/100g d.m. and 32.3 g/100g d.m., respectively. For the strain of *P. ostreatus* selected for this work, the total glucans content was found to be 36 g/100g d.m., with 35 g/100g d.m. corresponding to β-glucans. These values were used to calculate the yield of extraction (Table 2).

The contents of total glucans and β-glucans in the hot water extract W (obtained as described in Fig. 1) were 21.99 g/100g d.m. and 12.00 g/100g d.m., respectively corresponding to relatively low extraction yield of 12.33% and 7.57%. Indeed, hot water extraction of mushroom yields a fraction containing β-glucans, proteins, heteropolysaccharides and traces of starch, while the remaining β-glucans are not soluble (Synytsya et al., 2009).

The analysis of the aqueous washing phase (WW) showed that it contained a high amount of β-glucans that were lost (not reported). Indeed, the extract UW presented significantly higher contents of total glucans (27.46 g/100g d.m.) and β-glucans (21.84 g/100g d.m.) with respect to extract W and, consequently, higher extraction yields (21.34% for total glucans instead of 12.33% and 16.94% for β-glucans instead of 7.57%).

The protein content of *P. ostreatus* powder was 13.82 g/100g d.m. and this value was used to calculate the yield of extraction (Table 2). The contents of proteins in extract W and UW were not significantly different between extract W and UW, 14.12 g/100g d.m. and 14.08 g/100g d.m., respectively corresponding to extraction yields of 20.33% and 25.65%, which are significantly different because of the different dry matter content in the two extract. Likely a part of protein present in the *P. ostreatus* powder lost solubility upon heat denaturation occurring during extraction, while the recovered proteins could be the fraction that is closely associated to β-glucans (Synytsya et al., 2009). Ash contents of the extracts W and UW (9.90 and 15.44 g/100g d. m., respectively) were significantly different.

TABLE 2: Characterization of the extracts obtained from *P. ostreatus*.

Compositional parameters and FRAP values	**Extract**	
	W	**UW**
Dry matter (%)	$1.11^{b} \pm 0.10$	$1.40^{a} \pm 0.10$
Total glucans (g/100g d.m.)	$21.99^{b} \pm 1.12$	$27.46^{a} \pm 0.86$
***Total glucans yield** (%)*	$12.33^{b} \pm 0.63$	$21.34^{a} \pm 0.67$
of which		
β-glucans (g/100g d.m.)	$12.00^{b} \pm 1.41$	$21.84^{a} \pm 0.91$
***β-glucans yield** (%)*	$7.57^{b} \pm 0.78$	$16.94^{a} \pm 1.00$
Proteins (g/100g d.m.)	$14.12^{a} \pm 0.43$	$14.08^{a} \pm 0.30$
***Proteins yield** (%)*	$20.33^{b} \pm 0.56$	$25.65^{a} \pm 0.50$
Ash (g/100g d.m.)	$9.90^{b} \pm 0.14$	$15.44^{a} \pm 0.41$
FRAP (μmol Fe^{2+}/100g d.m.)	$42.25^{b} \pm 5.24$	$63.54^{a} \pm 1.08$

W and UW extracts were obtained as described in Figure 1. Data are average ± SD. Values in the same row with differing superscripts are significantly different (LSD, $p<0.05$).

Regarding the reducing power of the extracts, UW showed a higher value (63.54 μmol Fe^{2+}/100g d.m.) compared to W (46.01 mmol Fe^{2+}/100g d.m.) as observed for β-glucans. The polysaccharide-rich fraction of mushroom has been reported to have antioxidant activity (Li & Shah, 2016). Despite this fraction is obtained after ethanol washing, which can solubilize free phenolic compounds, it brings along phenolic acids which are bound through ester linkages. In fact, Carrasco-Gonzalez et al. (2014) reported that high contents

of coumaric acid and ferulic acid can be released from the fruiting bodies of *P. ostreatus* after alkaline hydrolysis.

3.2 Size distribution and physical stability of liquid emulsions

In a preliminary study, different formulations of O/W emulsions were tested at lab scale, using both the W and UW extracts, while maintaining the same percentage of dry matter (e.g. 40% w/w) but varying the amount of oil, β-glucans and MD, with the purpose to obtain a monomodal distribution with a median diameter (d50) around 2 μm (to ensure encapsulation within solids particles of about 20 μm) and a span value below 2, corresponding to relatively narrow distributions (Gharsallaoui, Roudaut, Chambin, Voilley & Saurel, 2007). The required average oil droplet size and span value were obtained when β-glucan/oil weight ratio was more than 0.014 and when MD/β-glucan weight ratio was lower than 468 (Gallotti et al., 2019). Moreover, size distribution must remain stable during the production process (at least 2 hours). For the four emulsions tested, monomodal oil droplet size distributions were obtained whatever the sunflower oil content (4% or 8% w/w) or the type of extract used (W and UW). Median diameters were below or around 2 μm with span values below 1.5, as expected; however, the emulsion prepared with higher oil content (E20UW) had the highest d50 (2.76 μm) (Table 3). As already observed by Christensen et al. (2001), emulsion size was also found to depend on the oil content, with smaller oil contents leading to smaller emulsion size. In fact, the higher the oil content, the lower the wall material content (for the same total solids content) and hence, the lower the amount of emulsifying agent available to cover the oil droplets, leading to faster droplets coalescence (Frascareli, Silva, Tonon & Hubinger, 2012). On the other hand, no significant differences were observed for the span, d10, d50 and d90 values at the time of preparation and after 2 h for all the emulsions (Table 3).

Hence, all the emulsions obtained by using *P. ostreatus* β-glucans can be considered physically stable, i.e, they showed emulsifying behavior as good as the AG control.

TABLE 3: Oil droplet size distribution in emulsions at the time of preparation (t = 0), after two hours (t = 2h) of rest at room temperature and for reconstituted emulsions.

Emulsion	Volume Size Distribution											
	d10 (µm)			d50 (µm)			d90 (µm)			Span		
	t=0	t=2h	rec	t=0	t=2h	rec	t=0	t=2h	rec	t=0	t=2h	rec
Ec	0.84^{c}	0.83^{c}	0.37^{d}	1.36^{b}	1.37^{b}	1.01^{c}	2.10^{a}	2.12^{a}	2.00^{a}	0.9^{a}	0.9^{a}	1.6^{a}
E10W	1.24^{d}	1.39^{d}	0.28^{e}	2.49^{c}	2.56^{c}	1.01^{d}	4.63^{a}	4.42^{a}	3.39^{b}	1.4^{a}	1.2^{a}	2.9^{a}
E10UW	$1.44^{c,d}$	$1.42^{c,d}$	0.75^{d}	2.50^{c}	2.59^{c}	2.11^{c}	4.19^{b}	4.51^{b}	6.62^{a}	1.1^{b}	1.2^{b}	2.8^{a}
E20UW	$1.57^{d,e}$	$1.66^{d,e}$	1.09^{e}	2.76^{c}	2.83^{c}	2.91^{c}	4.70^{b}	4.75^{b}	8.84^{a}	1.1^{b}	1.1^{b}	2.7^{a}

The formulation of Ec, E10W, E10UW and E20UW are reported in Table 1. d10, d50, d90 and span values in the same row with differing superscripts are significantly different (LSD, $p<0.05$).

It is noteworthy that the β-glucans in the E10W and E10UW emulsions were quantitatively the same but they were most likely different in their molecular weights, since the extract W contained just hot-water-soluble β-glucans and UW contained also β-glucans soluble in water at room temperature. Hence, both the W and UW extracts displayed good emulsifying behavior: however, since the extract UW had a higher extraction yield, it should be chosen over W. Besides β-glucans, the associated denatured proteins present in the hot-water-soluble extract from *P. ostreatus* could exert emulsifying properties as observed for various unfolded proteins (Voutsinas, Cheung, & Nakai, 1983).

3.3 Characterization of dry emulsions

The four emulsions were spray dried under the same conditions (e.g. inlet air temperature 170°C and feed flow rate of 32 $g.min^{-1}$). These conditions were expected to ensure a low drying rate to allow particle shrinkage during drying and avoid formation of hollow particles with entrapped air (Reineccius, 2004; Turchiuli, Gallotti, Hernandez Sanchez & Cuvelier, 2017). During drying of all emulsions, no fouling of the dryer chamber was observed.

The water content and the water activity of the four powders obtained were measured just after the production. For all of them, the moisture content (3.97-4.65 g/100g) was in the range of the minimum moisture specification of dried powder in the food industry which is between 3 and 4 g/100 g (Klaypradit & Huang, 2008). The moisture content of biopolymers may affect the accessibility of oxygen to the oil, thus, the low water contents usually associated with low a_w, might prevent lipid oxidation (Roccia, Martínez, Llabot, & Ribotta, 2014). The a_w levels of the samples were in the range 0.04-0.12, thus below the optimal range to reduced lipid oxidation, which has typically its lowest value at intermediate water activity value (e.g. 0.2-0.5), and increases whether the water activity increases above or decreases below this interval, as reported by Labuza (1970). Hence, it should be advisable to condition the powders in the proper environment before prolonged storage.

The four powders had fine particle size distributions. The median diameter ranged from 19.45 to 24.13 µm with span values between 1.4 and 1.8, corresponding to relatively straight particle size distributions (Table 4).

TABLE 4: Particle size distribution of powders.

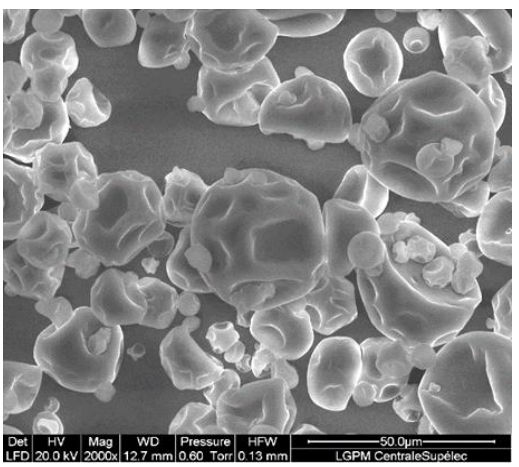
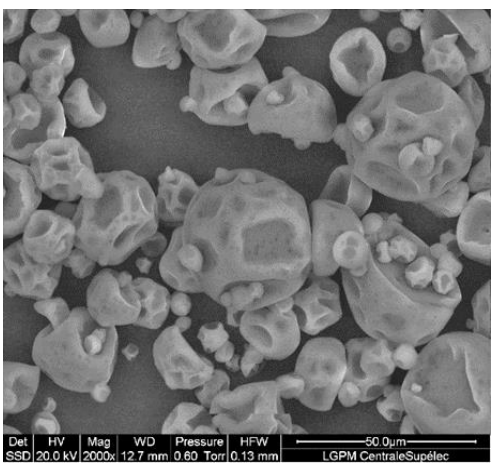
Powder	Size distribution			
	d10 (µm)	d50 (µm)	d90 (µm)	Span
Pc	9.04[a]	24.13[a]	52.69[a]	1.8[a]
P10W	8.13[a]	19.45[c]	40.24[b]	1.7[a]
P10UW	12.34[b]	22.29[b]	44.15[b]	1.4[b]
P20UW	11.12[b]	23.72[a,b]	51.30[a]	1.7[a]

Pc, P10W, P10UW and P20UW were obtained by spray drying of the Ec, E10W, E10UW and E20UW emulsions formulated as reported in Table 1. Values in the same column with differing superscripts are significantly different (LSD, $p<0.05$).

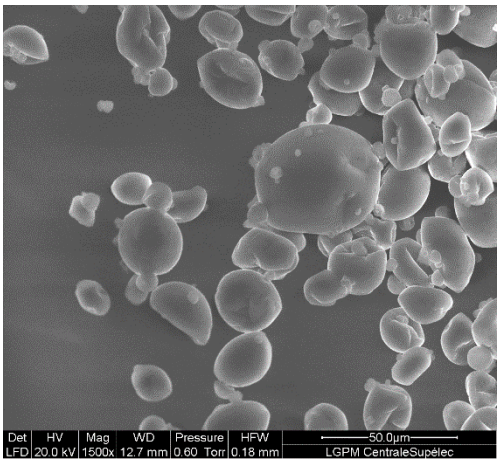
The particle morphology was studied by scanning electron microscopy using LFD detector (gas secondary electrons) (Fig. 2a). For the four powders, the size of the particles observed were similar and in agreement with the size distributions shown in Table 4. They consist mainly of particles of about 20 µm, with both some larger particles (diameter up to about 40 µm) and some smaller ones (diameter < 5 µm). The spray drying of initial liquid emulsion Ec resulted in a powder (Pc) with mainly shrunken particles with a spherical shape. The

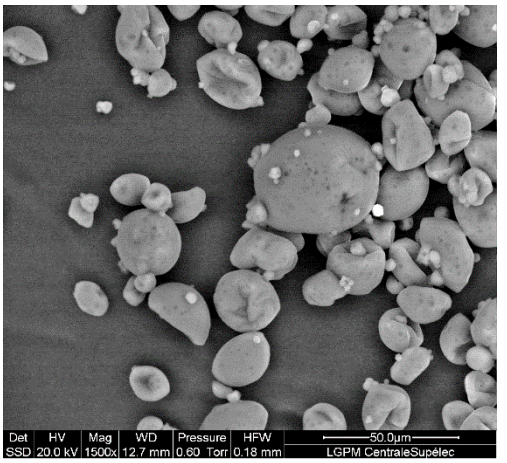
particle surface is concave and shriveled, which is typical of microcapsules produced by spray drying process under low drying rate conditions. No holes or pokes corresponding to the presence of non-encapsulated oil (surface oil) are visible. Particles in the other three powders (P10W, P10UW and P20UW) are comparable in size and size distribution, but they look more spherical with a more regular and smoother surface, also without holes and pokes.

The particle inner microstructure, throughout a thick surface layer of few micrometers, was observed using SSD detector (backscattered electrons) (Fig. 2b). A contrast between the different phases in the dry emulsion is observed due to the difference in the material properties (electron density). The dark spots correspond to material of lower electron density, i.e., the oil droplets, whilst the matrix creates the brightest areas. Contrast between MD and AG in Pc was not observed, which was to be expected due to the similarity of the two materials. P20UW displayed many oil droplets close to the surface, while the frequency of oil droplets in the other powders was lower, indicating that the oil droplets are located some distance away from the particle surface. The results suggest a better EE for Pc, P10W and P10UW, with no or a small amount of non-encapsulated surface oil compared to P20UW.

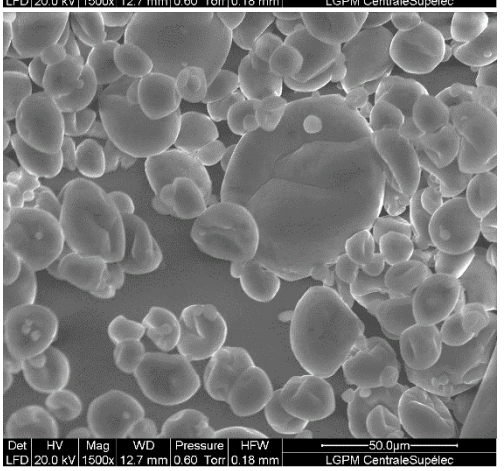
	(a) MORPHOLOGY	**(b) SURFACE MICROSTRUCTURE**
Pc		

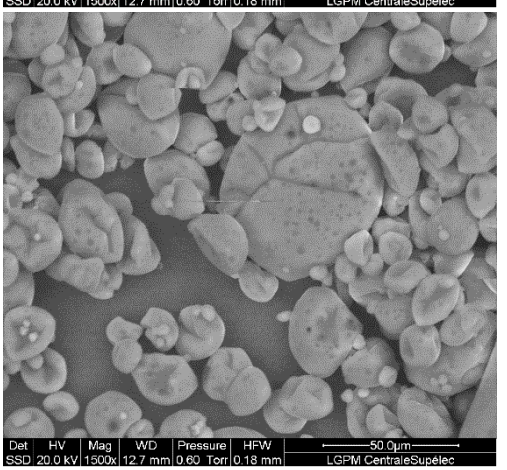
P10W



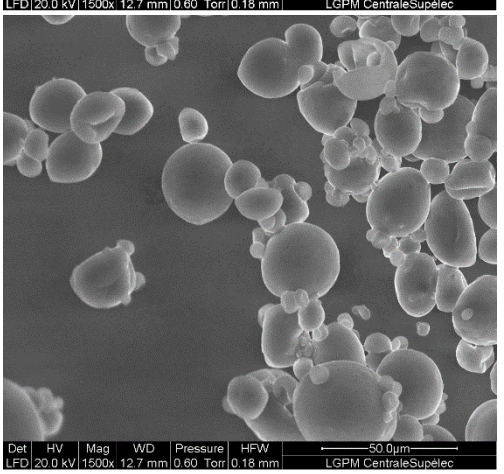


P10UW





P20UW



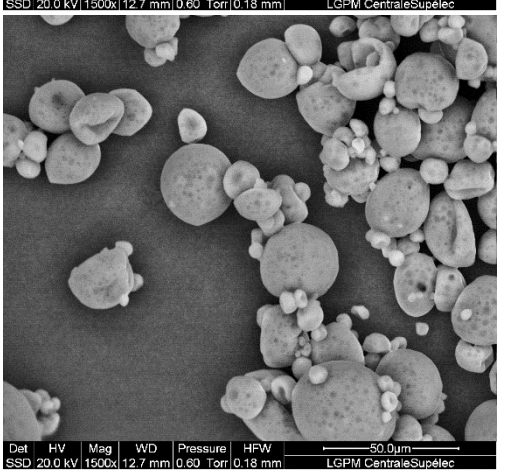


Fig. 2. Scanning electron microscope (SEM) micrographs of the Pc, P10W, P10UW and P20UW powders obtained by spray-drying of the Ec, E10W, E10UW and E20UW emulsions formulated as reported in Table 1.
Initial sample: (a) general view of the powder particles morphology (LFD detector) and (b) thick surface layer microstructure (SSD detector).

In order to observe the internal structure of the particles, the powders were crushed in a mortar to "break" the particles before observation. Anyway, plain shrunk particles were difficult to break and most of the broken particles that were observed were hollow particles. The sectioned particles (Fig. 3) show that the oil droplets, observed as holes in the solid shell, are distributed throughout the thickness of the shell, but not in the close vicinity of the surface. It therefore seems that there is no direct contact between the oil droplets encapsulated in the solid matrix and the air outside.

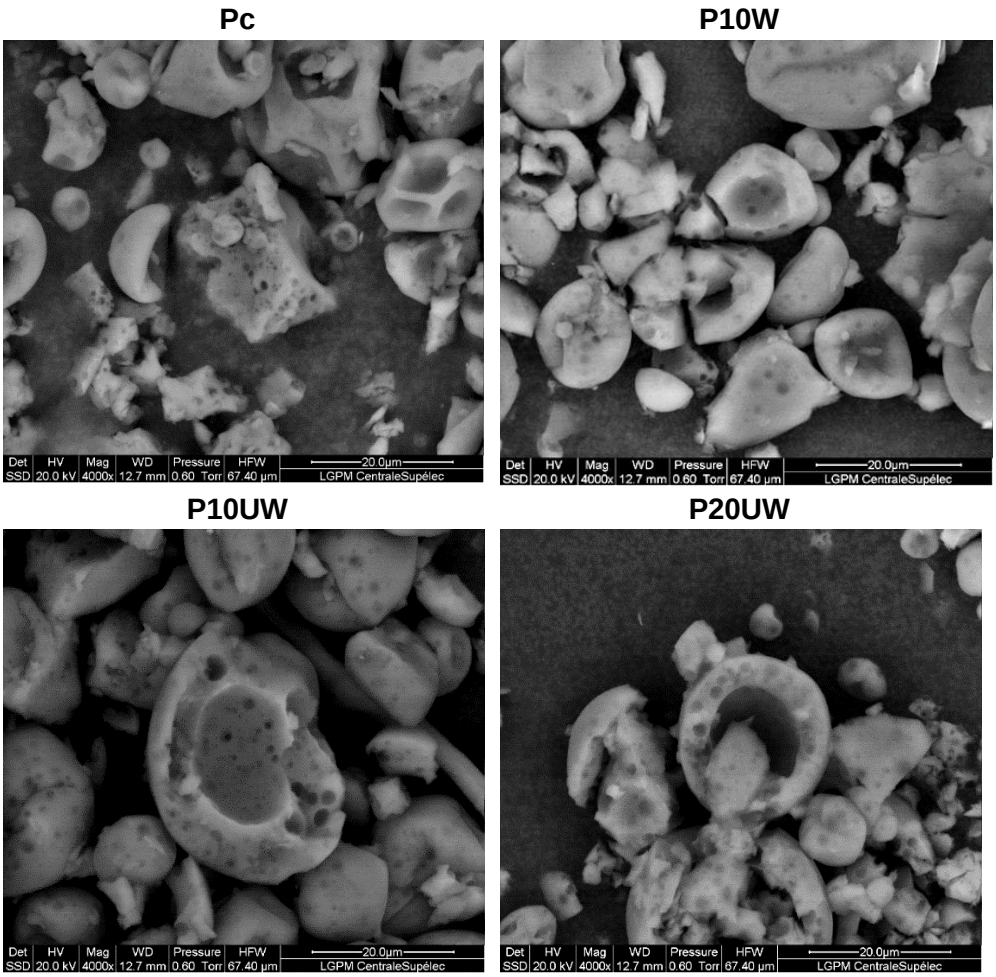


Fig. 3. Scanning electron microscope (SEM) micrographs of spray dried emulsions. Inner microstructure of crushed particles (SSD detector).

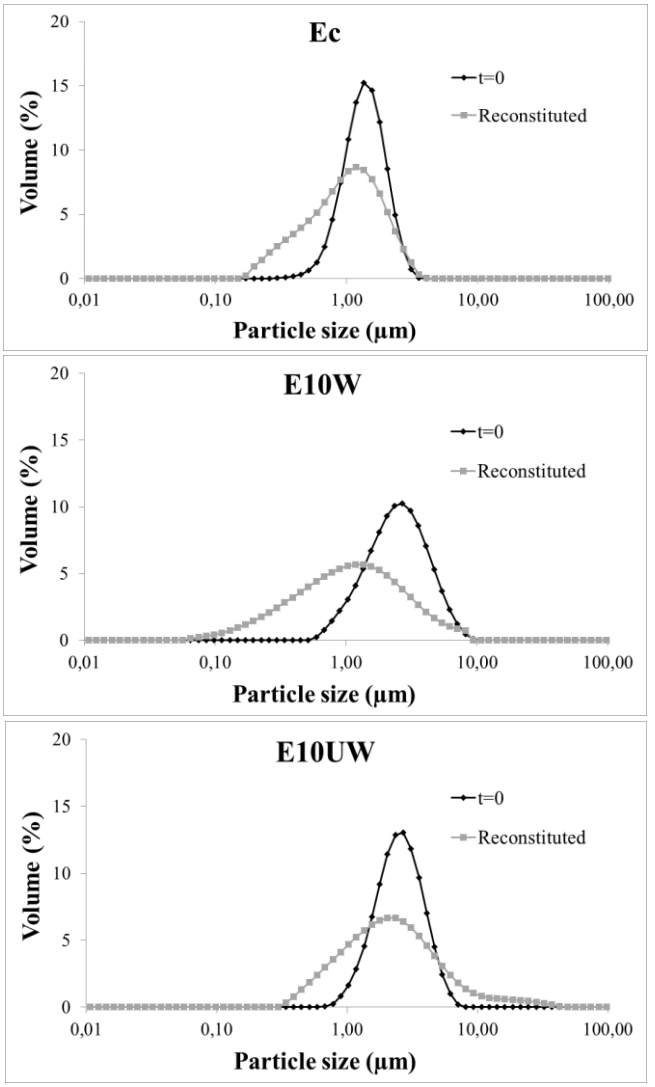
On the other hand, some holes appear at the level of the surface of the central cavity (vacuole), which can act as an oxygen reservoir, in some hollow particles present. The crust of these particles is approximately 5 to 10 μm thick; the thickness of the shell matrix is important for the oil protection in hollow particles since droplets will be located closer to the inner and outer surface in thinner shells leading to easier release and oxidation.

3.4 Powder reconstruction

Due to the high shear stress during atomization, spray drying is likely to cause changes in the emulsion structure with some coalescence or fragmentation of oil droplets that lead to a less efficient encapsulation and the modification of the oil droplet size distribution (Munoz-Ibanez et al., 2015). In order to study the influence of spray drying on the droplets size distribution of the emulsion, powders (dry emulsions) were dissolved in water to “reconstitute” the initial liquid emulsions (with same concentration). It was assumed that the oil droplets size distribution of reconstituted emulsions was representative of that of oil droplets encapsulated in the powder (Hernandez Sanchez et al., 2016). The oil droplet size distribution of initial O/W emulsions produced at pilot scale that were spray dried was monomodal for the four powders (Fig. 4).

The median diameter d50 was between 1.36 and 2.76 μm and span values below 1.4 for all of them (Table 3). These oil droplet size distributions are in agreement with those obtained at lab scale in a previous work (Gallotti et al., 2019) showing the adequacy between reproducibility of the lab and pilot scale protocols used for production of initial emulsions. In any case, the reconstituted emulsions showed slightly wider oil droplet size distributions (Fig. 4). As consequence, span values were larger compared to those of initial emulsions: increase of the span value from 0.9 to 1.6 for Pc, from 1.4 to 2.9 for P10W, from 1.1 to 2.8 for P10UW and from 1.1 to 2.7 for P20UW. For Pc and P10W, change in the span values was due mainly to the formation of some smaller oil droplets, whilst for P10UW and P20UW both smaller and larger oil droplets appeared in the reconstituted emulsions (Fig. 4). Formation of small

oil droplets is attributed to the oil breakup that occurs during the atomization of the emulsion under high mechanical stress (Munoz-Ibanez et al., 2015). The occurrence of large oil droplets in reconstituted emulsions, may correspond to some non-encapsulated oil present on the surface of the dry emulsion particles. However, the size of these droplets (d50 between 1 and 3 µm and d90 between 2 and 9 µm) remains compatible with a good oil encapsulation in the powder particles produced (Table 4).



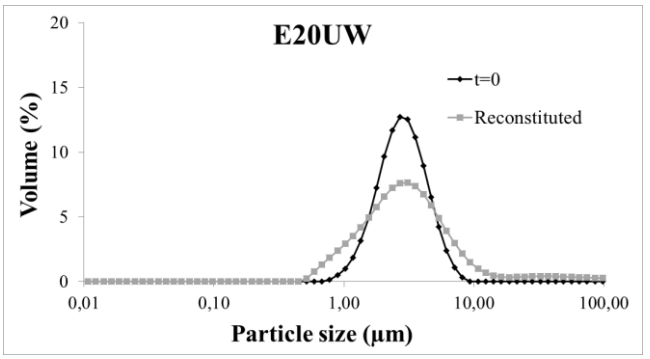


Fig. 4. Volume size distribution of oil droplets in initial liquid emulsions Ec, E10W, E10UW and E20UW formulated as reported in Table 1 and the reconstituted emulsions from the corresponding spray dried powders Pc, P10W, P10UW and P20UW.

3.5 Oil encapsulation efficiency

Different parameters can be considered to estimate the efficiency of oil encapsulation in powders.

First of all, the quantity of extractable oil at the surface of powder particles gives an estimate of the quantity of non-protected oil. For powders Pc, P10W and P10UW, prepared with 10% w/w of oil in dry matter, the surface oil contents measured (SO) were not significantly different and all inferior to 0.73 g/100 g powder corresponding to encapsulation efficiencies (EE) comprised between 92.7 and 95.3%. These values are in agreement with those obtained in a previous study, where the EE reported (for spray dried powders with 10% oil) was above 85% depending on the composition, initial emulsion size and oil droplet breakup during atomization (Hernandez Sanchez et al., 2016). Here, powder Pc showed a high encapsulation efficiency, probably due to its high AG content. The EE of other two powders with the same oil content was not significantly different from the control despite of the very low β-glucan content, demonstrating the high emulsifying properties of *P. ostreatus* β-glucan. The amount of SO measured in powder P20UW was significantly higher (2.71 g/100g of dry powder) with respect to the other powders, corresponding to only 86.5% of the total oil protected in the dry emulsion. The oil concentration is known to significantly influence the EE (Frascareli et al.,

2012). When there are more oil droplets dispersed in the solid matrix, they come closer to the surface at it was observed on the SEM micrographs.

The quality of the encapsulated oil also allows to estimate the efficiency of encapsulation. It was estimated here from the α-toc and CD contents in encapsulated oil just after spray drying, which are markers of lipids oxidation; in fact, CD can be produced during autoxidation (Frankel, 1998). The CD content in the oil evaluated before emulsification was 3.78 ± 0.19. After spray drying, CD was different for each powder but it is noteworthy that the oil encapsulated without β-glucans (in Pc) presented the highest amount of CD. Furthermore, CD content measured in the powder prepared with the extract UW was lower than in the one prepared with extract W. These results may be explained by the antioxidant power of the two extracts, which was found to be higher for the extract UW compared to W (Table 2). Despite its higher oil content and lower EE, the CD content of powder P20UW was low, confirming the protection regarding oxidation of lipophilic bioactive compounds provided by the extract used, with less formation of primary oxidation products. These findings are consistent with the α-toc contents measured. Indeed, α-toc starts to decrease the rate of lipid peroxidation in the propagation stage as it competes with unsaturated fatty acids for peroxyl radicals (Choe & Min, 2006). The control powder Pc showed the lowest quantity of α-toc (3.00 mg/100g of dry powder) while the presence of β-glucans with antioxidant properties in the other three powders provided protection during spray drying. To evaluate the retention of the oxidisable target (α-toc) during spray drying procedure, the actual content of the α-toc in the oil before spray drying was measured (605 mg/kg) in order to calculate the retention percentage, which was reported in Table 5.

It is noticeable that the samples with 10% of oil and β-glucan extracts had higher α-toc retention (95.7% and 80.9%) than the control (64.7%), supporting the hypothesis that the β-glucans can improve delivery of oxidisable target through encapsulation.

TABLE 5: Characterization of oil encapsulation efficiency in powders.

Oil parameters	Powders			
	Pc	P10W	P10UW	P20UW
SO *(g/100g dry powder)*	$0.48^{b} \pm 0.07$	$0.71^{b} \pm 0.17$	$0.73^{b} \pm 0.18$	$2.71^{a} \pm 0.16$
EE *(%)*	$95.3^{a} \pm 1.0$	$92.9^{a} \pm 1.7$	$92.7^{a} \pm 1.8$	$86.5^{b} \pm 0.8$
CD	$12.57^{a} \pm 1.01$	$9.22^{b} \pm 0.72$	$5.49^{c} \pm 0.58$	$7.60^{b,c} \pm 0.16$
α-toc *(mg/100g dry powder)*	$3.00^{d} \pm 0.07$	$3.73^{c} \pm 0.05$	$4.39^{b} \pm 0.01$	$5.96^{a} \pm 0.01$
α-toc retention (%)	$64.7^{c} \pm 1.0$	$95.7^{a} \pm 1.0$	$80.9^{b} \pm 1.1$	$65.5^{c} \pm 1.6$

Pc, P10W, P10UW and P20UW powders obtained by spray-drying of the Ec, E10W, E10UW and E20UW emulsions formulated as reported in Table 1. Data are average ± SD. Values in the same row with differing superscripts are significantly different (LSD, $p<0.05$).

The sample with 20% of oil obtained a value of α-toc retention (65.5%), similar to the control one; this could be explained by the fact that the amount of SO is almost four times higher than the others, thus more subjected to oxidation and less protected by the β-glucan extract antioxidant activity. In general, the values of α-toc retention were in the range of those reported previously for encapsulation by spray drying using different wall materials. Indeed, using soy protein as wall material, Nesterenko et al. (2014) found a retention for α-toc of 79.7%, which increased to 94.8% upon grafting of fatty acid chains to soy protein by acylation. Hategekimana et al. (2015) found the α-toc retention in the range 71.4 - 79.16% using octenyl succinic anhydride modified starch. Interesting, highly satisfactory α-toc retention were observed in this study using a natural extract from a sustainable source. Hence, the glucans rich fractions obtained from *P. ostreatus*, which are known to have antioxidant properties (Khan, Gani, Masoodi, Mushtaq, & Naik, 2017; Rathore, Prasad, & Sharma, 2017; Yan et al., 2019), gave a better protection to lipophilic bioactive compounds, with less formation of primary oxidation products and less depletion of α-toc.

4. CONCLUSIONS

The formulation of a low amount of β-glucans-rich extract form *P. ostreatus* (0.085-0.171% d.m.) with MD allowed producing stable O/W emulsions at pilot scale, with monomodal droplet size distribution and a median diameter of about 2 μm, as achieved for Ec made with a remarkable higher amount of AG (14.4%).

The efficiency regarding spray drying was tested with positive results since dry powders with d50 of about 20 μm were obtained without fouling of the dryer chamber. Moreover, the size distribution of the oil droplets in the reconstituted emulsions, (d50 between 1 and 3 μm and d90 between 2 and 9 μm) indicated that emulsion microstructure was retained despite the shear stress occurring during atomization.

The efficacy regarding protection of oil, evaluated by measuring both the amount of CD and α-toc was markedly higher for the wall material formulated with *P. ostreatus* rich-β-glucans extract, even when the amount of oil was doubled. The usage of emulsifiers with inherent bioactivity, such as *P. ostreatus* β-glucans, appears therefore to be a healthy and sustainable alternative to common emulsifiers such as AG.

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antioxidants

MDPI

Article

Impact of *Pleurotus ostreatus* β-glucans on oxidative stability of active compounds encapsulated in powders during storage and *in vitro* digestion

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ABSTRACT

Polyunsaturated fatty acids and α-tocopherol were encapsulated in powders by spray drying using maltodextrins DE 12 as wall material and different emulsifiers (Tween®20, acacia gum or β-glucans-rich extracts from *Pleurotus ostreatus*). The aim was to study the effects of the surfactants on: a) the oil droplet size distribution and α-tocopherol stability during *in vitro* digestion, and b) the oxidative stability during 15 days of accelerated storage. Acacia gum sample had the most stable particle size distribution up to the gastric phase, but showed a significant α-tocopherol degradation prior to the intestinal stage. On the contrary, β-glucan-samples displayed a bimodal distribution in the oral and gastric phases but retained α-tocopherol up to the beginning of the intestinal stage. At the end of intestinal stage, no α-tocopherol was found in the samples. The storage study showed that β-glucans improved the oxidative stability of the powders, which displayed 82% of α-tocopherol retention after 5 days under accelerated conditions (60 °C), corresponding to 310 days at 20 °C, while acacia gum and Tween® 20 did not delay α-tocopherol degradation. Results highlight the potential antioxidant activity of β-glucans used as emulsifying agent during *in vitro* digestion and accelerated aging conditions.

***Abbreviation List*:** PUFAs, polyunsaturated fatty acids; SO, sunflower oil; α-toc, α-tocopherol; O/W, oil-in-water; MD, maltodextrins; AG, acacia gum; GIT, gastrointestinal tract; TGs, triacylglycerols; MGs, monoacylglycerols; FFAs, free fatty acids; SSF, Simulated Salivary Fluid; SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid; RH, relative humidity; CD, conjugated dienes; SA, specific absorbance; DAD, diode array detector; LSD, least significant difference; SD, standard deviation.

1. Introduction

The importance of oils and their lipophilic compounds lies in their multifunctional role. They have an impact on the organoleptic properties of food products varying their taste, appearance, flavour, texture and shelf life, and they are also necessary for the maintenance of the function and structure of the human body and for the preservation of the well-being [1]. In particular, lipophilic compounds rich in large unsaturated hydrocarbon chains, named polyunsaturated fatty acids (PUFAs), are responsible for exercising various biological action, such as preserving cell-membrane fluidity, decreasing secretion of pro-inflammatory cytokines by monocytes/macrophages, reducing vulnerability to ventricular rhythm disorders of the heart, inhibiting inflammatory processes, preventing blood platelet aggregation, reducing triglyceride synthesis in the liver and improving functions of vascular endothelial cells [2]. Sunflower oil (SO) contains a considerably high amount (60%) of PUFAs, which are more prone to lipid oxidation than saturated ones, mainly due to their low activation energy for fatty acid radical's formation [3]; oxidation of PUFAs is damaging to cell function and leads to instabilities in membrane function and structure. Therefore, it is important to consume foods rich in antioxidants, since they are potentially able to quench or neutralize excess radicals. Vitamin E is the main lipid-soluble antioxidant in the cell antioxidant defence system and is entirely obtained from the diet. There are eight possible isomers of vitamin E, but α-tocopherol (5,7,8-trimethyltocol) (α-toc) is the most biologically important antioxidant *in vivo* [4]. SO contains up to

59 mg of α-toc per 100 g of oil, more than the other commonly consumed vegetable oils, i.e., palm, soybean and canola oil [5]. The major biological role of vitamin E is to protect PUFAs and other components of cell membranes from oxidation by being preferentially oxidized by free radicals. However, the amount of α-toc in biological membranes is approximately one part per 1000 lipid molecules, and the replenishment of α-toc is primarily achieved through dietary food components. Consequently, the protection of α-toc against factors promoting its degradation is critical to maintain efficacious concentrations on this bioactive compound in foods [6].

Encapsulation in powder improves the stability of the functional properties of lipophilic compounds by adding a physical barrier. The first step to encapsulate lipophilic compounds in powder consists in the preparation of a stable oil-in-water (O/W) liquid emulsion. Therefore, the choice of wall material and emulsifying agent is of great importance in order to maximize the incorporation and retention of the functional compounds [7]. Maltodextrins (MD), for example, are relatively low-cost polysaccharides with high solubility in water, low viscosity at high solids concentrations (e.g. up to 40–60% w/w dry matter), neutral taste and aroma and that acts as an effective wall material [7,8]. However, since MD have no interfacial properties, they must be used in association with surfactants, such as polyoxyethylene (20) sorbitan monolaurate (Tween®20), or emulsifiers, such as acacia gum (AG), to produce physically stable emulsions. Several researchers have studied the use of mixture of these coatings to encapsulate lipophilic bioactive compounds [1,8-10]. In addition to the physical protection provided by encapsulation, the usage of compounds with antioxidant properties in the wall material, such as β-glucans, can make a valuable contribution for the protection and preservation of food. Antioxidant activity of β-glucans derived from microorganisms such as *Paenibacillus polymyxa* and mushrooms such as *P. ostreatus* have been studied [11,12]. In a preliminary study, β-glucans-rich extracts form *P. ostreatus* were successfully used both for the preparation of stable O/W liquid emulsions and for the protection, during spray drying, of SO encapsulated in

them. The efficiency regarding oil protection was evaluated by measuring both the amount of conjugated dienes (CD) (as evaluation of the primary oxidation compounds) and α-toc after spray drying, with positive results [7].

Oxidative degradation of food lipids also occurs during digestion because of the pro-oxidant conditions of the gastrointestinal tract (GIT) (oxygen incorporated during mastication, low pH of gastric juice, and presence of reactive species like metallic ions). Moreover, the products of the enzymatic hydrolysis of food lipids are prone to oxidative degradation [13]. Consequently, the bioavailability of lipid-based components of nutritional interest depends on multiple factors such as the lipid content of the food, the unsaturation degree, and the initial oxidative status of the lipids, the presence of phenolic compounds or proteins in the food bolus, and processing conditions before ingestion, among others [13]. Oxidative loss of tocopherols during *in vitro* digestion has also been reported by Kenmogne-Domguia et al. [14], which could be prevented by the addition of protective compounds with antioxidant properties, like β-glucans rich extracts from *P. ostreatus.*

Besides the protection from degradation during digestion, it is crucial that a bioactive compound has a high bioavailability after ingestion, so that it can effectively deliver its positive biological effects [15]. Bioavailability of lipophilic vitamins, such as vitamin E, encapsulated in O/W emulsions is closely related to the absorption of dietary lipids.

First, these bioactive compounds must be released from the food matrix (i.e., the lipid phase surrounding the vitamins must be digested) [15,16]. Dietary lipids, like SO, are mainly composed of triacylglycerols (TGs) which are water-insoluble macromolecules that cannot be transferred from the intestinal lumen to the enterocytes. The ingested TGs must be emulsified and hydrolysed to monoacylglycerols (MGs) and free fatty acids (FFAs) to be absorbed in the GIT [17]. The pancreas is the main source of lipid-digesting enzymes, and the primary site of TGs hydrolysis is therefore the first part of the small intestine, the duodenum. However, the stomach also plays an important role in lipid digestion since its peristaltic movements induce the formation of O/W

emulsions [18], and gastric lipase can hydrolyse part of the TGs ingested [19,20]. When lipids enter the duodenum, bile and pancreatic juice are released. Pancreatic lipases hydrolyse TGs to diacylglycerols as intermediates, and MGs and FFAs as final products. Bile salts increase the efficacy of lipolysis, increasing the surface area of oil-water interfaces at which water-soluble lipase is active [21]. The products generated from lipid digestion interact with bile salts, phospholipids, and other lipophilic compounds to form mixed micelles, which are transported across the mucus layer to the brush border membrane of the enterocytes where they are absorbed [22]. Lipophilic vitamins like α-toc must be incorporated in the hydrophobic interiors of those mixed micelles to be absorbed by the enterocytes, and secreted into the blood stream via the lymphatic system. Bioaccessibility of lipophilic vitamins like α-toc therefore depends on the total amount of mixed micelles and their nature [23]. Variations may also be due to the chemical nature of the vitamin, since tocopherol esters must be further hydrolysed by digestive enzymes before absorption [24].

To improve the bioavailability as well as the protection from oxidation of lipophilic compounds, different techniques can be employed. Lipophilic contents could be entrapped in the double layer of liposomes; this method has the advantage to be therapeutic efficient but it is also characterized by several drawbacks, such as low process replicability, low encapsulation efficiency, and large particles size distribution [25]. Encapsulation techniques based on drying processes are often employed and, among them, spray drying is one of the most widely used for food industry due to its low-cost and flexibility [26]. Encapsulation by spray drying with β-glucans may increase the stability and availability of bioactive compounds; in fact, Ahmad et al. [27] discovered that the stability of saffron anthocyanins during passage through simulated GIT conditions was increased by using β-glucans extracted from barley. Also, the bioavailability of anthocyanins in the intestinal section was increased. However, since the source, molecular weight, molecular structure, and types of bonding interactions have effects on the physicochemical and nutraceutical

properties of β-glucans, further studies are required to better understand the behaviour of β-glucans from different sources, such as *P. ostreatus* mushroom.

Oxidative stability of oils has been defined as the resistance to oxidation not only during processing, but also during storage [28]. Resistance to oxidation can be expressed as the period of time required to reach the critical point of oxidation, whether it is a sensorial alteration or a rapid acceleration of the oxidative process. Oxidative stability is a significant indicator to determine shelf life and oil quality, since the low-molecular-weight off-flavour compounds produced through oxidation make oil undesirable to consumers or for industrial use as a food ingredient [29]. In order to implement lipophilic nutraceuticals enriched foods in the perspective of a value chain approach, long-storage stability is crucial; however, because of their high instability to oxidative deterioration, foods enriched with these compounds has been technically challenging [6,30].

In the first part of the study, the stability of α-toc in the GIT was investigated *in vitro* using the standardized INFOGEST method [31,32]. Additionally, the variations in size of the oil droplets throughout the different phases of the simulated GIT (oral, gastric and intestinal) were measured. The second objective of this work was to study the oxidative stability of SO, free or encapsulated in powder, with or without β-glucans rich extracts from *P. ostreatus*, correlated with α-toc degradation, in order to explore the potential protective effect exercised by the addition of a β-glucan-rich extracts on the oxidative status during storage. Our goal was to obtain a well-designed delivery system that can encapsulate bioactive lipophilic compounds in order to protect them from degradation during storage and digestion while ensuring satisfying bioavailability.

2. Materials and Methods

2.1. Materials

MD DE 12 (Glucidex®, Roquette, FR) were used as main wall material for the encapsulation process. One control emulsion (Ec_AG), was stabilized by adding AG (Instantgum AA, Nexira, France), while for the other control emulsion (Ec_Tween), Tween® 20 (Sigma Aldrich, FR) was used as surfactant [33]. Sample emulsions were stabilized with two β-glucan-rich extracts obtained from *P. ostreatus* (IoBoscovivo, IT) as described in a previous study [7] and labelled as (W) and (UW). Commercial SO containing 60% w/w polyunsaturated, 29% w/w monounsaturated and 11% w/w saturated fatty acids and 0.05% w/w α-toc (Cora, FR) was used as a model for lipophilic compounds encapsulation in powders. Free SO was also used for accelerated thermo-oxidation, together with the five powders Pc_Tween, Pc_AG, P10W, P10UW and P20UW, obtained after spray drying production in similar conditions as previously described [7,33]. Powders composition is described in Table 1.

Table 1. Composition of the powders used for the study and β-glucans and proteins content of dry matter in the extract.

Powder code	**Powder composition**					**Extract d.m. composition**	
	SO (%)	Tween® 20 (%)	AG (%)	MD (%)	Extract d.m. (%)	β-glucans (%)	Proteins (%)
Pc_Tween	10	0.3	---	89.7	---	---	---
Pc_AG	10	---	36	54	---	---	---
P10W	10	---	---	89	1	0.21	0.21
P10UW	10	---	---	89	1	0.22	0.14
P20UW	20	---	---	78	2	0.43	0.29

Extract d.m. is the dry matter of the W and UW hot water extracts from *P. ostreatus* obtained as previously described [34].

For in vitro digestion, α-amylase (A1031, measured activity ≈ 1330 IU/mg) from human saliva, pepsin (P6887, measured activity ≈ 2820 U/mg) from porcine gastric mucosa, pancreatine (P1750, measured activity ≈ 3.416 U/mg) from

porcine pancreas, bile porcine extract (B8631), Pepstatine-A (P5318), and Pefabloc (P76307) were obtained from Sigma-Aldrich (MO, USA). Standard α-toc (VWR International PBI, IT) was used for HPLC calibration. Different solvents were used to perform analysis: hexane (quality CHROMASOLV®) and isopropanol (quality CHROMASOLV Plus) were obtain from Sigma-Aldrich (FR). Tetrahydrofuran (THF), methanol (quality LC-MS), n-heptane and isooctane were purchased from Carlo Erba Reagents (FR). All other chemicals used were purchased from Sigma Aldrich (IT).

2.2. Methods

2.2.1. In vitro digestion

Reconstituted emulsions (Ec_AG, E10W, E10UW and E20UW from Pc_AG, P10W, P10UW and P20UW, respectively) were prepared by mixing 5 g of each powder sample with 7.5 mL of distilled water (corresponding to the same concentrations as the emulsions before drying), stirred for 5 min at room temperature and immediately analysed for oil droplet size distribution (1 mL, Section 2.2.2) and *in vitro* digestion (5 mL). The *in vitro* digestibility of these reconstituted emulsions was then immediately assessed according to the guidelines of the INFOGEST network [31,32]. The digestion process was divided in three phases, called oral, gastric and intestinal phases. For each phase, the composition and pH of the simulated digestive fluids were replicated according to the INFOGEST recommendations and enzyme characterization assays were performed to determine enzyme activities [31,32]. The temperature was kept at 37 °C during the entire digestion process and preheated solutions were used during the procedure to avoid temperature variations.

Oral phase

An amount of 9 g of reconstituted emulsion was mixed in a 50 mL conical tube with 7.2 mL of Simulated Salivary Fluid (SSF) stock solution, and pH was adjusted to 7.0 if needed (with a 1 M NaOH solution or a 1 M HCl solution,

accordingly). Next, 45 µL of a 0.3 M $CaCl_2$ solution, 0.5 mL of salivary amylase solution (75 IU/mL), and distilled water were added to reach a total volume of 18 mL. The tube was placed in a dry block incubator at 37°C and 400 rpm for 2 min. At that point, 6 mL of the sample were withdrawn and immediately analysed (Section 2.2.2) or mixed with a hexane/iso-propanol solution (3:1, v/v) to inactivate the enzymes before further analysis (Section 2.2.4 and 2.2.6). Finally, the pH of the remaining oral phase mixture was lowered to 3 to inactivate the amylases.

Gastric Phase

An amount of 9.6 mL of Simulated Gastric Fluid (SGF) stock solution was added to the oral phase mixture, pH was checked, and adjusted if needed, before adding 6 µL of a 0.3 M $CaCl_2$ solution, 0.5 mL of pepsin solution (2000 U/mL) and distilled water to reach a final volume of 24 mL. The mixture was incubated at 37 °C and 400 rpm for 2 h. At that point, 11 mL of sample were withdrawn and mixed with 110 µL of Pepstatin-A to inhibit the pepsins before further analysis. Finally, the pH of the remaining gastric phase mixture was increased to 7 to inactivate the enzymatic activity.

Intestinal Phase

7.4 mL of Simulated Intestinal Fluid (SIF) stock solution were added to the gastric phase mixture, pH was checked, and adjusted if needed, before adding 26 µL of a 0.3 M $CaCl_2$ solution, 1 mL of 10 mM bile extract, 3 mL of pancreatin solution (100 TAME U/mL) and distilled water to reach a final volume of 26 mL. The resulting mixture was incubated at 37 °C and 400 rpm for 2 h. At that point, 23 mL of sample were withdrawn and mixed with 230 µL of Pefabloc to inactivate the pancreatic enzymes before further analysis.

In vitro digestion of the reconstituted emulsions was repeated three times for each sample.

2.2.2. Oil droplet size distribution

The oil droplet size distribution of emulsions before and during *in vitro* digestion was measured by LASER light diffraction (Mastersizer 2000; Malvern, FR) in

wet mode (Hydro 2000) after dispersion in distilled water. For the oil droplets, the refractive index value used was 1.475. The number weighted size distribution was used to study the particle size distribution of the emulsions, as well as the surface area moment mean D[3,2] and the volume moment mean D[4,3]. The D[3,2] is mostly sensitive to the presence of fine particles in the size distribution, while the D[4,3] is the most sensitive to the presence of large particles in the size distribution; D[3,2] was also studied in order to estimate the impact of the surface area of particles on oil oxidation.

2.2.3. Accelerated thermo-oxidation

The spray dried powders were collected in polyethylene bags suitable for vacuuming, 0.20 mm thickness, and then stored under vacuum at -20 °C in the dark until use. In order to evaluate their oxidative stability and the protective effect exercised by β-glucan-rich extracts, an accelerated storage study was performed as follows: 30 g of each powder were equally distributed in three open glass Petri dishes (10 g powder/petri dish). The petri dishes were casually placed into a climatic test chamber (HC 0020, Vötsch Industrietechnik GmbH, DE) at 60 °C and 50% relative humidity (RH) during 15 days. Samplings were done on triplicate at different times. When analyses of some samples needed to be delayed for practical reasons, samples were stored in sealed plastic bags under vacuum at - 20 °C in the dark for few days. In a previous study, it was checked that no evolution regarding oxidation happened during frozen storage for powders [1]. In order to evaluate the oxidative stability of free oil, SO was equally distributed in three open glass Petri dishes, forming a thin layer and stored at the same conditions as the powders.

According to Lee et al. [3], activation energies of 79.496 kJ/mol and 83.680 kJ/mol were assumed for peroxide formation and degradation of tocopherols in SO, respectively. In order to predict storage at room temperature (20 °C) based on the data obtained at 60 °C, the Arrhenius law was applied, in order to find the acceleration factor of peroxide formation in SO (1):

$$\frac{k60°C}{k20°C} = 50 \quad (1)$$

and degradation of tocopherols in SO (2):

$$\frac{k60°C}{k20°C} = 62 \quad (2)$$

Hence, one day storage at 60 °C can then be considered equivalent to 50 days and 62 days for peroxide formation and degradation of tocopherols in SO, respectively, at 20 °C. Thus, in the present study a period of 15 days at 60 °C was chosen for the accelerated storage tests, pointing to notice quality deterioration of SO, simulating shelf life of 2 years and 26 days for peroxide formation in SO and of 2 years and 200 days for degradation of tocopherols in SO at 20 °C.

2.2.4. Oil phase extraction

The oil phase extraction from powders at different days of storage was performed according to the method described by Gallotti et al. [7]. To reconstitute the emulsions, 2 mL of purified water at 30 °C were added to 0.5 g of powder in a conical tube and agitated. Then, 50 mL of hexane/iso-propanol (3:1, v/v) were added to the reconstituted emulsion. The mixture was placed in an ultrasonic bath for 15 min and centrifuged at 1000×g for another 15 min at 20°C. After centrifugation, around 47 mL of organic phase were recovered, where 40 mL was used for α-toc analysis and 1 mL was used to measure CD. The oil phase extraction from oral, gastric and intestinal digestion of powders was performed according to the same method with few modifications. In brief, 2.5 mL, 5 mL and 10 mL of oral, gastric and intestinal phase, respectively, were withdrawn from the vials used for *in vitro* digestion (Section 2.2.1). These quantities were selected taking into account the dilution factor of every digestion phase. Then, 50 mL of hexane/iso-propanol (3:1, v/v) were added to the samples. The mixture was placed in an ultrasonic bath for 15 min and then centrifuged at 1000×g for 15 min at 20°C. After centrifugation,

around 47 mL of organic phase were recovered, where 40 mL were used for α-toc analysis.

2.2.5. Measurement of CD

For measurement CD in free SO, 1 drop (e.g. 15-20 mg) was weighed in a 10 mL volumetric flask adding isooctane to get to volume; then, the vial was vortexed for 60 s. The sample absorbance was measured using a UV–visible spectrophotometer (UVIKON 941 Plus, Serlabo Technologies, FR) and the specific absorbance (SA) was calculated according to the following equation (3):

$$\mathrm{SA} = \frac{Abs234nm}{Wg} \quad (3)$$

where *Abs234 nm* is the absorbance of the sample measured at 234 nm and *Wg* is the SO mass (g) in 100 mL of the solvent solution analysed.

For oil encapsulated by spray drying, measurements were made directly on the organic phase, which contained the extracted SO. For this purpose, 1 mL of the organic phase extracted as described above (Section 2.2.4) was diluted 1:6 by adding 5 mL of hexane/iso-propanol (3:1, v/v), and was vortexed for 30 s. The SA was calculated according to the following equation (4):

$$\mathrm{SA} = 6 * \frac{Abs234nm}{\frac{g\ Calculated\ Oil}{47\ mL} * 100\ mL} \quad (4)$$

where *Abs 234 nm* is the absorbance of the sample measured at 234 nm, *g Calculated Oil* is the calculated mass of oil contained in 0.5 g of powder and recovered in 47 mL of organic phase.

Results were expressed according to the following equation (5):

$$\Delta\mathrm{SA} = \mathrm{SA}(t) - \mathrm{SA}(0) \quad (5)$$

where SA(t) is the SA at a specific storage day (day t) and SA(0) is the SA at the beginning of storage (day 0).

2.2.6. Measurement of α-toc concentration

The chromatographic determination of α-toc was performed on a HPLC system as previously described by Gallotti et al. [7]. To measure the α-toc in the oil phase of powders, a precise volume (40 mL) of the organic phase, obtained as described above (Section 2.2.4), was evaporated in a flask by a rotary rotavapor (Heidolph Laborota 4000 efficient, WB eco, DE). Then, 5 mL of methanol were added and the flask was placed in an ultrasonic bath for 5 minutes. The solution was filtered through a nylon syringe membrane with 0.45 µm pore size and 10 µL of the sample were injected and analysed using a model Shimadzu LC-20 AD pump coupled to a model Shimadzu SPD-M20A photodiode array detector and an RF-20 AXS operated by Labsolution Software Shimadzu, JP. A 5 µm Sunfire C18 column (250 × 4.6 mm; Waters, IT) equipped with a C18 precolumn (Waters, IT) was used for the separation, performed by isocratic elution thermostated at 28°C, at a flow rate of 1 mL*min-1 with a runtime of 35 min, using methanol/water (95:5, v/v) as mobile phase. Fluorimetric detection was made at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. To measure α-toc content of free SO, one drop was weighed in a 10 mL volumetric flask and methanol was added to complete the volume. The flask was then vortexed for 60 s and placed in an ultrasonic bath for 5 min. The solution was filtered through a nylon syringe membrane with 0.45 µm pore size and 10 µL of the sample was injected and analysed as described for the powders. To measure the α-toc in the oil phase of digested powders, a precise volume (40 mL) of the organic phase was evaporated in a rotary rotavapor RE 120 (Buchi, CH). Then, 5mL of n-heptane was added to the extracted oil and the flask was vortexed for 60 s and placed in an ultrasonic bath for 30 s. The solution was filtered through a nylon syringe membrane with 0.45 µm pore size and put into vials. Then, 20 µL of the sample was injected and analysed with an HPLC system (Waters®, MA, USA) equipped with a pump (Waters® 2695) coupled with a UV– visible diode array detector (DAD) at 298 nm (Waters® 996). The stationary phase consisted in a bonded silica column 100 Diol (Lichrosphere, length 250 mm, internal diameter

4 mm, particle size 5 μm), thermostated at 25 °C and equipped with a precolumn (13 mm) with similar characteristics. The mobile phase was a mixture of n-heptane/tetrahydrofuran (96.15/3.85, v/v) eluted isocratically at a flow rate of 1 mL/min with a runtime of 12 min. A calibration curve was built with a purified standard was used for the identification and quantification of α-toc.

The degradation of α-toc was expressed according to the following equation (6):

$$\alpha - \text{toc degradation } (\%) = 100 * \frac{C0 - Ct}{C0} \quad (6)$$

where C0 is the concentration of the component at the beginning of storage (day 0) and Ct is the concentration measured at a specific storage day (day t).

2.2.7. Statistical analysis of data

Experimental data were investigated using one-way ANOVA with the least significant difference (LSD) as a multiple range test, and by linear regression analysis using Statgraphics 5.1 (STCC Inc., MD, USA). These results are reported as the average of triplicate values ± standard deviation (SD).

3. Results

3.1. Effect of in vitro digestion on emulsion structure

Particle size distribution of the four reconstituted emulsions was measured before *in vitro* digestion and after each stage of the INFOGEST protocol. The emulsions had a different structure prior to digestion. Ec_AG had the smallest oil droplets (D[3,2] = 0.81 ± 0.01 μm) and E20UW the largest (D[3,2] = 2.64 ± 0. μm) (Table 2). This was expected since E20UW had a higher oil content than the other samples, which is known to influence emulsion size [35]. Oil droplets in the three emulsions stabilized with the β-glucans-rich extracts from *P. ostreatus* were larger than the oil droplets in the emulsion stabilized with AG, as described in a previous study [7]. After the simulated oral phase, no significant changes were observed in the particle size distribution of Ec_AG

(Table 2), meaning that the salivary amylase did not affect the structure of the emulsion. Particle size distribution were slightly shifted to lower values for E10W and E10UW (Fig. 1b, c), and an increase in D[4,3] was measured for E20UW (Table 2). These results suggest that these emulsions were less stable than Ec_AG after exposure to the oral phase conditions. This may be related to the content in MD of the original powders (Table 1). Salivary amylase in the simulated oral fluid may have partially hydrolysed MD used as wall material, leading to the disruption of some oil droplets (E10W, and E10UW) or the coalescence of the largest less stable oil droplets (E20UW).

Table 2. Oil droplet size distribution statistics in reconstituted emulsions before *in vitro* digestion and after the oral, gastric and intestinal phase of the INFOGEST protocol.

Emulsion reconstituted	**Digestion stage**	**Particle size**	
		D (3,2)	**D (4,3)**
Ec_AG	Before	0.81 ± 0.01 A a	1.15 ± 0.01 A a
	Oral	0.75 ± 0.05 A a	1.13 ± 0.03 A a
	Gastric	0.77 ± 0.06 A a	1.13 ± 0.03 A a
	Intestinal	0.68 ± 0.15 A a	299.15 ± 100.12 B a
E10W	Before	1.76 ± 0.01 A b	2.58 ± 0.36 A a,b
	Oral	1.67 ± 0.04 A b	3.46 ± 0.43 A a
	Gastric	1.53 ± 0.24 A b	4.19 ± 1.58 A a,b
	Intestinal	0.80 ± 0.10 B a	305.13 ± 83.79 B a
E10UW	Before	2.23 ± 0.02 A c	4.16 ± 0.90 A b,c
	Oral	1.88 ± 0.31 A b	5.34 ± 3.60 A a
	Gastric	2.01 ± 0.33 A b	17.28 ± 1.46 B c
	Intestinal	1.83 ± 0.56 A a	407.13 ± 37.51 C a
E20UW	Before	2.64 ± 0.05 A d	4.37 ± 0.38 A c
	Oral	2.45 ± 0.22 A c	16.81 ± 6.63 B b
	Gastric	2.68 ± 1.10 A c	9.53 ± 3.08 B b
	Intestinal	8.39 ± 3.05 B b	233.05 ± 27.77 C a

In the same column, significant differences ($p < 0.05$) are indicated by capital letters (A, B, C) when comparing different digestion stages, and by lower-case letters (a, b, c) when comparing different emulsions. Sample codes are explained in Section 2.2.1.

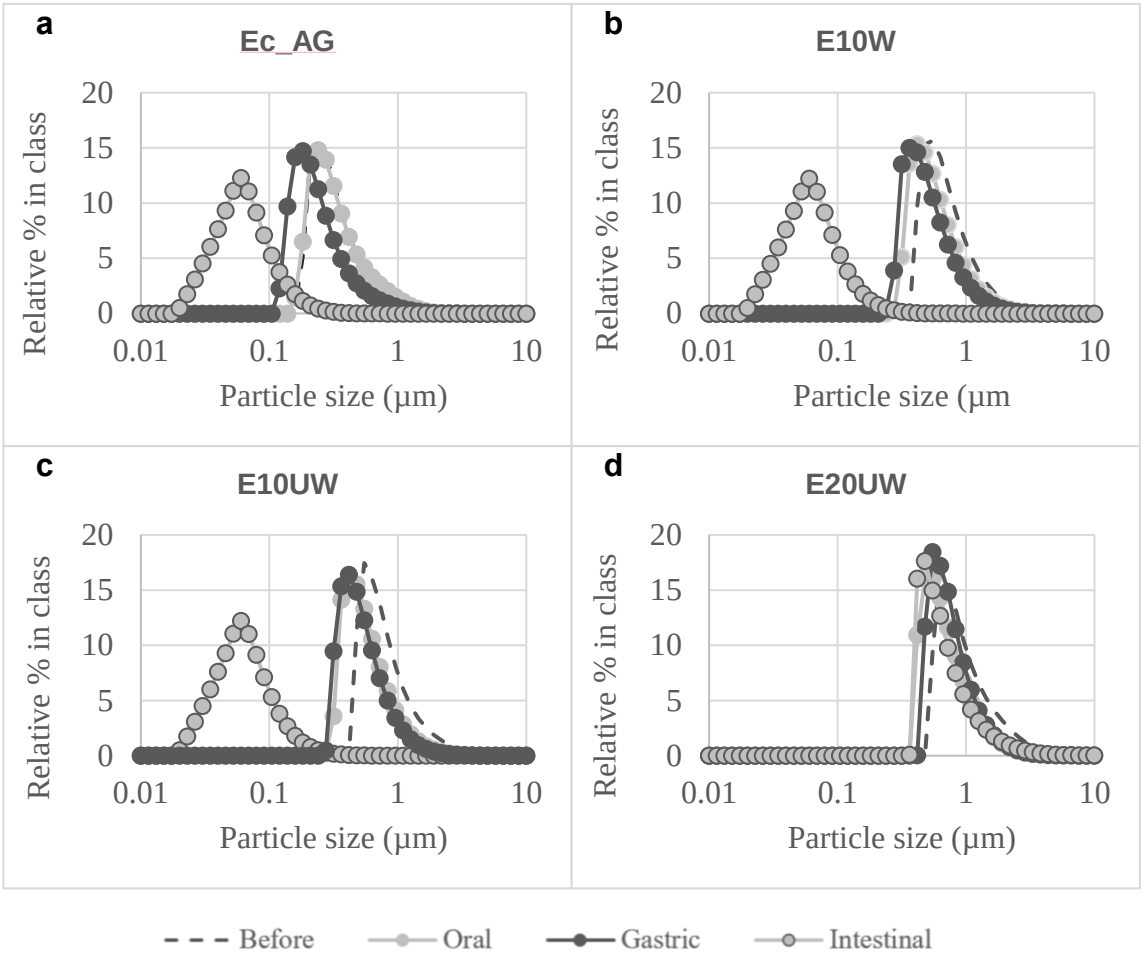

Figure 1. Effect of *in vitro* digestion on the number weighted particle size distributions of SO reconstituted emulsions stabilized with AG **(a)**, β-glucan-rich extracts washed **(b)**, and unwashed **(c, d)**.
Sample codes are explained in Section 2.2.1.
Results presented are representative data for three separate experiments.

After the simulated gastric phase, the particle size distribution of Ec_AG was slightly shifted to lower values (Fig. 1a), but no significant changes were observed in D[3,2] and D[4,3] (Table 2). These results agree with previous studies on emulsions stabilized by AG [36,37]. AG is an amphiphilic polysaccharide-based emulsifier, which attaches on the surface of the oil droplets and forms a thick interfacial layer resisting aggregation over a wide range of conditions, like highly acidic conditions, through strong steric

repulsions [37-39]. Similar results were observed for E10W after exposure to the gastric phase conditions (Fig. 1b, Table 2). Consequently, for the control sample (Ec_AG) and the sample containing the W extract (E10W), the structure of the emulsion that reached the intestinal phase was similar to the structure of emulsion before *in vitro* digestion. In contrast, an increase in D[4,3] was measured for E10UW after the gastric phase (Table 2), meaning that the amount of large oil droplets in the emulsion increased. This partial coalescence is probably due to the protonation of carboxyl groups on the polar regions the emulsifier (i.e., proteins associated to β-glucans in the mushroom extracts) under highly acidic conditions, leading to a decrease in electrostatic repulsions between the oil droplets. However, the majority of the oil droplets were still resistant to aggregation (Fig. 1c), suggesting that they were mostly protected by steric repulsions. No further changes were observed for E20UW after exposure to the gastric phase conditions (Table 2).

At the end of the *in vitro* digestion, a significant decrease in particle size was observed for Ec_AG, E10W and E10UW (Fig. 1a, b, c), which can be attributed to the hydrolysis of the oil droplets by pancreatic enzymes and the formation of mixed micelles by bile salts and lipid digestion products. The mixed micelles produced from the *in vitro* digestion of Ec_AG, E10W, and E10UW were similar in size (mode ≈ 60 nm). Tan et al. [37] also observed the formation of mixed micelles after *in vitro* digestion of β-carotene loaded corn oil O/W emulsions stabilized with AG. On the other hand, the D[3,2] value measured for E10UW at this stage was higher than for E10W. Therefore, it appears that E10UW was less digested by the pancreatic lipases than E10W. This may be related to irreversible coalescence in the gastric phase, since the size of the oil droplets is known to influence the kinetics of lipolysis. Larger droplets have a smaller surface area available for interaction with lipase molecules, resulting in a decrease in the rate of lipolysis [40,41].

No significant changes were observed in the particle size distribution of E20UW at the end of the *in vitro* digestion (Fig. 1d). But an increase in D[3,2] was noted (Table 2), probably due to partial droplet coalescence. These

results suggest a reduction in the physical stability of E20UW during this last phase of digestion. Bile salts and phospholipids present in the simulated intestinal fluid may have displaced some of the β-glucans from the oil droplet surfaces, leading to droplet coalescence and a decrease in the rate of lipolysis. E20UW was also probably the less stable emulsion due to its higher oil content. Indeed, when increasing the oil content, the wall material content decreases (for the same total solids content), which may lead to faster droplets coalescence [42]. Similar results were reported by Jeanes et al. [16] for O/W emulsions made with corn oil, α-toc acetate and quillaja saponins. However, they also pointed out that the overall influence of lipid digestion on oil droplets is complicated and may results in an increase or a reduction of their size, since the digestion products of lipolysis may accumulate at the oil–water interface or move into the surrounding aqueous phase, liable on their molecular weight and the concentration in bile salts and phospholipids [22,23].

Overall, both W and UW extracts from *P. ostreatus* were able to stabilize the emulsions during the first phases of the digestion *in vitro*. More coalescence was observed in sample E10UW than in sample E10W, which may be related to the different molecular weights of the β-glucans in the extracts [7].

3.2. Effect of in vitro digestion on α-toc stability

Lipophilic bioactive compounds, such as α-toc, are usually situated inside of the lipid droplets and, in order to be released, the surrounding TGs have to be digested. After being released from the droplets, they have to be incorporated into the hydrophobic areas within the mixed micelles; if not, they will precipitate or form a separate layer [43]. To assess the suitability of Ec_AG, E10W, E10UW and E20UW as emulsion-based delivery systems for lipophilic compounds, presence of α-toc in the oil phase were examined after *in vitro* oral, gastric and intestinal digestion (Fig. 2).

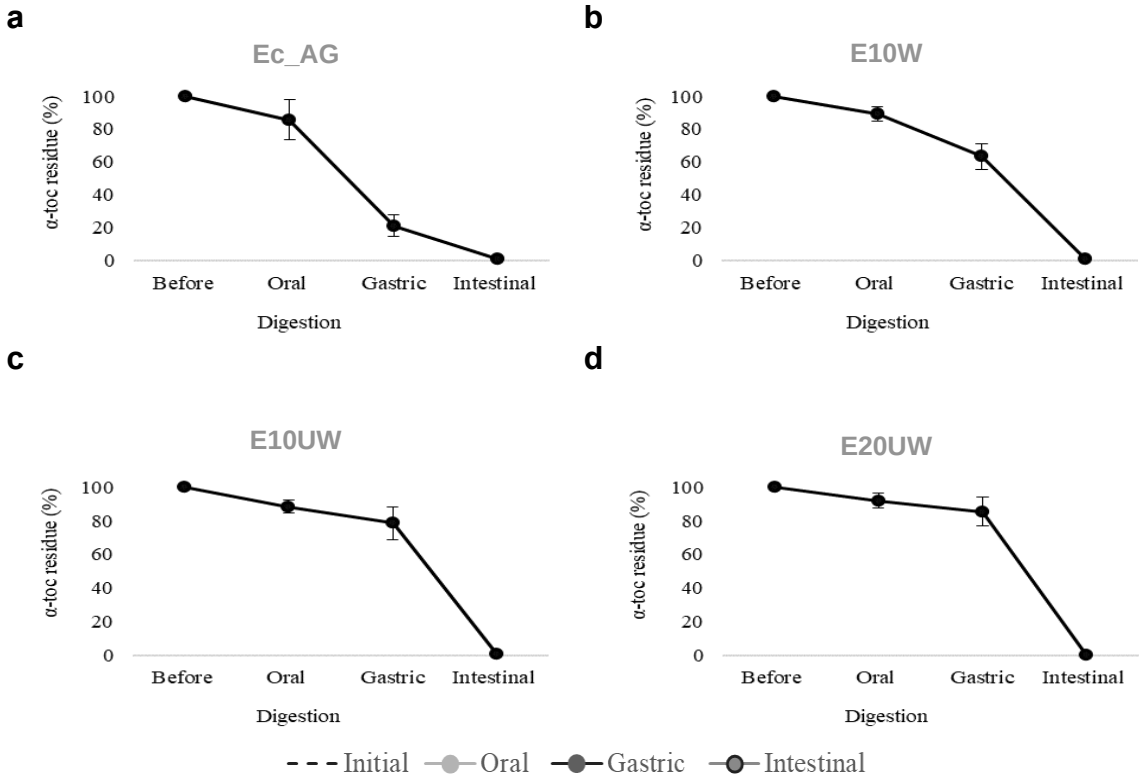

Figure 2. Effect of *in vitro* digestion on the vitamin E degradation in SO reconstituted emulsions stabilized with AG **(a)**, β-glucan-rich extracts washed **(b)**, and unwashed **(c, d)**.
Sample codes are explained in Section 2.2.1.
Results presented are representative data for three separate experiments.

During mastication, oxygen can be incorporated and triggers oxidative degradation of lipophilic compounds within the food matrix. Hence, the possible influence of the oxygen on the incidence of lipid oxidation cannot be excluded, since it is a mayor limit for *in vitro* and *ex vivo* models [13]. However, in all samples there was no significant decrease of α-toc.

While no significant changes were observed in the particle size distribution of Ec_AG, α-toc concentration in this sample significantly ($p < 0.05$) decreased compared to the samples with β-glucans. The α-toc residue was 21% in Ec_AG, 63% in E10W, 79% E10UW and 86% in E20UW. Such difference could be explained by the fact that β-glucans acted as antioxidant and

protected α-toc against the low pH of the simulated gastric juice, which is a well-known pro-oxidant condition of the GIT [13].

Since most nutrients and vitamins are absorbed at the intestinal level for additional utilization in the body, α-toc should be preserved in encapsulated particles and should not be released in the stomach [44]. At the beginning of the simulated intestinal phase, the residual amount of vitamin E in the digesta of E10W, E10UW and E20UW was not significantly different from the amount recovered in the oil phase before digestion. Therefore, these samples had a better vitamin E stability during the first two phases of *in vitro* digestion compared to the control sample Ec_AG. However, after 2 h of intestinal digestion, no α-toc was found in any of the four samples (Fig. 2).

It is possible that the presence of reactive species like metallic ions activated some oxidative degradation phenomena; moreover, the products of the enzymatic hydrolysis of food lipids are prone to oxidative degradation. Since large oil droplets have a smaller surface area available for lipolysis and oxidation, different results were expected in samples containing UW extracts. However, no α-toc was found in the digesta of these samples either. Thus, no correlation between changes in size of the oil droplets and α-toc stability was observed.

However, it is not simple to explain this phenomenon, as physical stability and bioactive bioaccessibility are elaborated processes influenced by several factors. It is well known that polysaccharides can interact with lipase, bile acids, calcium ions and other various digestive components [45]. Therefore, Lv et al. [43] made assumptions that the AG interacts with bile salts and/or free fatty acids, thus decreasing the incorporation of α-toc into the mixed micelles or causing the latter to precipitate and thereby not be detectable after the oil phase extraction. This may have occurred here with *P. ostreatus* β-glucans, as an increase in turbidity was observed in the samples after the intestinal stage. Clearly, further *in vivo* studies are required to better understand the results obtained using this *in vitro* model, especially regarding the intestinal step.

3.3. Oxidative stability during storage

Oxidative stability of oils has been defined as the resistance to oxidation during processing and storage [28]. For comparison purposes, free SO, the control powders Pc_AG and Pc_Tween, and powders made with *P. ostreatus* β-glucans, were stored at 60 °C and 50% RH in air in order to study the CD and antioxidant (α-toc) content under thermo-oxidation conditions. The CD are formed by the rearrangement of the hydroperoxide double bonds during oxidation, thus representing the primary degradation products of oil [46]. The CD content of free SO and of the different powders is reported in Figure 3.

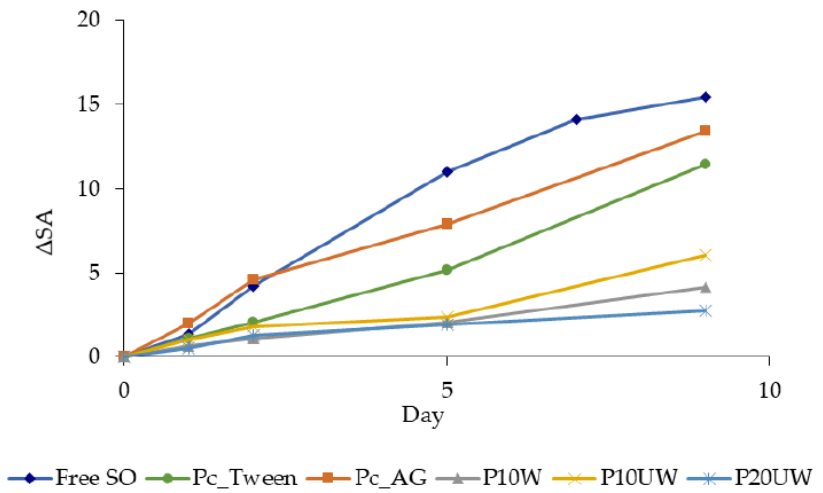

Figure 3. CD (expressed as ΔSA =SA(t) – SA(0) value) of Free SO and of spray dried emulsions, determined at different storage times up to 9 days. Sample codes are explained in Table 1.

Although spray drying encapsulation was expected to protect PUFAs from oxidation during accelerated storage, evolutions of CD content for the control powders Pc_Tween and Pc_AG showed a fast increase during the first days of storage (Fig. 3), up to 11.5 and 13.4, respectively, in 9 days (Table 3). The CD content in the free oil reached 15.5 in 9 days and then the oil appeared as a resin, indicating that polymerization occurred. However, spray drying encapsulation prevented the polymerization of encapsulated oil. The presence

of β-glucan-rich extracts gave higher protection compared to the control powders, made with emulsifier with no recognized antioxidant activity (Fig. 3). The CD of P10W and P20UW increased similarly, both reaching 2 after 5 days, and 4.2 and 2.8 after 9 days, respectively. P10UW also reached a CD value around 2 after 5 days, then it slightly increased up to 6 at day 9 of storage, which is about half the CD values of the control powders. The storage of free oil was stopped at 9 days because further analyses were not possible since the oil could not be dissolved into isooctane. Conversely, the storage study continued for an additional 6 days with the powders only (Table 3). Along the 15 days of storage, the amount of CD for all samples increased; however, β-glucans clearly acted as an antioxidant. The maximum CD levels for P10W and P10UW reached at 15 days was 9.6 and 11.3, respectively, showing no significant differences, while P20UW reached the maximum of 6.6 (Table 3). Consequently, for P10W, P10UW and P20UW, the increase of CD content during the first days of aging was slower suggesting that β-glucan-rich extract played a role in the prevention of the formation of CD from peroxyl radicals, thus slowing down the propagation stage of oxidation. Regarding the control powders, Pc_Tween reached the maximum of CD content after about 12 days of aging, followed by a stabilization (CD around 15), while Pc_AG had a notable decrease immediately after reaching the maximum CD level (Table 3). In SO, when the concentration of hydroperoxides is significant, secondary oxidation products are formed [28]. Thus, in Pc_AG, the CD level fell because all the hydroperoxides were oxidized to aldehydes and ketones, while this did not happen in the other powders during 15 days of storage.

Table 3. CD content and degradation of α-toc in free SO and in powders, determined at different storage times up to 15 days.

	Free SO			Pc_Tween			Pc_AG			P10W			P10UW			P20UW		
Day	**CD (ΔSA)**																	
0	0.0 ± 0.0	A	a	0.0 ± 0.0	A	a	0.0 ± 0.0	B,C	a	0.0 ± 0.0	A	a	0.0 ± 0.0	A	a	0.0 ± 0.0	A	a
1	1.4 ± 0.2	A	a	1.1 ± 0.1	A,B	a	2.0 ± 0.9	B,C	a	0.6 ± 0.6	A	a	1.0 ± 0.7	A,B	a	0.6 ± 0.4	A	a
2	4.2 ± 0.6	B	b,c	2.1 ± 0.2	B	a,b	4.6 ± 1.5	C	c	1.2 ± 0.5	A	a	1.8 ± 0.7	A,B	a,b	1.4 ± 0.7	A,B	a
5	11.0 ± 1.2	C	c	5.4 ± 0.4	C	a,b	7.9 ± 1.5	C,D	b,c	2.1 ± 0.5	A	a	2.4 ± 0.3	B	a	2.0 ± 0.2	A,B	a
9	15.5 ± 0.5	D	c	11.5 ± 0.3	D	a,b,c	13.4 ± 5.9	D	b,c	4.2 ± 1.1	B	a	6.1 ± 0.9	C	a,b	2.8 ± 0.2	A,B	a
12	n.d.			15.1 ± 0.6	E	d	-5.7 ± 0.4	A	a	7.0 ± 0.2	C	c	7.0 ± 0.8	C	c	4.0 ± 0.4	B,C	b
15	n.d.			15.4 ± 0.8	E	d	-3.3 ± 1.3	A	a	9.6 ± 0.1	D	c	11.3 ± 0.8	D	c	6.6 ± 0.1	C	b
Day	**Degradation α-toc (%)**																	
0	0 ± 0	A	a	0 ± 0	A	a	0 ± 0	A	a	0 ± 0	A	a	0 ± 0	A	a	0 ± 0	A	a
1	8 ± 15	A	a,b	1 ± 8	A	a	27 ± 6	B	b	3 ± 1	A	a	10 ± 5	A,B	a,b	7 ± 4	A	a,b
2	12 ± 11	A	a	22 ± 9	B	a	64 ± 3	C	b	8 ± 4	A	a	12 ± 4	A,B	a	16 ± 5	A	a
5	42 ± 11	B	b	50 ± 2	C	b	87 ± 2	D	c	17 ± 5	B	a	18 ± 3	B,C	a	17 ± 7	A	a
9	99 ± 0	C	c	67 ± 5	C	b	95 ± 1	D	c	18 ± 3	B	a	20 ± 5	B,C	a	25 ± 6	A	a
12	n.d.			95 ± 1	D	b	97 ± 1	D	b	20 ± 2	B	a	28 ± 3	C,D	a	28 ± 3	A	a
15	n.d.			98 ± 1	D	b	97± 1	D	b	27 ± 4	B	a	37 ± 3	D	a	29 ± 7	A	a

CD and degradation of α-toc (expressed as 100*(C0-Ct)/C0) in Free SO and in spray dried emulsions, determined during storage. Sample codes are explained in Table 1. n.d. = not detectable. Different capital letters (A, B, C, D, E) in the same column were used to designate significant difference (LSD, $p < 0.05$) for different storage time (same sample), and different lower-case letter in the same row (a, b, c, d) were used to designate significant difference among different samples (same storage time).

To further study the behaviour of SO concerning oxidation, the time-course of α-toc consumption in free and encapsulated SO was analysed (Table 3). α-toc was consumed progressively with different rates depending on the sample. After 9 days of aging, the consumption of α-toc in free SO was completed (99%), while spray drying encapsulation with Tween 20 delayed the degradation (67%) and the presence of *P. ostreatus* β-glucans slowed it down between 18 and 25%. In the other control powder, made with AG, the consumption of α-toc was even faster than in free SO, with higher percentages of degradation since the first days of storage. In a previous study on β-carotene stability in O/W emulsions with different droplet sizes, it was demonstrated that the degree of degradation of this oxygen-sensitive target increases with a decrease in mean particle diameter. This effect was attributed to the increased surface area of the smallest droplets with respect to the larger droplets and the bulk lipid phase [47]. Hence, the fast oil CD formation in Pc_AG, similar to the free oil and the even faster decrease of α-toc content with respect to the free oil can be explained by the fact that Pc_AG had the smallest D[3,2] (9.39 ± 0.36 μm) and hence it was more accessible to oxygen. Conversely, Pc_Tween had higher D[3,2] (16.10 ± 0.97 μm) and provided better oil protection than Pc_AG. Interestingly, P10W, P10UW and P20UW with D[3,2] of 11.09 ± 4.69, 17.50 ± 1.28 and 14.63 ± 5.71 μm were able to retain a significant percentages of α-toc through storage under accelerated conditions. After 5 days of storage, the retention of α-toc in P10W, P10UW and P20UW was 82%. As explained under the Material and Methods section, the predicted variation of α-toc is 62 times slower at 20 °C than under the accelerated conditions and hence 82% retention can be expected after 310 days at 20 °C, i.e., approximately one year. At the end of the storage, α-toc was completely consumed in the control powders and in free SO, while in both P10W, P10UW and P20UW the percentage of α-toc residue was about 70%. The powders made with β-glucan-rich extracts showed the lowest α-toc degradation values during storage, with no significant differences between them, confirming that the presence of an emulsifying agent with inherent

antioxidant activity can help in the protection of bioactive compounds susceptible to oxidation.

5. Conclusions

The purpose of this study was to determine the influence of different emulsifying agents on the stability of encapsulated SO, rich in PUFAs and vitamin E, using *in vitro* digestion and an accelerated storage. The powders obtained by adding the β-glucan-rich extracts appeared to have better oxidative stability, with low α-toc degradation and production of CD, whereas those stabilized by the commonly used emulsifier (i.e., AG and Tween 20) were prone to oxidation. The β-glucan-emulsions had similar vitamin E degradation profiles during *in vitro* digestion, whereas degradation was faster in the AG-emulsion in the gastric phase. This phenomenon was linked to the antioxidant properties of β-glucans, thereby inhibiting the oxidation phenomena until the beginning of the intestinal phase. This study has proved for the first time the potential antioxidant activity of β-glucan used as emulsifying agent under *in vitro* gastrointestinal digestion and accelerated aging conditions. However, no α-toc was found at the end of the intestinal phase in none of the digested samples, probably because of some oxidative degradation phenomena. Structure of the emulsions during *in vitro* digestion depended on the emulsifier used and the oil content. Emulsions were better protected from aggregation during the simulated oral and gastric phases by AG, but the mushroom-derived emulsifiers were also able to fairly stabilize the oil droplets. Both types of emulsifiers led to the formation of mixed micelles during the simulated intestinal phase, but when the oil content was increased the stability of the emulsion decreased, and large oil droplets were observed instead of mixed micelles. Further studies are needed to better understand the fate of vitamin E encapsulated in O/W emulsions. In particular, the kinetics of α-toc degradation during the intestinal phase and the presence of oxidation products during digestion should be studied in order to design emulsion-based delivery systems robust enough to last through food production, storage, and

digestion; moreover, *in vivo* animal feeding studies are required to confirm the results of this *in vitro* study.

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CHAPTER 4

GENERAL CONCLUSION, IMPLICATIONS and FUTURE DIRECTIONS

The concepts proposed in this PhD thesis were to explore the use of *P. ostreatus* as a sustainable food ingredient to address the needs of populations with endemic nutritional deficiencies as well as the needs of populations at risk or affected by some chronic diseases.

The breadsticks developed could be conveniently sized in the right amount and provide a significant percentage of the recommended daily level of both vitamin D_2 and dietary fiber, since these products could be easily consumed as snacks during the day. The liking results demonstrated that even if the acceptability decreased with increasing concentration of mushroom powder, all the samples formulated with this vitamin D_2 source, which is more sustainable than animal sources, were clearly well accepted. A future perspective of study could be to add *P. ostreatus* powder in other food products and investigate consumers' responses in other specific target populations. To address the need of population having severe vitamin D_2 deficiency, UV irradiation can be applied on *P. ostreatus* biomass to enhance its natural level of this vitamin. However, process monitoring should be implemented to accomplish the target value of vitamin D_2 in the final product and to avoid excessive and toxic levels. In the perspective of a value-chain approach, drying of mushroom, which is notably an energy-consuming operation, should be followed by long-time storage at room temperature, i.e., with no further energy input, at aw levels around 0.11. Moreover, kinetics data for vitamin D_2 degradation in mushroom powder provided can be used as a basis to design the best formulation strategy to deliver this vitamin with functional foods. After investigation on the effect of UV irradiation on the activities of *A. bisporus* and *P. ostreatus* against glycation and oxidative processes, which are responsible for human diseases, the results obtained indicated that there was not a decrease in antiglycation activity, while there was a slight decrease in antioxidant activity. Considering the varied biodiversity among mushrooms, even if the study included the two most

cultivated mushroom species, future researches are required to extend knowledge on the effects of UV irradiation on other mushroom properties.

Low amount of β-glucans-rich extracts from *P. ostreatus* allowed producing stable O/W emulsions, as achieved using a remarkable higher amount of another emulsifier. The efficiency regarding spray drying was tested with positive results. The efficacy regarding protection of oil was markedly higher for the powders with *P. ostreatus* rich-β-glucans extract, even when the amount of oil was doubled. Hence, the glucans rich fractions obtained from *P. ostreatus*, which are known to have antioxidant properties, gave a better protection to lipophilic bioactive compounds, with less formation of primary oxidation products and less depletion of α-toc after spray drying process. Moreover, for the first time, the potential antioxidant activity of β-glucan from *P. ostreatus* used as emulsifying agent under *in vitro* gastrointestinal digestion and accelerated aging conditions has been proved. The effects of the *P. ostreatus* extracts on protection against oil droplets aggregation and oxidation during *in vitro* digestion were positive up to the beginning of intestinal phase. Regarding the intestinal step, the stability of the emulsions decreased and no residual α-tocopherol was found. Therefore, further *in vivo* studies are needed to understand better the *in vitro* results obtained. During 15 days of accelerated storage the oxidative stability of the powders was improved in presence of β-glucans and compared to common emulsifier with no inherent bioactivity. Overall, the usage of emulsifiers with inherent bioactivity, such as *P. ostreatus* β-glucans, appears to be a healthy and sustainable alternative to common emulsifiers.

In conclusion, the results indicated that *P. ostreatus* and its compounds can be used as sustainable food ingredients for both therapeutic strategies and to stabilize oxidizable target during the various passages of food production, storage and consumption.

CHAPTER 5

APPENDICES

SCIENTIFIC PRODUCTS

Papers with Impact Factor

- Lavelli, V., Proserpio, C., **Gallotti**, **F.,** Laureati, M., Pagliarini, E., 2018. Circular reuse of bio-resources: the role of *Pleurotus* spp. in the development of functional foods, Food Func., 9, 1353-1372. https://doi.org/10.1039/C7FO01747B
- Proserpio, C., Lavelli, V., **Gallotti, F.,** Laureati, M., Pagliarini, E., 2019. Effect of vitamin D_2 fortification using *Pleurotus ostreatus* in a whole-grain cereal product on child acceptability, Nutrients, 11, 2441. https://doi.org/10.3390/nu11102441
- Pedrali, D., **Gallotti, F.,** C., Proserpio, Pagliarini, E., Lavelli, V., 2020. Kinetic study of vitamin D_2 degradation in mushroom powder to improve its applications in fortified foods, LWT - Food Sci. Technol., 125, 109248. https://doi.org/10.1016/j.lwt.2020.109248
- **Gallotti, F.,** Lavelli, V., Turchiuli, C., 2020. Application of *Pleurotus ostreatus* β-glucans for oil-in-water emulsions encapsulation in powder, Food Hydrocolloids, 105, 105841. https://doi.org/10.1016/j.foodhyd.2020.105841
- **Gallotti, F.,** Lavelli, V., 2020. The effect of UV irradiation on vitamin D_2 content and antioxidant and antiglycation activities of mushrooms, Foods, 9(8), 1087. https://doi.org/10.3390/foods9081087
- **Gallotti, F.,** Lavoisier, A., Turchiuli, C., Lavelli, V., 2020. Impact of *Pleurotus ostreatus* β-glucans on oxidative stability of active compounds encapsulated in powders during storage and *in vitro* digestion, Antioxidants, 9, 1219. https://doi.org/10.3390/antiox9121219

Book chapters

- **Gallotti, F.,** Turchiuli, C., Lavelli, V., 2019. Production of stable emulsions using β-glucans extracted from *Pleurotus ostreatus* to encapsulate oxidisable compounds. In: Petrotos, K., Leontopoulos, S. (Eds.). Proceeding of the 4th International Conference of food and biosystem engineering, Crete Island, University of Thessaly (Greece), pp. 201-208.
- Lavelli, V., **Gallotti, F.,** Proserpio, C., Pedrali, D., Frigerio, B., Laureati, M., Panno, L., Pagliarini, E., 2019. *Pleurotus ostreatus* as a source of vitamin D_2 and functional foods for a young population. In: Petrotos, K., Leontopoulos, S. (Eds.). Proceeding of the 4th International Conference of food and biosystem engineering, Crete Island, University of Thessaly (Greece), pp. 209–216.
- Lavelli, V., **Gallotti, F.,** Pedrali D., 2021. Applications of compounds from grape processing by-products: formulation of dietary fiber and encapsulated bioactive compounds, 12 pp. Chapter 17 In: Food Waste Recovery (2nd Edition), Galanakis, C., Ed. Elsevier, in press.

Oral communications

- **Gallotti, F.,** Proserpio, C., Laureati, M., Pagliarini, E., Lavelli, V. From food waste to food proteins. Advanced School on “Food Proteins”, Bergamo (Italy), 2-4 May 2018.
- **Gallotti**, **F.,** Turchiuli, C., Lavelli, V. Production of stable emulsions using β-glucans extracted from *Pleurotus ostreatus* to encapsulate oxidisable compounds. 4th International Conference on Food and Biosystems Engineering, Agia Pelagia, Heraklion, Crete Island (Greece), 30 May – 2 June 2019.
- Lavelli, V., **Gallotti**, **F.,** Proserpio, C., Pedrali, D., Frigerio, B., Laureati, M., Panno, L., Pagliarini, E. *Pleurotus ostreatus* as a source of vitamin D_2 and functional foods for a young population. 4th International Conference on Food and Biosystems Engineering, Agia Pelagia, Heraklion, Crete Island (Greece), 30 May – 2 June 2019.
- **Gallotti**, **F.,** Proserpio, C., Pedrali, D., Frigerio, B., Barbarito, S., Casaretta, F., Laureati, M., Pagliarini, E., Lavelli, V. *Pleurotus* as new ingredient for functional foods. Dare Valore ai Residui Agro Alimentari (on-line), 30 July 2020.
- **Gallotti**, **F.** Design of functional foods with new ingredients from *Pleurotus ostreatus*. 25th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology (on-line), 18 September 2020.
- **Gallotti**, **F.,** Lavelli, V., Almeida, G., Turchiuli, C. Application of β-glucans from *Pleurotus ostreatus* for lipophilic compounds encapsulation in powder. 19th International Conference "Life sciences for sustainable development" (on-line), 24-25 September 2020.

Poster presentations

- Cattaneo, C., Lavelli, V., Proserpio, C., **Gallotti**, **F.,** Laureati, M., Pagliarini, E. Consumers' attitude towards food by-products and novel technologies. 8th European Conference on Sensory and Consumer Research, Verona (Italy), 2-5 September 2018.
- **Gallotti**, **F.** Design of functional foods with new ingredients from *Pleurotus ostreatus* grown on agri-food waste. 23rd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Oristano (Italy), 19-21 September 2018.
- Proserpio, C., Lavelli, V., **Gallotti**, **F.,** Laureati, M., Pagliarini, E. Assessing liking and food neophobia for vitamin D_2 enriched breadsticks among school aged children. 13th Pangborn Sensory Science Symposium, Edimburgh (United Kingdom), 28 July – 1 August 2019.
- Pasquali, M., Scarafoni, A., Colombo, E. M., Muratore, C., **Gallotti, F.,** Lavelli, V. The activity of *Pleurotus ostreatus* extracts against pathogenic *Fusarium* spp. 87th Annual Meeting of the Mycological Society of America, Minneapolis, Minnesota (United States of America), 10-14 August 2019.
- **Gallotti, F.** Production of functional ingredients using bioactive compounds from *Pleurotus ostreatus*. 24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Florence (Italy), 11-13 September 2019.
- **Gallotti, F.,** Lavelli, V., Turchiuli, C. Development of stable emulsions from lab to pilot scale for spray drying encapsulation using *P. ostreatus* β-glucans as emulsifier. 34th EFFoST International Conference (on-line), 10-12 November 2020.

CONGRESSES, WORKSHOPS, SUMMER/WINTER SCHOOLS

- Challenges of our era summit, BASE, Milan (Italy), 6-7 March 2018.
- Advanced School on “Food Proteins”, Bergamo (Italy), 2-4 May 2018.
- 23rd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Oristano (Italy), 19-21 September 2018.
- 4th International Conference on Food and Biosystems Engineering, Agia Pelagia, Heraklion, Crete Island (Greece), 30 May – 2 June 2019.
- 24th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, Florence (Italy), 11-13 September 2019.
- “Future Scenarios and new training strategies to enhance skills and competences for an innovative and sustainable food system” (on-line), 22 April 2020.
- Dare Valore ai Residui Agro Alimentari (on-line), 30 July 2020.
- 25th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology (on-line), 18 September 2020.
- 19th International Conference "Life sciences for sustainable development" (on-line), 24-25 September 2020.
- “International INFOGEST Webinar on Food Digestion” (on-line), 7 October and 4 November 2020.
- “Food Structure & Functionality Online Mini Symposium” (on-line),20 October 2020.
- 34th EFFoST International Conference (on-line), 10-12 November 2020.

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Ci sono soltanto due possibili conclusioni:
se il risultato conferma le ipotesi, allora hai appena fatto una misura;
se il risultato è contrario alle ipotesi, allora hai fatto una scoperta.

Enrico Fermi

La science n'a pas de patrie, parce que le savoir est le patrimoine de l'humanité, le flambeau qui éclaire le monde.

Louis Pasteur