TESIS DOCTORAL

Doctorado en Ciencia, Tecnología y Gestión Alimentaria

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Fungal biomass valorization for obtaining functional food-related materials

Valorización de biomasa fúngica para la obtención de materiales funcionales de interés en alimentación

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A l’ Emma

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Abstract

This doctoral thesis was aimed at providing fundamental and practical knowledge related to the valorisation of underexploited mushroom biomass, in order to understand the structure-function relationship of β-glucan aqueous extracts in terms of immunoregulatory, antioxidant and antiviral capacity as well as technological (gelling and emulsifying) properties. It was also the aim of this PhD thesis to explore the feasibility of mushroom biomass to develop compostable food packaging materials.

Initially, a sequential fractionation process, involving several consecutive cold or hot aqueous and alkaline treatments, was applied to widely consumed mushrooms *(P. ostreatus, L. edodes and G. frondosa*), in order to understand how the initial differences in composition and cell wall architecture affected β-glucan extraction. Based on the results, the potential application of β-glucan aqueous extracts from *Pleurotus* genus was deeply analysed, thus exploring how the composition of the extracts and structural complexity of β-glucans affected their immunoregulatory capacity. The results evidenced that both *Pleurotus ostreatus* and its stipes showed the best results, with a much higher immunostimulant activity than the other explored *Pleurotus* species.

In the second part of the thesis, functional (antiviral and antioxidant) and technological (gelling and emulsifying) properties of purified and unpurified β-glucan aqueous extracts from *Pleurotus ostreatus* and its stipes were evaluated. The purification process, as expected, increased the carbohydrates content (greater in those obtained from the stipes), which resulted in a greater viscosity and gelling capacity. Furthermore, the extract obtained from the stipes showed a strong antiviral activity against murine norovirus, probably ascribed to the higher structural complexity of β-glucans. Although the presence of proteins in the β-

glucan aqueous extracts enhanced their emulsifying properties, it depended on the accessibility of the protein to adsorb at the O/W interphase, which also affected the viscosity of the resulting emulsions.

Chitin and β-glucans are two of the major carbohydrates of mushrooms, having a great potential in the formation of packaging materials. Thus, in the last part of this thesis, the feasibility of mushroom waste biomass to develop biodegradable and compostable food packaging materials was investigated. The results showed that the composition of mushroom biomass and the processing temperatures had an impact on the physicochemical properties of the developed films and, all of them were biodisintegrated under composting conditions according to ISO 20200.

Therefore, this PhD thesis represents a significant step forward in the understanding of the discarded mushroom biomass (whole biomass and stipes) for valorisation purposes and highlights its suitability to develop new cost-efficient functional ingredients and packaging materials for food and food packaging applications.

Resumen

El objetivo de esta tesis doctoral ha sido proporcionar conocimientos fundamentales y prácticos relacionados con la valorización de la biomasa de setas, para comprender la relación estructura-funcionalidad de extractos acuosos ricos en β-glucanos, en términos de capacidad inmunorreguladora, antioxidante y antiviral, así como las propiedades tecnológicas (gelificantes y emulsionantes). El objetivo de esta tesis doctoral también ha sido explorar la viabilidad de dicha biomasa en el desarrollo de materiales compostables para el envasado de alimentos.

Inicialmente, se aplicó un proceso de extracción secuencial, que implicaba varios tratamientos consecutivos tanto acuosos con y sin temperatura como alcalinos, aplicados a setas de gran consumo (*P. ostreatus*, *L. edodes* y *G. frondosa*), con la finalidad de comprender cómo las diferencias iniciales en la composición y la arquitectura de la pared celular afectaban a la extracción de β-glucano. A partir de los resultados obtenidos, se analizó en profundidad la aplicación potencial de extractos acuosos, ricos en β-glucanos, del género *Pleurotus*, explorando cómo la composición de los extractos y la complejidad estructural de β-glucanos afectaban a su capacidad inmunorreguladora. Los resultados evidenciaron que tanto *Pleurotus ostreatus* como sus estipes mostraron los mejores resultados, con una actividad inmunoestimulante mucho mayor que las otras especies de *Pleurotus* exploradas.

En la segunda parte de esta tesis, se evaluaron las propiedades funcionales (antivirales y antioxidantes) y tecnológicas (gelificantes y emulsionantes) de los extractos acuosos de β-glucano, purificados y sin purificar, de *Pleurotus ostreatus* y sus estipes. El proceso de purificación, como era de esperar, incrementó el porcentaje en carbohidratos (con un mayor aumento en los estipes), lo que se tradujo en una mayor viscosidad y capacidad gelificante. Además, el extracto obtenido de los estipes mostró una fuerte actividad antiviral frente a norovirus

murino, probablemente atribuida a la mayor complejidad estructural de sus β-glucanos. Aunque la presencia de proteínas en los extractos acuosos de β-glucanos potenció sus propiedades emulsionantes, esta propiedad fue dependiente de la accesibilidad de la proteína para adsorberse en la interfase O/W, lo que también afectó a la viscosidad de las emulsiones resultantes.

La quitina y los β-glucanos son dos de los principales carbohidratos de las setas, y tienen un gran potencial en la formación de materiales de envasado. En la última parte de esta tesis, se investigó la viabilidad de la biomasa de residuos de setas para desarrollar materiales de envasado de alimentos biodegradables y compostables. Los resultados mostraron que la composición de la biomasa de champiñón y las temperaturas de procesado tuvieron un impacto en las propiedades fisicoquímicas de los films desarrollados y, todas fueron biodesintegrables en condiciones de compostaje según la norma ISO 20200.

Por lo tanto, esta tesis doctoral representa un importante avance en la valorización de la biomasa de setas (seta entera y estipes) y pone de relieve su idoneidad para desarrollar nuevos ingredientes funcionales y materiales de envasado para aplicaciones alimentarias y de envasado de alimentos.

Resum

L'objectiu d'aquesta tesi ha sigut proporcionar els coneixements fonamentals i pràctics relacionats amb la valorització de la biomassa de bolets, per a comprendre la relació estructura-funcionalitat d'extractes aquosos rics en β-glucans, en termes de capacitat immunoreguladora, antioxidant i antiviral, així com de les propietats tecnològiques (gelificants i emulsionants). L'objectiu d'aquesta tesi ha estat també l'exploració de la viabilitat d'aquesta biomassa pel desenvolupament de materials compostables per a l'envasat d'aliments.

Inicialment, es va aplicar un procés d'extracció seqüencial, que implicava diversos tractaments consecutius, tant aquosos amb temperatura i sense, com alcalins, aplicats a bolets de gran consum (*P. ostreatus*, *L. edodes* i *G. frondosa*), per tal de comprendre com les diferències inicials en la composició i l'arquitectura de la paret cel·lular afectaven l'extracció de β-glucà. A partir dels resultats obtinguts, es va analitzar en profunditat la potencial aplicació d'extractes aquosos, rics en β-glucans, del gènere *Pleurotus*, explorant així com la composició dels extractes i la complexitat structural dels β-glucans afectaven a la seua capacitat immunoreguladora. Els resultats van evidenciar que tant *Pleurotus ostreatus* com les seues estipes mostraren els millors resultats, amb una activitat immunoestimulant molt més gran que la obtinguda per a les altres espècies de *Pleurotus* explorades.

A la segona part d'aquesta tesi, es van avaluar les propietats funcionals (antivirals i antioxidants) i tecnològiques (gelificants i emulsionants) dels extractes aquosos de β-glucà, purificats i sense purificar, de *Pleurotus ostreatus* i les seues estipes. El procés de purificació, com calia esperar, va incrementar el percentatge en carbohidrats (amb un major augment en els estipes), cosa que es va traduir en una major viscositat i capacitat gelificant. A més, l'extracte obtingut dels estipes va mostrar una forta activitat antiviral contra norovirus murí, probablement atribuïda

a la complexitat estructural més gran dels seus β-glucans. Tot i que la presència de proteïnes en els extractes aquosos de β-glucans va potenciar les seues propietats emulsionants, aquesta propietat va ser dependent de l'accessibilitat de la proteïna per adsorbir-se a la interfase O/W, cosa que també va afectar la viscositat de les emulsions resultants.

La quitina i els β-glucans són dos dels principals carbohidrats dels bolets, i tenen un gran potencial en la formació de materials d'envasament. Així, a la darrera part d'aquesta tesi, es va investigar la viabilitat de la biomassa de residus de bolets per desenvolupar materials biodegradables i compostables d'envasat d'aliments. Els resultats van mostrar que la composició de la biomassa de bolets i les temperatures de processament van tenir un impacte en les propietats fisicoquímiques de les pel·lícules desenvolupades i, totes elles van ser biodesintegrables en condicions de compostatge segons la norma ISO 20200.

Per tant, aquesta tesi doctoral representa un important pas endavant en la valorització de la biomassa de bolets (sencers i estipes) i posa en relleu la seua idoneïtat per desenvolupar nous ingredients funcionals i materials d'envasament per a aplicacions alimentàries i d'envasat d'aliments.

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Abbreviations

UNEP: United Nations Environment Programme.

FAO: Food and Agriculture Organization of the United Nations.

TNF-α: Tumor Necrosis Factor alpha.

IHNV: Infectious Hematopoietic Necrosis Virus.

IL-: Interleukin.

IFN-: Interferon.

LDL: Low-density lipoprotein.

O/W: Oil-in-Water.

I. INTRODUCTION

1.1. Biomass valorisation from food waste

According to the United Nations Environment Programme's (UNEP) Food Waste Index Report 2021, one third of all food produced in the world is lost or wasted each year (United Nation Environment Programme, 2022). FAO's "The State of Food and Agriculture" report (2019) shows that about 14% of the world's food is lost after it is harvested and before it reaches the shops. And according to The Food Waste Index report (UNEP), 17% of our food ends up being wasted in retail and by consumers, mainly in households (PNUMA, 2021). While in less developed countries losses occur mainly between harvesting and processing due to problems such as inappropriate use of inputs or lack of infrastructure, in developed countries 40% of losses are generated at trade and consumer level, meaning that food is wasted further up the chain, with a higher added value ("FAO Publ. Cat. 2022," 2022). These large amounts of food waste are largely due to the quality standards of the industry and shops, as well as the consumer's own expectancies towards the external appearance of the products (Motelica et al., 2020).

Food loss and waste negatively affects climate, food security and the sustainability of our agri-food systems. All this has become a major problem as the prolonged COVID-19 pandemic, the climate crisis and the war in Ukraine have contributed to rising food prices, deteriorating food security and nutrition globally. Food loss and waste accounts for 8-10% of global greenhouse gas (GHG) emissions, contributing to extreme weather events such as droughts and floods ("FAO Publ. Cat. 2022," 2022).

In 2015, the United Nations (UN) adopted the 2030 Agenda for Sustainable Development, which established 17 Sustainable Development Goals (SDGs) to promote prosperity while protecting the planet. One of the targets of these goals is, by 2030, to substantially reduce waste generation through prevention, reduction, recycling, and reuse (United Nations, 2022). For all these reasons, nowadays, there

is a great interest in the recycling and recovery of this food waste, also as a consequence of its high availability and broad composition. Food waste normally contains interesting biopolymers such as starch, proteins, lipids, cellulose and other microelements, making them appealing for compound valorisation of interest in many different applications, such as food and feed ingredients, nutritional supplements, biopolymeric materials or even for the production of biofuels and bioenergy (Tropea, 2022). These applications can help transform the generalised linear economy model towards the desired circular economy model.

On one side, the transformation of food waste into animal feed, as substitutes for part of the cereal grains and plant-based protein sources, could reduce food competition between humans and animals and alleviate the increased demand for animal feed because of increased animal consumption in the future, as well as having a beneficial impact on the economic benefits. On the other hand, the use of food waste as a liquid biofuel, like biodiesel, bioethanol or biooil is also being explored. However, the fact that food waste collection is still an unorganized sector and that it is a non-standard resource with a variable and complex composition are challenges that so far hinder its use as a source of biofuel or animal feed (Karmee, 2016; Rajeh et al., 2021). So, even though recycling food waste as animal feed can bring significant environmental and health benefits, this requires consumer and industry support as well as policy changes and investment in food waste collection infrastructure, so that together with composting, anaerobic digestion for biogas production, or landfill disposal are less preferable options in order of priority (Salemdeeb et al., 2017).

Another strategy that has been identified to reduce food waste has been the valorisation of these wastes as a source of bioactive compounds for use as functional food ingredients or nutraceuticals. This requires a compositional study and analysis of the potential of each type of waste, but it can result in obtaining ingredients or extracts with high added value. This could be the case for some compounds from the industrial processing of plant products, a valuable source of

folic acid or phenolic compounds (Melini et al., 2020). In a study on the utilization of green walnut husk waste, glucans and pectin were isolated from dried walnut husks by alkaline and acid extractions and characterized. The results suggested that this waste material could be a source of glucans and pectin, with potential use in the food, cosmetic and pharmaceutical fields (La Torre et al., 2021). The work from (Trombino et al., 2021) shows the potential value of the recovery of carotenoids, and especially lycopene (one of the most potent antioxidants), from tomato waste.

The compounds extracted from food waste are also being investigated as food texture modifiers, as some of them are able to form hydrogels, act as emulsifiers, or can be used as food packaging additives of materials. Depending on the extraction conditions, the biopolymers extracted can also have bioactive properties, thus giving rise to bioactive materials great added value. For example, in a study from (Méndez et al., 2023) on the valorisation of persimmon discards, a food-grade antiviral coating was developed from polyphenol-rich pectin extracts and applied on blueberries, showing improved antiviral and physicochemical properties compared to commercial citrus pectin.

On the other hand, in the field of food packaging, the competition of these biodegradable materials is often sought against other materials that are not biodegradable or that are formed from highly polluting materials, such as plastics. Paper-based food packaging accounts for 31.9% of the total value and dominates as a biodegradable material, but unfortunately, plastic food packaging continues to dominate worldwide due to its superior properties and lower prices, with the associated environmental and pollution problems. Agencies such as the Food and Drug Administration (FDA) or the European Commission (EC) are trying to shift the market towards more sustainable solutions, to reduce the use of traditional plastic packaging in favour of bio-based polymers (EC, 2019). For instance, (Otoni et al., 2018) developed biodegradable biocomposites from minimal carrot processing waste with hydroxy-propyl-methyl-cellulose as a ligand and high-

pressure microfluidized cellulose fibers as mechanical reinforcement, resulting in biodegradable biocomposites with properties suitable for food packaging applications. Another example could be the formation of cellulose-rich materials from residual biomass of *Posidonia oceanica* leaves or the waste generated after the extraction of agar from the *Gelidium sesquipedale* algae (Benito-González et al., 2019, Martínez-Sanz et al., 2020) to obtain sustainable and low-cost materials.

1.1.1. Mushroom waste

There are more than 14,000 species of mushrooms of which about 2,000 are edible, and of these, about 300 have bioactive properties. They are also considered a nutritious and healthy food, due to their composition rich in high quality protein, fibre, phenolic compounds, lipids rich in essential fatty acids, minerals, vitamins and lectins (Strong et al., 2022). In addition, not only excellent nutritional profile but also health benefits have been reported, due to their bioactive properties mainly attributed to the presence of fungal polysaccharides such as β-glucans. Probably due to all these mentioned reasons, mushroom consumption has significantly increased in recent years. In 2021, the global mushroom market size was valued at $50.3 billion, projected to expand at a growth rate of 9.7% per annum from 2022 to 2030. The Asia Pacific region led the mushroom market in 2021, accounting for more than 78.6% of global revenue. This is mainly due to China, Japan, Malaysia, India, and Australia. In particular, China is the largest producer with an annual per capita consumption of up to 10 kg, closely related to the use of various mushroom species in traditional Chinese medicine. On the other hand, Europe is one of the main consumers of this product, with a level of imports valued at about $183 million in 2019 (*Mushroom Market Size & Analysis Report, 2022-2030*, 2022).

Some of the most widely consumed mushrooms worldwide are *shiitake* (*Lentinus edodes*), oyster mushroom (*Pleurotus ostreatus*) and common white button mushroom (*Agaricus bisporus*). Specifically, in China, the country with the highest production, among the most demanded edible mushrooms there are several types of *Pleurotus* (*P. eryngii, P. citrinopileatus, P. djamor, or P. ostreatus*), *Agaricus bisporus* (button mushroom) and *Grifola frondosa* (*maitake*) among others.

However, as a consequence of increased mushroom production, more waste is generated, both from the substrate in which the mushrooms are grown and from their discards. A large number of mushroom by-products and their disposal could become a problem for mushroom producers due to difficulties in storing or reusing them. For every kg of edible mushroom processed, the industry produces 5 kg of spent mycelium substrate, by-products of mushroom production, spent growth substrate or waste mushroom medium (Wan Mahari et al., 2020). Taking into account the waste generated by discards, depending on the type of cultivation and mushroom, the amount of waste generated ranges between 5 and 20 % of the production volume, mainly coming from the removal of excess length of stipes and units with discolorations or gross size variations (Ramos et al., 2019). Therefore, this thesis will present various recovery options aimed at mitigating environmental hazards and recovering value-added products from this agri-food waste.

1.2. Chemical composition and structure of mushrooms biomass

The term mushroom is used to refer to the fruiting body of higher fungi, which is formed from underground mycelia (hyphae) through the process of fructification so they are above ground, and usually have a lifespan of 10-14 days (Kalač, 2009). These fruiting bodies, also well-known as mushrooms, are complex forms

distinguished by a cap, a stipe and a hymenophore (spore-bearing surface), which provide support and protection for the developing spores (Varga et al., 2019).

This thesis will mainly focus on the mushrooms *Pleurotus spp.*, *Agaricus bisporus* (*brunnescens* and *hortensis*), *Lentinula edodes* and *Grifola frondosa*, see Figure 1.

Oyster mushrooms (*Pleurotus spp.*) natural habitat is tropical and subtropical forests. In central Europe, these species are found on trunks and stumps of deciduous trees but can also be cultivated on various lignocellulosic waste substrates. Its fruiting bodies are usually found as part of clusters of several smaller and larger specimens growing from a common base and arranged in the form of a cluster. They have been found to have a characteristic β-glucan, pleuran, with immunostimulant and anticancer effects (Zawadzka et al., 2022).

White button mushroom (*Agaricus bisporus hortensis*) is the most cultivated variety of the genus *Agaricus bisporus*. The interior is white, the cap is hemispherical, whitish, with scales and the stipe is cylindrical (Ramos et al., 2019). On the other hand, Portobello mushroom (*Agaricus bisporus brunnescens*) is the fully mature form of the common cultivated white button mushroom (*Agaricus bisporus hortensis*). It is an extremely large, dark brown mushroom with an open, flat cap. The natural habitat of both is forests, but they are often grown on compost (Wang et al., 2018).

Lentinula edodes, well known as Shiitake, is a brown mushroom native to China with concentric ring-shaped formations of different shades on the cap. It is traditionally cultivated in log beds, although it has been widely replaced by cultivation in sawdust. It contains a characteristic β-glucan in its cell wall, lentinan, a 1,3-linked-β-glucan with a triple helix conformation, which has been used clinically as an anti-tumour polysaccharide and immunomodulator (Nam et al., 2021; Zheng et al., 2021).

And finally, *Grifola frondosa*, commonly known as Maitake, is an important grayish-looking medicinal mushroom, with a characteristic β-glucan, grifolan. In nature it grows around the stumps of broadleaf trees or trunks although it is usually

grown in bags in a substrate consisting of sawdust supplemented with wheat bran. It is a particularly popular mushroom in Asia not only for its taste and texture, but also because it has long been considered a medicinal mushroom (Song et al., 2018; Wu et al., 2021).

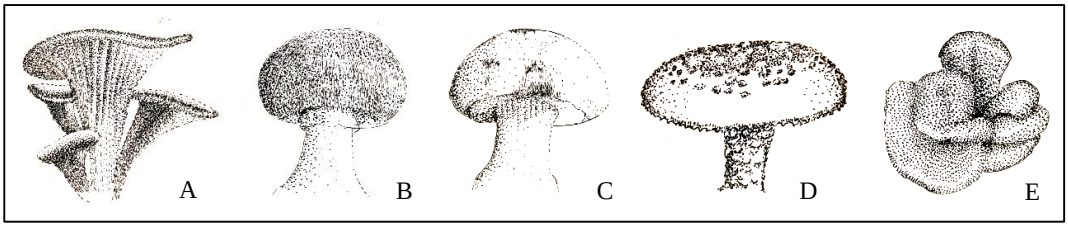

Figure 1. Different species of studied mushrooms. *Pleurotus ostreatus (A), Agaricus bisporus brunnescens (B)* and *hortensis (C), Lentinula edodes (D)* and *Grifola frondosa (E)*. Illustration reproduced with permission of the author Jeremías Pérez.

1.2.1. Mushroom cell wall

Mushrooms are a source of bioactive compounds such as antioxidant, antitumour, antiviral, antimicrobial, hepatoprotective, antidiabetic, hypolipidemic, hypotensive and immunomodulatory agents, with medicinal effects, mainly ascribed to some of their polysaccharides, polyphenols, sterols (ergosterol), tocopherols, vitamins, minerals and proteins (Landi et al., 2022). Mushrooms contain a high percentage of proteins rich in essential amino acids, digestible carbohydrates, and non-digestible carbohydrates such as chitin, mannans and β-glucans, that perform diverse biological activities, or carbohydrate complexes such as glycoproteins, or lectins, being one of the main compounds responsible for their bioactivity. Although fungal polysaccharides are mostly linear and branched glucans, with glycosidic linkages, as (1→3), (1→6)-β-glucans (the major ones) or (1→3)-α-glucans, some of them are heteroglycans composed of arabinose, mannose, fructose, galactose, xylose, glucose, and glucuronic acids (Thatoi et al., 2018).

Their lipid content is mainly composed of essential fatty acids (linoleic and linolenic), polyunsaturated fatty acids higher than saturated and monounsaturated fatty acids. Regarding their micronutrient content, significant amounts of potassium, calcium and sodium and trace elements such as iodine, fluorine, copper, zinc, mercury, and manganese have been found among its compounds. In particular, some species such as the oyster mushroom (*Pleurotus spp.*) contain significant amounts of vitamins B, such as thiamine, riboflavin, niacin, pyridoxine, pantothenic acid and folic acid, as well as vitamin D and provitamin D2 (Kalač, 2012; Zawadzka et al., 2022).

The distribution of the main bioactive compounds of mushrooms through their cell wall is of vital importance to understand their bioavailability or the different extraction processes necessary to obtain these compounds of interest. According to (Ehren et al., 2020; Kang et al., 2018), who focused their study on the sugar composition of the mushroom cell wall, the innermost rigid part of the cell wall consists of α- and β-(1,3)-glucan, β-(1,3)-(1, 6)-glucan and different forms of randomly oriented chitin, highly branched β-(1,4)-chitin and single-stranded chitin, aged into a highly branched β-(1,3)-(1,6)-glucan, together with that which would form the alkali-insoluble part of the cell wall. In contrast, on the outer part of the cell wall, an α-(1,3)-glucan is found forming a more dynamic layer together with glycoproteins covering a matrix of linked β-glucans, mainly consisting of β-(1,3)-glucan, β-(1,3)-(1,6)-glucan, β-(1,6)-glucan and polymeric mannose, where xylans and galactans can also be found.

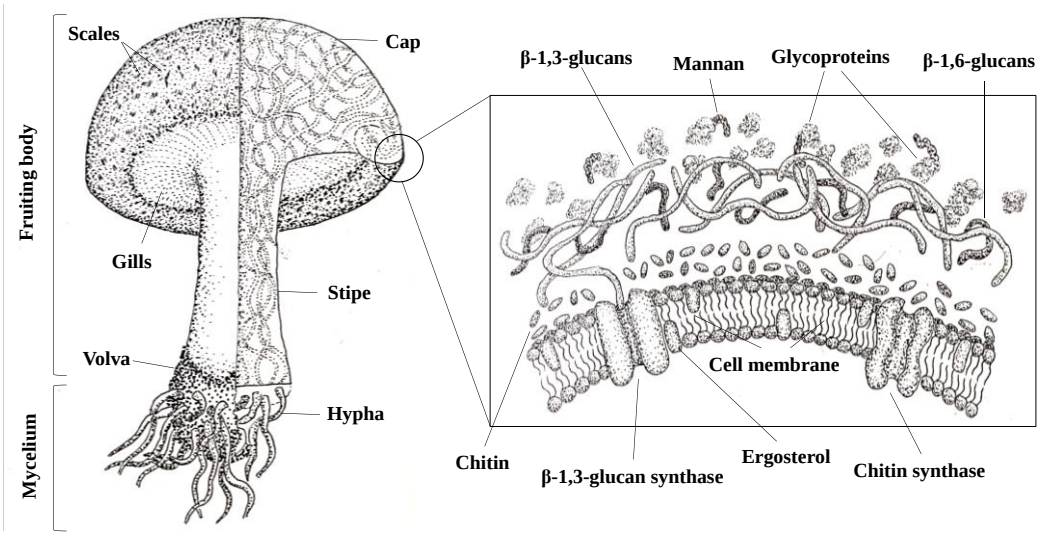

Figure 2. Structure of mushrooms (left) and their cell wall (right). Illustration reproduced with permission of the author Jeremías Pérez.

1.2.2. Main compounds considered in this thesis

1.2.2.1. β-glucans from mushrooms

Although the major biologically active components of interest in mushrooms belong to several structural classes, polysaccharides dominate the trend in terms both of research and of marketed nutraceutical/pharmaceutical/cosmeceutical ingredients, with special interest in β-glucans, mainly due to their relationship to the innate immune system function. Mushroom β-glucans are long- or short-chain polymers formed by glucose units via β-1,3 linkages (forming linear structures) with β-1,6 branching (Figure 3A), unlike β-glucans from cereals, which, on the contrary, have neither 1,6 linkages nor branching (Cerletti et al., 2021). They can modulate innate and adaptive immune responses by binding to various immune cell membrane receptors, such as dectin and Toll-like receptor, activating multiple signalling pathways that involve macrophages, monocytes, neutrophils, and

dendritic, natural killer, B and T cells. So, β-glucans can stimulate the release of cytokines such as TNF-α and various interleukins (Lu et al., 2020). The heterogeneity of β-glucans will influence their bioavailability and interactions with immune cells mainly due to their different solubility, size, structure, supramolecular conformation and degree of branching (Chanput et al., 2012). The higher bioactivity of these polysaccharides is usually related to a higher molecular weight. Although lower molecular weight β-glucans with high bioactivity have also been described, one of the reference β-glucans in terms of bioactive properties is lentinan, with a structure containing two branches of (1→6)-β-glucopyranoside in side chains for every five residues of (1→3)-β-glucose in the linear biopolymer backbone, with a molecular weight between 400-800 KDa (C.Ooi & Liu, 2012). In the work of (Ren et al., 2018), lentinan from mycelia of *Lentinus edodes* was extracted. This β-glucan was composed of a β-(1→3)-glucan backbone with side-branching units of -(1→6)-glucosyl terminated by mannosyl and galactosyl residues. This lentinan exhibited antiviral activity, through direct inactivation and inhibition of viral replication, to IHNV at a concentration of 100 μg/mL β-glucan. In addition, it was observed to significantly down-regulate the expression level of TNF-α, IL-2 and IL-11, up-modulating the expression levels of IFN-1 and IFN-γ.

Mushrooms have been widely used as a food ingredient, not only for their flavour and aroma, but also for their texturizing capacity. Specifically, in the study of mushroom β-glucans, not only very interesting properties have been observed in terms of bioactivity, but also interesting physicochemical properties have been found for technological applications in the food, pharmaceutical or cosmetic industry, such as moisture retention, foam formation, gel formation or emulsifying capacity (Zhu et al., 2016), and these properties can also be affected by the molecular weight, water solubility, structure, branching degree, and food matrix (Du et al., 2019). For example, in the work from (Chen, 2012), β-glucan from mushrooms with high moisture retention was used in eye drops to alleviate xerophthalmia. These β-glucans had a molecular weight between 35 to 2000 KDa.

Moreover, the application of chemical, enzymatic and physical methods to modify the molecular structure of β-glucan poses a significant impact on its solubility, viscosity, and rheological properties. In fact, a lower molecular weight of β-glucans has been related to the formation of gels more quickly and with lower elasticity than those obtained with β-glucans of higher molecular weight (Du et al., 2019).

1.2.2.2. Chitin from mushrooms

Mushrooms have a significant content of the polysaccharide chitin, which is found in higher concentration in their stems. This polysaccharide is structural to the fungal cell wall, composed of β-(1,4)-linked N-acetyl-D-glucosamine units and intimately cross-linked with β-1,3-glucan. This intimate intertwining is of great importance in the extraction, yield, and functional properties of mushroom chitin and β-glucan (Alimi et al., 2023; Zhang et al., 2020). Depending on the source of chitin, this polysaccharide can present two configurations, α and β. The β-chitin is easily converted to α-chitin by alkaline treatment followed by water washing. There is a third form, γ-chitin, which would be a combination of the α and β structures. α-chitin is the most resistant and abundant form, and is found in insects, crustaceans and fungi, while β-chitin can be extracted from squid feathers, and γ-chitin from fungi and yeasts (Kumirska et al., 2010).

Chitin can sometimes be limited in its applications due to its insolubility, so it is sometimes subjected to a deacetylation process, by which it is converted into its derivative, chitosan, being both compounds very valuable in food and pharmaceutical applications, mainly due to their bioactive properties. Some of these applications have been as a component in the formulation of functional foods or materials (Papadaki et al., 2019). Due to the different physicochemical properties of chitin, such as its high crystallinity, insolubility in some solvents or

its degree of acetylation, its applications in food may be limited, but on the other hand it may have interesting properties as a packaging additive (for material reinforcement) or as a packaging material in itself (Alimi et al., 2023). In the work by (Mat Zin et al., 2022), fungal chitin nanofibers were extracted by a mild alkaline process for the development of sustainable materials in the field of nanocomposites. It was observed that the presence of glucan in chitin helped to bind the individual chitin fibres together offering toughness while the chitin crystalline chain offered stiffness. Overall, this study suggests the potential of fungal chitin nanopaper in the development of sustainable materials in the field of nanocomposites. In another work by (Kaya et al., 2022), composite films were prepared by combining different concentrations of curcumin with chitin and glucan complexes extracted from *Agaricus bisporus* as a promising biodegradable active packaging material to improve the shelf life of meat products due to their antioxidant and antimicrobial properties.

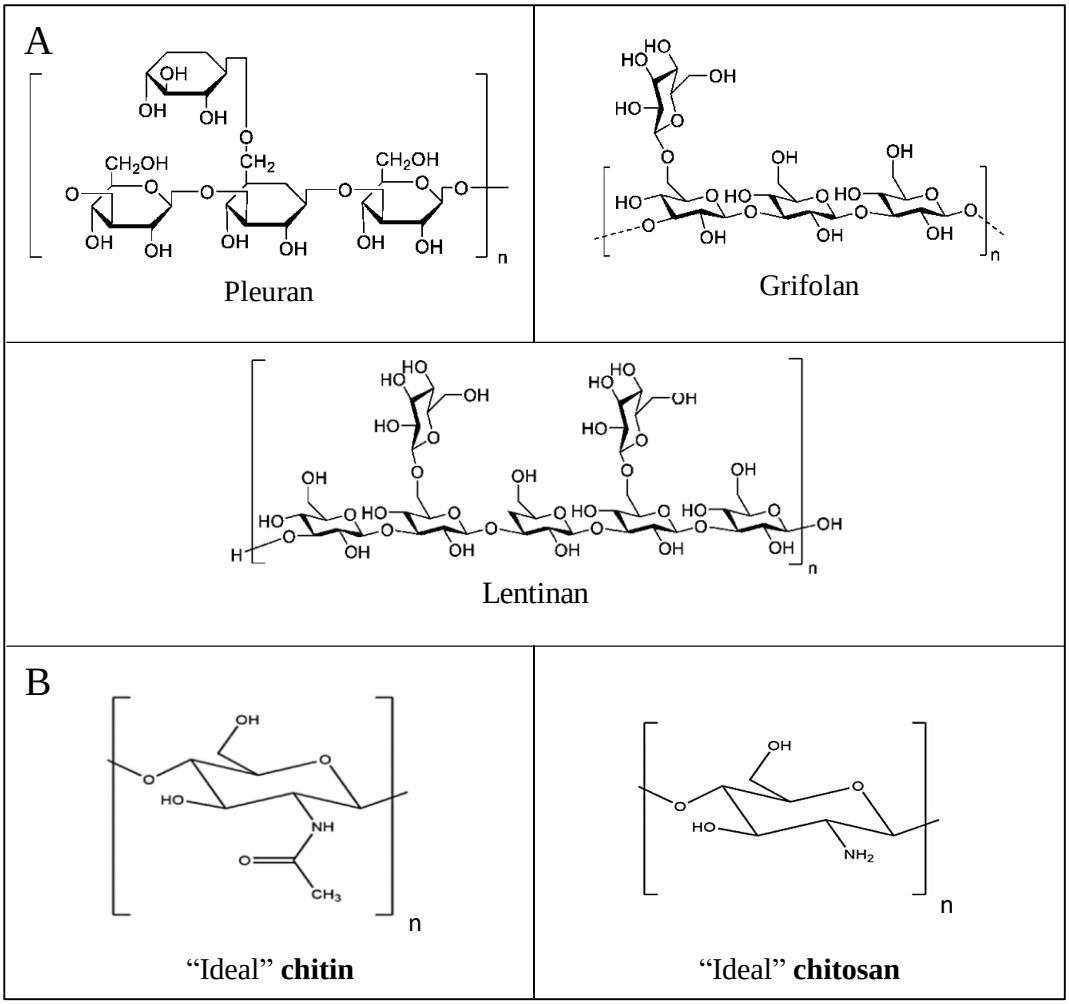

Figure 3. (A) Different structures of characteristic mushroom β-glucans (Cerletti et al., 2021), and (B) mushrooms chitin units (left) and chitosan units, as an example of chitin deacetylation (right) (Jones et al., 2020).

1.3. Main extraction methods of relevant compounds (β-glucans and chitin) from mushroom biomass

Several extraction methods have been used to obtain the different compounds of interest from mushrooms. The matrix of this food is complex and contains different compounds of interest with functional properties. It is important to extract these compounds in order to obtain purer fractions, without interferents and, thus, achieve a greater potential for application. To achieve these objectives, it is necessary to have a good knowledge of the structure of the mushrooms cell wall and different interactions and bonds that may exist in it, as we have seen in the previous sections. In addition, in recent years, thanks to the advance in new technologies, novel extraction methods have been developed that can help the more traditional methods to obtain higher extraction yields or even replace them with the aim to use more effective and environmentally friendly methods.

1.3.1. Traditional methods

There are numerous works in which the extraction of polysaccharides has been carried out using different methods. The traditional and most commonly used method, specifically for the extraction of β-glucans, is hot water under constant agitation (Vezaro et al., 2022; Zhu et al., 2016). Although sometimes this is not a very effective method because of not being aggressive enough to break the cell walls of the mushrooms. However, depending on the species, both bioactive and functional compounds may be extracted. It has the advantage of being a non-toxic and non-polluting solvent that in most studies is eliminated by evaporation or freeze-drying. Using hot water extraction, easily soluble compounds are obtained, having the great advantage for later use, covering a broad number of possible applications, as bioactive compounds for example. But on the other hand, large

volumes of water and long extraction times are generally needed (Hwang et al., 2019; Vezaro et al., 2022).

Other traditional methods reported for the extraction of β-glucans have been those using acidic or alkaline solvents. Chemical methods are more aggressive and can extract more tightly bound compounds but, on the other hand, they can lead to degradation of the β-glucan, and subsequently involve sample separation treatment (dialysis) and solvent disposal (Liu et al., 2021). It is very common to find several of these methods sequentially applied to obtain purer fractions, as the application of certain solvents promotes the extraction of different compounds. Often an organic solvent, usually EtOH, acetone or chloroform: methanol mixtures, is first applied to remove apolar compounds (lipids, phenols and terpenes). Subsequently, the solvent-insoluble residue is usually subjected to aqueous extraction at room temperature or with heat, depending on the fractions of interest. And then, the remaining residue is usually treated with basic aqueous solutions (NaOH or KOH), with NaBH4 to protect the reducing end and avoid degradation of the polysaccharide chains, and with varying conditions of temperature and concentration, depending on the recalcitrance of the fractions of interest (Ruthes et al., 2015; Y. X. Wang et al., 2020).

On the other hand, in mushrooms it has been observed that most commonly β-1,3-glucans with (1→6) branching are associated with chitin by covalent bonds. So, due to the crystalline nature of chitin and its conformation within the cell wall, this carbohydrate will be more difficult to extract than other mushroom compounds. In fact, the most commonly used methods are alkaline, usually NaOH (1M). But while in other sources of chitin, such as crustacean shells, pure chitin can be obtained after alkaline treatment, chitin-β-glucan complexes are mainly obtained from fungal sources. These complexes may also be very useful for various functional applications, as it will be detailed later, but in order to obtain purer chitin, acidic glucan degradation treatments have to be employed (Jones et al., 2020).

1.3.2. New extraction methods

Compared to conventional methods, over time, new, faster, more efficient or complementary, simpler and environmentally friendly extraction techniques have been proposed, such as enzyme-assisted extraction, pulsed electric fields, ultrasound, microwave, subcritical and supercritical fluid extraction and pressurised solvent extraction (Roselló-Soto et al., 2016; Ruthes et al., 2015). These new techniques are increasingly used; however, they are often more costly than conventional extractions. It is required to have a use that justifies this additional cost and, in many cases, it is necessary to optimize the process depending on the food matrix on which they are applied and the compounds of interest to be extracted. For example, in the case of extractions using enzymes, good results can be obtained, but the more specific working conditions and the difficulty in separating substrate and enzyme must be considered. On the other hand, pressurised solvent extraction requires more complex devices, fact which increases processing costs (Liu et al., 2021). In a work from (Barbosa et al., 2020) a novel methodology was developed for the recovery of an extract rich in antioxidant polysaccharides with pharmacological and food potential from *Pleurotus ostreatus* using a combined hot water and supercritical CO_2 extraction system. In the (Hwang et al., 2019) study, different methods based on new technologies were performed to see how they affected the extraction of bioactive compounds from mushrooms. It was observed that the yield of β-glucans as well as phenolic compounds was higher with the application of high temperature pressure than with hot water while the triterpenoid content was increased in the extracts when using ultrasound. In another work (Yiyong Chen et al., 2010), the use of ultrasound and microwave as alternative methods to other traditional methods was proposed. The results showed a higher efficiency in ultrasound/microwave-assisted extraction of bioactive polysaccharides from *Inonotus obliquus* mushroom compared to traditional hot water extraction.

1.4. Functional and technological applications of mushrooms biomass

1.4.1. Bioactive capacity

As mentioned above, mushrooms are not only consumed as food for their great taste and aroma and for their nutritious character but are also highly valued for their content of bioactive compounds, such as lectins, polysaccharides, phenolic and polyphenolics, terpenoids, ergosterols, and volatile organic compounds (see Table 1). In this work we will focus on the main bioactive compounds that can be found in the cell walls of mushrooms, polysaccharides, and proteins.

Polysaccharides

Among the different bioactive compounds widely studied in fungi, polysaccharides have been ascribed as being responsible for many bioactivities of some species. Several biological functions have been reported for them including anticancer, antihypoxic, anti-inflammatory, antilipidemic, antimicrobial, antioxidant, hypoglycaemic and immunomodulatory activity. These polysaccharides can have almost unlimited structural diversity, depending on their monomers and linkages (Wang et al., 2017). In fact, most of the bioactivity in mushrooms has been related to glucans (glucose homopolysaccharides), but significant bioactivity has also been observed in heteropolysaccharides. The most studied monosaccharide compositions in fungi include glucose, mannose, galactose, xylose, arabinose, rhamnose and fucose, along with less frequent ones such as fructose, ribose, glucuronic acid, galacturonic acid and N-acetyl-glucosamine (Friedman, 2016; Ruthes et al., 2016). Chitin and β-glucan have a diverse range of physiological functions. In a study of (Zhang et al., 2022), where

the composition and bioactivity of a chitin-glucan complex extracted from *Coprinus comatus* were studied, it was observed that this complex had prebiotic activity and significantly enhanced the production of butyric acid. Diets supplemented with β-glucan have been linked to improved immunity against upper respiratory infections, seasonal allergies, osteoarthritis and obesity, among others (Yadav & Negi, 2021). In some cases, polysaccharides high in mannose, rhamnose and fucose appear to be responsible for bioactivity. On the other hand, those containing high levels of uronic acid show significant antioxidant activity. Furthermore, arabinose, xylose, mannose and galactose were the sugars most strongly related to macrophage stimulatory activities (Wang et al., 2017).

Proteins

Certain mushroom proteins and peptides have antitumor, immunomodulatory, antimicrobial and anti-inflammatory properties. It has been shown that mushroom proteins can affect the composition of the microbiota, the production of bacterial metabolites and have positive effects on the intestinal mucosa. In particular, some bioactive peptides from edible mushrooms are being recognized for their antihypertensive, antioxidant and antibacterial properties (Yadav & Negi, 2021). Species such as *Pleurotus ostreatus*, *Ganoderma applanatum* and *Agaricus bisporus* produce lectins, a glycoprotein that binds to the surface of cells promoting antitumor, antimicrobial and antiproliferative activities. Mushroom lectins have been attributed in numerous studies with antiproliferative, antitumor, hypotensive, immunomodulatory, among others. There have even been cases where more than one lectin has been isolated from the same mushroom, with completely different properties. These properties have been related to the interaction of these lectins with cell wall glycoproteins, glycolipids and polysaccharides (Hassan et al., 2015; Ma et al., 2018). Lectins possess many shallow binding sites that are hydrophilic and the interaction with carbohydrates

and lectins is usually weak, but their binding strength and specificity are enhanced in the form of oligomers. Thus, they are formed from several similar or identical monomers and each of these monomers binds to the same type of carbohydrate. These simultaneous bonds increase the affinity and apparent binding strength, being glucose and galactose two of the elemental sugars for the bioactivity of these lectins (Singh et al., 2015).

Table 1. Bioactivity reported for different mushroom species studied in this thesis. Studies have been carried out in the last 5 years.

Species	Bioactive compound	Functionality	Reference
Pleurotus ostreatus	Protein hydrolysates	Antioxidant activity, renin angiotensin-converting enzyme inhibitory, antiproliferative activities for cervical carcinoma cell line	(Goswami et al., 2021)
Pleurotus ostreatus	Phenols and flavonoids	Antimicrobial activity against *Candida albicans*, *Staphylococcus aureus*, *Micrococcus luteus* and *E. coli*	(Hamad et al., 2022)
Pleurotus ostreatus	β-glucan	Induction of an innate immune response against breast and lung cancer cells	(EL-Deeb et al., 2019)
Pleurotus ostreatus	Ethanol extracts	Antioxidant, antibacterial and anticancer properties	(Mishra et al., 2022)
Pleurotus eryngii	Water-soluble polysaccharides	Anti-obesity and LDL cholesterol-lowering effects in obese mice	(Nakahara et al., 2020)
Pleurotus pulmonarius	Water-soluble carbohydrate and protein	Enhancement of innate immune parameters and serum bactericidal activity against *S. agalactiae* in red hybrid tilapia fish	(Ching et al., 2021)
Pleurotus ferulae	Water-soluble polysaccharides	Significant antioxidant activities in vitro and in vivo	(Wang et al., 2020)
Pleurotus citrinopileatus	Polysaccharides (arabinose, galactose, glucose, xylose, mannose and glucuronic acid)	Antitumor effect. Protection of immune organs and improve inflammation and anemia	(Wang et al., 2021)
Pleurotus citrinopileatus	β-glucan (450 KDa)	Anti-inflammatory properties	(Minato et al., 2019)
Pleurotus djamor	Water-soluble galactoglucan	Antioxidant activity	(Maity et al., 2021)

Species	Bioactive compound	Functionality	Reference
Pleurotus ostreatus and *Lentinula edodes*	Carbohydrates, phenolic compounds, flavonoids, and proteins	Antiviral, antitumor and antioxidant capabilities	(Elhusseiny et al., 2021)
Lentinula edodes	β-glucan	Dietary supplementation with β-glucan as effective prevention in cognitive decline associated with obesity	(Pan et al., 2021)
Lentinula edodes	Polysaccharides	Polysaccharide-rich fractions from *Lentinula edodes* in the immature phase showed higher bioactivity, with higher molecular weight and protein content	(Wang et al., 2020)
Lentinula edodes	Lentinan	*In vitro* pulmonary immunomodulatory and cytoprotective effects	(Murphy et al., 2020)
Lentinula edodes	Ergosterol and β-glucan	Hypocholesterolemic effect	(Morales et al., 2019)
Lentinula edodes	Purified glucan fractions	*In vitro* hypocholesterolemic, immunomodulatory antioxidant and antitumoral activities	(Morales et al., 2020)
Lentinula edodes	Polysaccharides	Immunostimulatory activity	(Li et al., 2023)
Grifola frondosa	Heteropolysaccharide consisted of arabinose, mannose and glucose	Enhancer for the improvement of type 2 diabetes and regulator of the intestinal microbiota in diabetic individuals	(Chen et al., 2019)
Grifola frondosa	Acid-soluble polysaccharide, (1 → 3)-β-D-Glc and (1 → 3)-α-D-Man	Protective antitumor effect in mouse thymus and spleen	(Yu et al., 2020)
Grifola frondosa	Heteropolysaccharide	Significant protective action against nonalcoholic fatty liver disease	(Li et al., 2019)
Grifola frondosa	Polysaccharides	Hypoglycemic and hypolipidemic with modulated gut microbiota effects in diabetic mice	(Guo et al., 2020)

Species	Bioactive compound	Functionality	Reference
Grifola frondosa	Polysaccharide-protein complex	Immune stimulating activity. Antitumoral effect. Regulatory capacity of the composition and abundance of the intestinal microbiota through the production of short-chain fatty acids	(Zhao et al., 2023)
Grifola frondosa	α-glucan	Inhibition of tumor growth by enhancing immune responses	(Masuda et al., 2023)
Agaricus bisporus hortensis	Lectin and mannose-binding proteins	Anti-proliferative activities toward cancer cells and immunostimulatory effect	(Ismaya et al., 2020)
Agaricus bisporus hortensis	Carbohydrates, phenolic compounds, flavonoids, and proteins	Antioxidant activity and antiviral activities	(Elhusseiny et al., 2021)
Agaricus bisporus hortensis	Polysaccharides (glucose as the dominant monosaccharide)	Immune stimulating activity	(Liu et al., 2020)
Agaricus bisporus hortensis	Ergosterol and linoleic acid	Antioxidant and hypolipidemic effect, improved insulin sensitivity and glucose homeostasis in obese mice on high-fat diet	(Das et al., 2022)

1.4.2. Applications as texturizers

One of the qualities for which mushrooms are valued as food formulation ingredients is their texture modification. They are used, for example, in creams and sauces or as a substitute ingredient in the preparation of meat products, as they contain a high percentage of dietary fibre, easily digestible proteins and meat-like texture, so they can serve as substitutes for salt, phosphates, proteins and fats in the formulation of these products (Pérez Montes et al., 2021; Yuan et al., 2022; Zahari et al., 2022). Some of the compounds responsible for these modifications in texture are fungal biopolymers, that have gained interest as useful additives for the food industry in the form of thickeners, gelling and film-forming agents, stabilisers, texturisers and emulsifiers, and also as tools to improve the bioavailability for the delivery of hydrophobic or poorly soluble nutraceuticals (Choudhary, 2020). Specially mushroom β-glucans can have the potential to increase water viscosity, which is related to their molecular weight, chemical structure, concentration, and the food matrix on which they are applied, providing food products with improved rheological properties (Sovrani et al., 2017). That is why they can contribute to various forms of application in the food and pharmaceutical industry, depending on their chemical structure and intermolecular interactions. The texture, flavour, quality, and shelf life of foods will be influenced by the addition of these polysaccharides, so rheological studies have been applied to determine the viscosity and gelling properties of glucans, with the aim of using these molecules as food additives (Abreu et al., 2019). In addition, chitin nanomaterials from fungi have immense potential for applications in the nutraceutical and food industry and are a potential structural reinforcement agent to improve the mechanical properties of composites such as films, hydrogels, and aerogels (Zhang et al., 2020).

1.4.2.1. Gelling properties

Due to their great capacity in the rheological modification of foods, different polymers formed by polysaccharides and/or proteins, known as hydrocolloids, are used in the formation of viscous dispersions or water-based gels for food applications. The main functional properties that they are able to provide, affect thickening, gelling, but they can also affect emulsification, stability or control in the formation of ice or sugar crystals on different food matrices (Saha & Bhattacharya, 2010). The main objective is to affect the viscosity and texture of the food in order to achieve certain desired sensory properties. Among the properties of these polymers are some that have the ability to form gels, which implies the formation of a three-dimensional network as a result of the association or cross-linking of the polymeric chains, with water trapped inside. The application of these hydrogels in food products is intended to specifically affect the properties of hardness, cohesiveness, elasticity or chewiness (Wee et al., 2018). One of the most studied and used gelling hydrocolloids is gelatin, due to its functional properties. Gelatin melts at a relatively low temperature, is slow-setting and provides elasticity and clarity in the formulations of milk desserts, spreadable creams, confectionery, among others. However, the high price, as well as the problems derived from its animal origin, cause some limitations for its use in the food industry. Other alternative gelling products to gelatin have been used, such as alginate, pectin, carrageenan, gellan gum and agar. But replacing gelatin is very difficult due to its unique properties (Fenton et al., 2021; Mardani et al., 2019; Saha & Bhattacharya, 2010).

On the other side, fractions rich in mushroom polysaccharides have been shown to have gelling properties as well (see Table 2), without the need for purification and without the addition of certain ions or other non-gelling polysaccharides (Zhang et al., 2011). The work carried out by (Sovrani et al., 2017) shows that two aqueous fractions obtained from *Pholiota nameko*, one unpurified (rich in mannose,

galactose and glucose) and another purified fraction rich in β-glucans, showed similar shear-thinning behaviour and gel-like structure, so that it was not necessary to isolate the β-glucan polymer to achieve some desirable rheological properties. It appears that one of the mechanisms for the formation and support of gel-like structures by mushroom polysaccharides could be hydrogen bonds formed between them (Bao et al., 2018). The evaluation of polysaccharides fractions' gelling or thickening properties can be the key to the improvement of the manufacture, distribution, storage, and consumption of food products (Xu et al., 2016).

1.4.2.2. Emulsifying effect

Emulsions are dispersions prepared from the mixture of two immiscible liquids composed of an oily and an aqueous phase stabilized using surfactants, which reduce possible coalescence and Ostwald ripening processes, avoiding phase separation (Campelo et al., 2023). The preparation of stable emulsions is one of the most challenging processes in the food industry. The nutritional and functional values of mushrooms encourage their application or that of their extracts in the food industry as additives, supplements, and emulsifiers (Sovrani et al., 2017). Among the different types of emulsifiers used, biopolymers have received great attention in recent years due to the growing interest in the use of natural and sustainable food ingredients and some of the most studied natural macromolecules have been β-glucans, vegetable proteins or pectin (Umaña et al., 2021). The stability of the emulsions can be enhanced by viscosity and steric hindrance in addition to the interaction of polysaccharides with proteins (Jung et al., 2022). Ideal characteristics for emulsion stabilization using macromolecules include their high molecular weight, amphiphilic character, thickening effect, presence of few ionizable groups and surface activity (Campelo et al., 2023). It seems that most of

the emulsifying power of fractions obtained from mushrooms comes from their polysaccharide content (Table 2). As seen in the work carried out by (Campelo et al., 2023), emulsions were stabilized using polysaccharides from the fungus *Agaricus blazei Murill* and morphological analysis suggested the deposition of thin polysaccharide layers around the oil droplets. Moreover, the work performed by (Umaña et al., 2021) showed the stabilizing capacity for O/W emulsions of mushroom by-products obtained from *Agaricus bisporus* rich in glucose (β-glucans), mannose and proteins.

1.4.3. Biodegradable packaging films

In recent decades, the use of plastic materials at an alarmingly increasing rate has raised concerns related to their waste management and environmental pollution. Therefore, this has generated a growing interest in obtaining biopolymers with biodegradable character and suitable technological properties for replacing synthetic plastics. Extraction of these biopolymers from renewable natural resources is seen as the most sustainable and long-term solution (George et al., 2020).

The formation of film-like materials from different biomasses has been studied, but specifically the use of fungal biomass has been less explored. Mushrooms and their residues contain structural carbohydrates, such as β-glucans and chitin, which may be of great interest in the development of film-like biodegradable materials (Hong & Ying, 2019). The use of fungal chitin as reinforcement in the formation of different biomaterials has been reported but as previously mentioned, it is difficult to obtain pure fractions of chitin from mushroom's biomass. The structure and properties of fungal chitin nanocrystals mainly depend on the extraction protocols by which it has been obtained, the allomorphic form of chitin, or its composition and its interaction with proteins or glucans (Mushi, 2021). Chitin is

usually found as part of chitin-glucan complexes that act by reinforcing the structure of nanomaterials obtained from fungal biomasses (Kaya et al., 2022). Sometimes these film-like materials have been used not only as packaging material, but also as carriers of bioactive compounds (Table 2). For example, in (Ju & Song, 2020) study, the objective was to manufacture biodegradable films based on water-soluble polysaccharides from the *Tremella fuciformis* mushroom, containing peanut skin extract, for use as bioactive ecological packaging material with antioxidant activity. On the other hand, Zhang's work (Zhang et al., 2020) focused on the development of biodegradable/edible films based on insoluble dietary fibres derived from dried stems of aged *Lentinus edodes* and *Flammulina velutipes*. This study demonstrated the ability to form film-like materials from the insoluble fibre of these by-products, showing great potential for its application in the field of edible packaging.

Table 2. Texturizers and film-forming applications from mushrooms. Studies have been carried out in the last 5 years.

Species	Material forming compounds	Material applications	Reference
Pleurotus ostreatus	Polysaccharides from *Pleurotus ostreatus* powder	Emulsion stability of sausages as a phosphate alternative	(Jung et al., 2022)
Pleurotus ostreatus	Mushroom β-glucan and oat protein conjugates	Glycosylated oat protein emulsion for β-carotene encapsulation	(Zhong et al., 2019)
Pleurotus ostreatus	β-glucan with associated proteins	Food emulsifier as a physical protection to functional, oxygen sensitive lipophilic ingredients by microencapsulation	(Gallotti et al., 2020)
Pleurotus eryngii	Mushroom polysaccharide and soy protein conjugates	Protein-polysaccharide conjugates-stabilized emulsions for β-carotene encapsulation	(Hu et al., 2021)
Lentinula edodes	β-glucan	Hydrogels for topical use	(Menezes et al., 2022)
Lentinula edodes	Mushroom polysaccharide and gelatin	Film for food packaging applications	(Guo et al., 2023)
Agaricus bisporus and *Pleurotus ostreatus*	Mushroom powder	Partial replacement of pork backfat and salt of frankfurters sausages	(Cerón-Guevara et al., 2020)
Agaricus bisporus	Polysaccharides and proteins	Stabilization of oil-in-water emulsions	(Umaña et al., 2021)
Agaricus bisporus	Chitin-glucan complex and curcumin	Films for food packaging with bioactive properties (antioxidant activity, inhibitory effect against *E. coli*)	(Kaya et al., 2022)
Agaricus bisporus	Mushroom powder, sorbitol, and agar-agar	Films as a source of delivery of active compounds	(Garcia et al., 2022)

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II. OBJECTIVES

The general objective of this thesis was to carry out a biomass valorisation of different types of mushrooms to find applications of food interest both functional and in the formation of materials.

To achieve this general objective, several specific objectives were established:

1. To **better understand the structure of the mushroom cell wall** and the release of its compounds **by different widely used extraction methods**.

2. **To find** high added-value applications for **extractable mushroom compounds with reported bioactivity**.

3. **To develop functional ingredients**, for applications in food formulations.

4. **To obtain materials of interest in food packaging** for the recovery of direct mushroom residues.

These general objectives were materialized in 6 individual studies, which have been structured in 3 chapters.

III. RESULTS

This section includes the results from 6 individual studies, which have been structured in 3 chapters:

Chapter 1. Structure and bioactivity study of different fractions obtained from mushrooms biomass

1.1. Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol.

1.2. Composition, structural properties and immunomodulatory activity of several aqueous *Pleurotus* β-glucan-rich extracts.

Chapter 2. Relationship between structure and functional properties of mushroom biomass as texturisers for food applications

2.1. Antiviral and technological properties of β-glucan-rich aqueous fractions from *Pleurotus ostreatus* waste biomass.

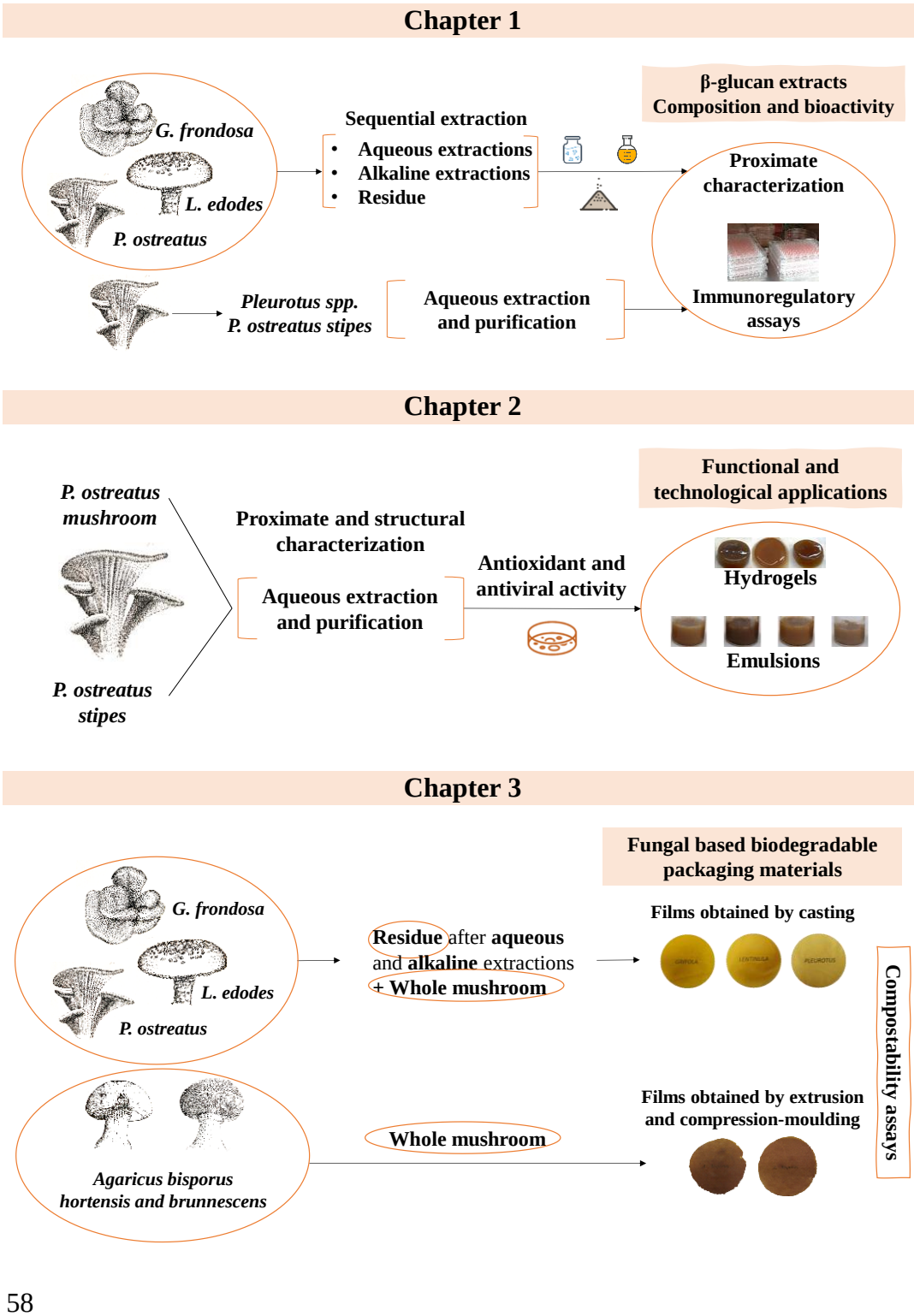
2.2. Emulsifying properties of β-glucan-rich extracts obtained from *Pleurotus ostreatus* mushroom and stipes.

Chapter 3. Use of mushrooms waste biomass for the formation of biodegradable film-like materials for food packaging applications

3.1. Ultrasound-treatment as a promising strategy to develop biodegradable films obtained from mushroom waste biomass.

3.2. Feasibility of *Agaricus bisporus* waste biomass to develop biodegradable food packaging materials.

Thesis flow chart



Introduction of the results

This PhD thesis contributes towards increased understanding in mushroom valorisation for obtaining novel food ingredients and food packaging materials. The PhD work combines research activities dealing with sequential extraction, characterization and assessment of immunostimulatory activity of several β-glucan extracts (Chapter 1); assessment of the obtained mushroom extracts as gelling and emulsifying ingredients with additional functional properties such as antioxidant and antiviral activities (Chapter 2); and development of novel biodegradable and compostable packaging materials (Chapter 3). Specifically, each chapter deals with one of the specific milestones defined above and includes two specific SCI papers already published or being currently under review.

The production of mushrooms has been on an increasing trend in the last years due to their excellent nutritional properties (being rich in valuable proteins, minerals, and dietary fibres such as β-glucans). However, its increased production and consumption is also leading to an increase volume of mushroom by-products (including stipes and mushroom discards that do not comply with commercial standards). As commented above, mushrooms are a rich source of interesting carbohydrates, specially the β-glucans, which have a tremendous potential for being used as functional ingredients. The structural properties of β-glucans, in terms of branching degree, molecular conformation, molecular weight and their interaction with other compounds, will determine their solubility and extractability.

Promoted by global policies fostering a circular economy, several research activities are devoted to understanding the composition of discarded mushroom biomass (whole mushrooms and stipes) and explore their potential applications. Therefore, a proper characterization is needed to ascertain their potential. The integration of valorisation strategies for these substrates (whole biomass and its stipes), would minimize waste generation and achieve a higher degree of

valorisation for mushroom biomass, while developing new cost-efficient ingredients. Therefore, **Chapter 1** is focused on performing a thorough characterization of the different streams generated during a sequential fractionation process involving several consecutive cold or hot aqueous and alkaline treatments applied to widely consumed mushrooms (*P. ostreatus*, *L. edodes* and *G. frondosa*), in order to understand differences in the cell architecture of the different mushroom species, which will be considered when designing adequate valorisation strategies. Since *Pleurotus* genus had a more accessible type of β-glucans than the other widely consumed mushrooms together with the fact that their cultivation and further processing generate greater residual volumes (part of stipes), in the second part of this chapter, water-soluble β-glucans-rich fractions of various *Pleurotus* species and of the stipe of *P. ostreatus* were obtained and, the impact of their compositional and structural differences on the immunostimulatory activity deeply analysed.

It is well-known that mushrooms have been widely used as food ingredients not only for their fibre and protein-rich composition, low fat and low salt content, but also for their ability to modify the texture of certain foods, such as creams and sauces. These texturizing properties can be mainly ascribed to their protein and β-glucan components. Therefore, **Chapter 2** was aimed to evaluate the technological applications of β-glucans aqueous fractions. Although there are highly branched β-glucans which need more aggressive treatments, water-soluble fractions (easily extractable β-glucans), could be a more eco-sustainable option in the production of new functional ingredients. It is hypothesized that different composition and structural properties will have different technological implications. Therefore, this chapter provides fundamental knowledge to understand the gelling and emulsifying properties of β-glucans aqueous extracts obtained from one of the most consumed mushrooms, *P. ostreatus* and its residue (stipes). Furthermore, the

impact of β-glucan structural complexity on the functional properties (antiviral and antioxidant capacity) was also investigated.

Apart from being promising sources of new functional ingredients, mushrooms are also rich in chitin which, together with β-glucans, are the major constituents of polysaccharides from mushrooms' cell walls and they have a great potential for being used as packaging materials. **Chapter 3** is aimed at exploring different mushroom biomasses and their residues generated after aqueous and alkaline treatments, as sources of biodegradable and compostable packaging films in order to understand how the processing conditions and composition of the raw material affect physicochemical properties and compostability of the developed films.

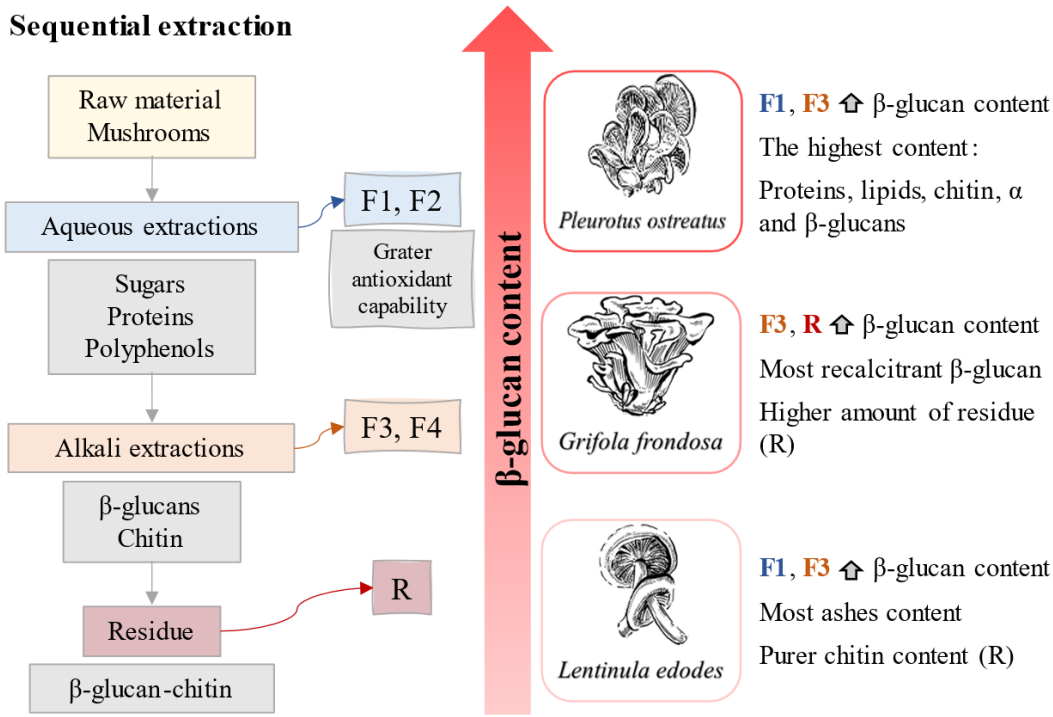
CHAPTER 1

Structure and bioactivity study of different fractions obtained from mushrooms biomass

1.1. Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol.

1.2. Composition, structural properties and immunomodulatory activity of several aqueous *Pleurotus* β-glucan-rich extracts.

1.1. Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol.



This section is an adapted version of the following published research article:

Zaida Pérez-Bassart, Maria Jose Fabra, Antonio Martínez-Abad & Amparo López-Rubio, 2023.

"Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol"

Food Chemistry, volume 402, 134207.

Abstract

In this work, a sequential fractionation protocol (cold and hot aqueous and alkaline extractions) and detailed compositional analysis (gross composition, monosaccharide analysis, FTIR, TGA) was applied to three relevant mushrooms in terms of global production (*Grifola frondosa*, *Lentinula edodes* and *Pleurotus ostreatus*) aiming to understand what is preferentially extracted during fractionation and how β-glucan extraction is affected by mushroom source. Room temperature aqueous extracts showed highest overall yields (56.3-82%) consisting of proteins, sugars and polyphenols. β-glucan content was highest in P. ostreatus and was concentrated in the more soluble fractions. On the contrary, a recalcitrant β-glucan in G. frondosa was mainly present in the residue (7.38%). L. edodes showed β-glucan populations distributed along aqueous and alkaline extracts, higher abundance of non-glucan polysaccharides and higher chitin purity (47.78%) in the residue. This work sets the basis for the rational design of extraction processes aiming to valorise mushroom biomass.

1. Introduction

Edible mushrooms are a good source of interesting carbohydrates, proteins, vitamins, antioxidants and minerals with low fat levels and low caloric content, thus making them a nutritious and healthy food source (Kalač, 2012). Probably linked to the wide recognition of their nutritional and health benefits, the economic importance of mushrooms is also growing, with a considerable global production increase from 7.5 million tonnes in 2009 to 11.8 million tonnes in 2019 (Schill et al., 2021). Market requirements of uniform size, shape and colour for these type of foods result in the removal of excess stipe length and rejection of mushroom biomass with slight discoloration and gross size variations (Ramos et al., 2019). This discarded biomass could be upcycled to obtain added-value compounds, in line with current circular economy practices.

The fungal cell wall is mainly formed by two types of structural polysaccharides, a rigid chitin fibrillary structure, and a matrix-like structure consisting on β-glucans, α-glucans and glycoproteins (Ruthes et al., 2016). Fungal glucans comprise structurally different polymers of D-glucopyranose (D-Glcp), which, despite of their simple monosaccharide composition, show a large diversity regarding molecular mass, anomeric configuration, position and sequence of glycosidic bonds along the chain, branching degree, branch composition and chain conformation (de Jesus et al., 2018). According to the anomeric structure, two main groups of fungal glucans are found, i.e. α-D-glucans and β-D-glucans, the last ones being the most abundant polysaccharides in fungal cell walls (Synytsya et al., 2009). β-glucans consist of β-(1→3) and (1→6) linkages with a huge structural diversity, being lineal or branched, and amorphous or microfibrillar structures (Bai et al., 2019), which can be embedded in the crystalline chitin (Ifuku et al., 2011). The interest in fungal polysaccharides, and especially in β-glucans, derives from their proven biological activity, e.g. antioxidant, antiinflammatory, anticancer, anti-diabetic, antimicrobial, antilipidemic, hypoglycemic, and immunomodulatory activities (Bai et al., 2019), thus making them attractive as active ingredients with potential applications in food, medicine, pharmacy, cosmetics, chemical and feed industries (Mirończuk-Chodakowska et al., 2021).

Specific fungal species, such as *Grifola frondosa, Lentinula edodes*, and *Pleurotus ostreatus*, are even considered medicinal, being recommended for therapeutic applications (Badalyan et al., 2019). *G. frondosa* (Maitake) has been widely used as a traditional food supplement in China, Japan and Korea (Ji et al., 2019) because of the recognized health beneficial effects from its polysaccharides, including antioxidant and antitumor activity amongst others. Specifically, a characteristic β-glucan isolated from *G. frondosa* may serve as a macrophage activator by increasing cytokine production and TNF-α expression (Wu et al., 2021). On the other hand, β-glucans present in *L. edodes* and *P. ostreatus* (two of

the most widely used and cultivated mushroom varieties), also have pharmacological activities, with proven antibacterial, antiviral and anti-inflammatory capacity amongst others (Bai et al., 2019). Other bioactive compounds such as polyphenols present in the mushroom biomasses could also contribute to the reported antioxidant or antimicrobial activity (Smolskait et al., 2015).

Of course, these bioactivities depend on the β-glucan structure which, at the same time, is influenced by source and extraction conditions. Therefore, extraction of β-glucans from different fungal biomass has been widely explored, using a plethora of methods, but mainly applying treatments based on aqueous or alkaline treatments, but without considering the inherent recalcitrance of the different biomass sources. Aqueous and alkaline extractions are the most widely used methods for β-glucan extraction (Ruthes et al., 2016). Specifically, NaOH treatments are a widely recognized simple, efficient, and low-cost purification method for obtaining water-insoluble glucan fractions with different anomeric configurations and degrees of branching (de Jesus et al., 2018). Although specific names describing bioactive β-glucan have been coined for the different species, e.g. lentinan, grifolan, pleuran, etc., these sometimes refer to fractions obtained under completely different extraction conditions, which explains great differences in the extent of their bioactive effects, both qualitatively and quantitatively (Mirończuk-Chodakowska et al., 2021). While a subject scarcely investigated, knowledge on the inherent recalcitrance of the different polysaccharide components is crucial to design protocols for the targeted extraction of bioactive polysaccharides.

In this work, a sequential fractionation protocol was applied to *G. frondosa*, *L. edodes* and *P. ostreatus* and a complete compositional analysis was performed to all fractions obtained, with emphasis on the distribution of β-glucan, chitin or other polysaccharide components and their antioxidant activity. This comparative study thus aimed at shedding some light on the inherent recalcitrance and organization of

polysaccharide components in these relevant species, understanding what components were preferentially extracted along the sequential protocol, key for the design of valorisation strategies.

2. Materials and methods

2.1. Fungal biomass production

Fungal biomass from *G. frondosa*, *L. edodes* and *P. ostreatus*, grown at the Instituto de Investigación y Tecnología Agroalimentaria (IRTA, Spain), was used as feedstock (see photos in Figure S1 from the Supplementary Material). The main characteristics of these species are also provided in the Supplementary Material and details of fruit body production are provided elsewhere (Aranaz et al., 2021). Briefly, 4 L of a standard substrate for the commercial production of edible mushrooms contained in polypropylene bags with ventilation windows (Sac O2®, Deinze, Belgium) were autoclaved at 100 ºC for 2 h and then inoculated with commercial inoculum of the different species (Deinze, Belgium; https://www.mycelia.be/en). After eight weeks of incubation at 22–25 ºC needed for mycelium growth, the culture bags were transferred to flowering (or fruiting) rooms at 15 ± 3 ºC and 80–90% RH to induce mushroom production and kept for another two weeks, being finally harvested.

2.2. Reagents

Reagents NaOH (pure), potassium persulfate and EtOH (96%) were obtained from PanReac AppliChem. Lentinan (min. 30% purity) was purchased at Carbynth Biosynth (UK). Other materials and reagents used, specifically HCl (37%), KH_2PO_4 (≥ 99.0% purity), $HNa_2O_4P{\cdot}2H_2O$ (≥ 99.0% purity), shrimp shells chitin, sodium borohydride (NaBH4), Folin-Ciocalteu reagent, sodium carbonate, gallic acid, potassium persulfate, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphate buffered saline (PBS), 6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox), trifluoroacetic acid (TFA),

fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid were purchased from Sigma-Aldrich. Yeast β-glucan (49% purity) was obtained from Megazyme.

2.3. Sequential extractions

For each mushroom, a fractionation protocol was performed (depicted in Figure S2 in the Supplementary Material). 25 g of freeze-dried raw material were taken, and 300 mL of distilled water were added at room temperature (RT) under agitation for 7 h. Each sample was then centrifuged 15 minutes at 8000 rpm and 4ºC and the precipitate was subjected twice more to aqueous extractions at room temperature with a volume of 150 mL, under agitation for 7 h. The aqueous fractions obtained from these extractions were concentrated and then freeze-dried, resulting in fraction F1. The precipitate obtained from the F1 extraction was then subjected to an aqueous treatment at 100 °C with reflux a total of 3 times, with a volume of 300 mL of distilled water in the first incubation and twice more with a volume of 150 mL. The soluble part resulting from these 3 extractions was again concentrated and freeze-dried as the F2 fraction. The precipitate was subjected to a first alkaline treatment at room temperature overnight with 150 mL of 1 M NaOH and 0.05% $NaBH_4$ to avoid polysaccharide oxidation or "peeling" at the reducing end (Leong et al., 2021). The suspension was centrifuged 15 minutes at 8000 rpm and4ºC and the supernatant was reserved for precipitation with 1:3 (v/v) ethanol to remove low molecular weight components, and subsequently neutralized by washing with 0.4 M HCl in ethanol first, followed by another ethanol washing. The neutralized precipitate was left to air dry and was subsequently freeze-dried, as fraction F3. The remaining precipitate was subjected to a second alkaline treatment, under the same conditions to promote the extraction of the less soluble β-glucans from the mushrooms, which after the first alkali treatment (F3) are expected to be more accessible. The supernatant fraction was treated as with F3 and named as F4. The final residue was named as R.

Prior to all analyses, F3, F4 and R fractions were dialyzed through a molecular weight cut-off of 100-500 Daltons (SpectrumLabs), to remove the salts used in all extraction and neutralization steps, and were finally freeze-dried.

All extractions were made in triplicate and further analyses in triplicate were performed with each of these to evaluate reproducibility of the whole extraction process.

2.4. Raw material gross characterization

Moisture content was determined gravimetrically, by weight difference before and after a freeze-drying process, resulting in the percentage of water loss (AOAC, 2022).

For the quantification of ashes, samples of known weight were placed in a muffle furnace at 550 °C until complete calcination. The difference in weight at room temperature was recorded and the result was expressed as a percentage of weight lost compared to the initial sample (AOAC, 2022).

The total nitrogen content was determined using an elemental nitrogen analyser (rapid surplus N analyser) based on the modified Dumas method. As chitin also contributes to the total nitrogen, a factor of 4.38 was applied (Kalač, 2012).

Lipid determination was based on Soxhlet extraction (Somashekar et al., 2001), starting from 5 g of biomass and using 250 mL of hexane as solvent for 8 hours. The resulting extract after the treatment was dried and the lipid content estimated gravimetrically.

2.5. Antioxidant capacity

Antioxidant capacity was determined by the ABTS method (2.2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)(Re et al., 1999). 0.192 g of ABTS and 0.033 g of potassium persulfate were dissolved in 50 ml of PBS (phosphate buffered saline) at pH 7.4 and left to stir overnight. Prior to the determination, $ABTS^{\cdot+}$ radical cation was diluted in PBS for an initial absorbance of about 0.70 ± 0.02 at

734 nm. The determination of the free radical scavenging activity was performed by mixing 230 µL of the $ABTS^{\cdot+}$ solution with 20 µL of each sample and measuring the absorbance after 6 minutes' incubation at room temperature and darkness. The experiments were done in microplates and the absorbance at 734 nm was measured in a CLARIOstar spectroscopy equipment (BMG LABTECH). A calibration curve was built using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity of the samples is expressed as µmol Trolox equivalents (TE)/g fraction. All the determinations were performed in triplicate.

2.6. Total phenolic compounds

The total phenolic content of mushrooms and their fractions were determined using the assay based on the Folin-Ciocalteu method as modified by Singleton (Singleton et al., 1999). 1000 µL of 1/10 diluted Folin-Ciocalteau reagent were mixed with 200 µL of sample and allowed to incubate for 5 minutes. Then, 800 µL of sodium carbonate (75 mg/mL) were added and the samples were incubated in a bath at 40 °C for 30 minutes. The absorbance values were measured at 760 nm. A calibration curve was performed using gallic acid as standard, and the total polyphenol concentration was expressed as mg gallic acid/g sample.

2.7. Carbohydrate composition

The monosaccharide composition was evaluated after acid hydrolysis from the raw materials and different fractions to convert the polysaccharides into monosaccharides according to (Aranaz et al., 2021). Briefly, 1-2 mg sample were added 1 mL of 2 M of trifluoroacetic acid (TFA) and kept at 120 °C for 3 hours. Samples were dried with a stream of air at 40 °C for complete evaporation of the TFA and re-suspended in deionized water. The monosaccharides were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex) using a CarboPac™ PA1 column. Samples of

known concentrations with mixtures of fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid were used for calibration. All experiments were carried out in triplicate.

2.8. α-glucans and β-glucans content

According to (McCleary & Draga, 2016) the α-glucan content was determined upon enzymatic hydrolysis with amyloglucosidase plus invertase (Megazyme, Ireland). Generated glucose was quantified with a glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-aminoantipyrine; Megazyme, Ireland). The absorbance of all solutions was measured at 510 nm against the reagent blank. The values of glucan contents were expressed as g of glucose per 100 g of dry matter. The β-glucan content was calculated by subtracting the α-glucan glucose from the total glucose content as determined by TFA hydrolysis and made in triplicate.

2.9. Chitin and protein content

To determine the chitin content, hydrolysis was carried out in 6 M HCl at 100 ºC for 7 hours. The hydrolysis time was set after some preliminary optimization trials (see Supplementary Material) based on the method described in (Ekblad & Nasholm, 1996) and selected conditions were found to reach maximum glucosamine recovery. In contrast to most previous works determining the released glucosamine by colorimetric methods (Vetter, 2007), glucosamine was in this case quantified by HPAEC-PAD with a CarboPac™ PA1 column.

The D-glucosamine nitrogen (NGlc) content was obtained according to:

$$NGlc = \frac{WGlc \cdot 14}{MGlc}$$

Where WGlc was the % w/w of D-glucosamine content obtained from the above-mentioned hydrolysis, and MGlc was de D-glucosamine molecular mass (Gomba et al., 2015).

The total N_2 content in all fractions was determined using an elemental nitrogen analyzer (rapid surplus N analyzer) based on the modified Dumas method. The protein nitrogen was calculated as the difference between the total nitrogen from the elemental analysis and D-glucosamine nitrogen, and by applying the correction factor for proteins in foods of 6.25.

2.10. Fourier-transform infrared (FT-IR)

Infrared analysis was performed at 21 ºC using a Jasco 4100 FT-IR spectrometer. The spectra were taken at 4 cm^{-1} resolutions in a wavelength range of 600–4000 cm^{-1} with a minimum of 32 scans. The results were processed using Origin Pro 2019 software.

2.11. Thermogravimetric analysis (TGA)

Thermogravimetric curves (TG) were obtained from a made with a TGA550 from TA Instruments, equipped with a Pt/Rh furnace with a maximum temperature of 1000 °C and an autosampler. The samples were heated in platinum capsules in the range of 10 to 600 °C with a heating rate of 10 °C/min under air atmosphere. Derivative TG curves (DTG) were plotted to express the weight loss rate as a function of temperature using Origin Pro 2019 software.

2.12. Statistical Analysis

Data analysis of results was carried out using Statgraphics. One-way analysis of variance (ANOVA) was done to determine the significant differences between samples, at a significance level of $P < 0.05$.

3. Results and discussions

3.1. Raw material characterization

Initially, a characterization of *G. frondosa, L. edodes* and *P. ostreatus* biomass was carried out and the results are compiled in Table 1. The moisture content was around 87-88 % with no significant differences between samples and within

moisture content values (67.2 to 91.5%) previously reported for mushrooms (Manzi et al., 2004).

The ash content ranged between 6-7% (Table 1) for all three species, again without significant differences between them and in accordance to previously reported values, which are reported to vary between 5 and 12% on a dry matter basis and usually below 10% (Kalač, 2012).

As commented in the introduction, mushrooms generally have low lipid contents, which may range from 0.1 to 16.3% and usually between 1 and 4% of the dry matter (Kalač, 2012). As observed in Table 1, *P. ostreatus* was the mushroom with greatest lipid content (5.3%), followed by *L. edodes* (4.6%) and *G. frondosa* (4.2%).

Regarding the protein content, the three mushrooms contained 23-26% based on dry weight, *P. ostreatus* being again the one with greatest protein content within the margin in accordance with previous reports (González et al., 2021), and *G. frondosa* (Wu et al., 2021) showing the lowest (Table 1). In general, edible mushrooms are considered a good source of protein, with protein content values reported between 19 and 35% (Ramos et al., 2019;).

The amount of protein was initially determined by applying a corrective factor of 4.38 to the total nitrogen content, as previously suggested in the literature (Kalač, 2012). In order to confirm the adequacy of the factor applied, chitin content was also quantified by chromatography methods (see section 2.8) and its nitrogen contribution subtracted from the total nitrogen. The remaining nitrogen was considered to come solely from protein and a factor of 6.25 was then applied. Both calculation methods yielded results with no significant differences amongst them, thus confirming the suitability of the applied factor for estimating protein content in whole mushrooms.

In order to complete this first gross biomass characterization with other relevant compositional data, the glucans and chitin content were also evaluated for the three mushrooms. *P. ostreatus* had significantly higher α and β-glucan content and

higher chitin content than *G. frondosa* and *L. edodes* (Table 1), all values within the range reported for wild mushroom species (2-8.5 dwt% for chitin, less than approximately 10% for α-glucan and <60.8 % for β-glucan) (Vetter, 2007). This positive correlation seems logical, as it is generally considered that a large part of the β-glucans present in the cell walls of mushrooms is bound to chitin. However, for valorisation purposes, it is not only the composition that matters, but how the different components are structurally organized in the cell walls, how strong their interactions are and, thus, how easily it is to extract the fractions of interest, i.e. understanding the recalcitrance of the various biomass sources.

Finally, the total polyphenol content and antioxidant capacity of the three mushroom species were also determined. As observed in Table 1, *P. ostreatus* showed greatest polyphenol content and antioxidant capacity, while the lowest content and related antioxidant capacity was found in *G. frondosa*. The significantly greater antioxidant capacity of *P. ostreatus* could also partially be ascribed to the greater β-glucan content of this mushroom, as these biologically active carbohydrates also possess antioxidant properties (Bai et al., 2019).

With the aim of shedding some light on the inherent recalcitrance and organization of the β-glucans in these relevant mushrooms and gather useful information for process optimization and the development of future targeted extraction protocols, the three biomass sources were fractionated (see Figure S1 in the Supplementary material) and the side streams obtained after each extraction step deeply characterized as shown below.

Table 1. Raw material initial characterization expressed as mean values ± standard deviations. Antioxidant capacity was expressed as µmol Trolox equivalents (TE)/g of sample, and polyphenols content as mg of gallic acid equivalents (GA)/ g of sample. Moisture is expressed as g/100 g of fresh mushroom and the other results are expressed as g/100 g of dried mushroom.

	G. frondosa	***L. edodes***	***P. ostreatus***
Moisture %	88.4 ± 2.0 [a]	87.0 ± 3.3 [a]	88.6 ± 4.1 [a]
Ash %	6.9 ± 0.2 [a]	7.2 ± 0.2 [a]	6.7 ± 0.3 [a]
Lipid %	4.2 ± 0.1 [a]	4.7 ± 0.2 [ab]	5.3 ± 0.6 [b]
Protein %	22.7 ± 2.1 [a]	24.5 ± 1.5 [a]	26.1 ± 2.4 [a]
Chitin %	5.09 ± 0.09 [a]	5.32 ± 0.06 [ab]	6.38 ± 0.30 [b]
α-glucans %	3.31 ±0.23 [a]	0.28 ± 0.04 [b]	7.57 ± 1.01 [c]
β-glucans %	25.9 ±1.4 [a]	23.3 ± 5.4 [a]	38.7 ± 2.1 [b]
Antioxidant capacity (µmol TE / g)	63.36 ± 3.57 [a]	99.81 ± 0.39 [b]	137.35 ± 9.39 [c]
Polyphenols content (mg GA/g)	16.04 ± 0.23 [a]	18.40 ± 1.22 [b]	20.11 ± 1.00 [b]

Different letters in the same row mean that the values are significantly different.

3.2. Extraction yields of the different fractions

As previously explained in the materials and methods section, a sequential extraction consisting on consecutive cold and hot water extraction steps (giving raise to fractions F1 and F2) followed by two alkaline extractions (F3 and F4, respectively) was applied to the three tested species (cf. Figure S2). The biomass yields obtained after each of the extraction steps were quantified and the results are compiled in Table 2. As observed in this table, the greatest yields were obtained after the first extraction step carried out using water at room temperature (F1). In fact, 56-82% of the mushroom biomass was easily solubilized in water without the need of applying heat treatments. Significant differences were, however observed between the three mushrooms, *G. frondosa* being much more recalcitrant than the

other two species, fact which was also reflected in this mushroom having the greatest residual (R) yield (cf. Table 2).

Applying a second extraction step with hot water (F2) only seemed to be useful in the case of *G. frondosa*, again pointing out the higher recalcitrance of even water-soluble components, in which around 8% of the biomass was solubilized. For the other two mushroom species, the yields obtained were relatively low and, thus, in principle, this second step could be skipped when designing a valorisation scheme for these specific mushrooms. Direct comparison with previous research works is not straightforward as, generally, extractions were focussed on obtaining carbohydrates (Morales, Smiderle, et al., 2019; Su et al., 2017) or specific β-glucan structures (Liu et al., 2014). However, low polysaccharide yields (between ~2.5 and 3.1% of the initial wet biomass) were also obtained from *G. frondosa* after aqueous extraction, thus confirming the recalcitrant nature of this mushroom (Su et al., 2017).

Table 2. Yields and protein content of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*, expressed as a percentage by weight of the initial dry samples. Antioxidant capacity (in µmol Trolox equivalents (TE)/g of sample) and polyphenols content (mg of gallic acid equivalents (GA)/g of sample) in mushroom fractions.

	F1	**F2**	**F3**	**F4**	**R**
Yield (%)					
G. frondosa	56.3 ±3.6 [a]	7.9 ± 0.2 [a]	5.6 ± 0.8 [a]	4.3 ± 1.1 [a]	15.9 ± 0.6 [a]
L. edodes	82.0 ± 4.2 [b]	5.0 ± 1.5 [b]	6.2 ± 0.7 [a]	2.2 ± 0.5 [a]	8.2 ± 1.5 [b]
P. ostreatus	76.5 ± 0.3 [b]	2.6 ± 0.5 [c]	8.3 ± 0.6 [b]	2.2 ± 0.8 [a]	8.6 ± 0.6 [b]
Antioxidant capacity (µmol TE/g)					
G. frondosa	279.95 ± 0.38 [ab]	292.94 ± 4.31 [a]	6.18 ± 3.25 [a]	6.61 ± 0.43 [a]	11.62 ± 0.08 [a]
L. edodes	250.30 ± 20.87 [a]	277.99 ± 7.84 [ab]	90.65 ± 0.27 [b]	78.01 ± 1.89 [b]	4.27 ± 1.02 [b]
P. ostreatus	294.54 ± 6.78 [b]	275.89 ± 3.06 [b]	32.13 ± 4.94 [c]	51.49 ± 1.70 [c]	12.96 ± 2.20 [a]
Polyphenols content (mg GA/g)					
G. frondosa	41.84 ± 1.80 [a]	44.42 ± 0.97 [a]	4.22 ± 0.62 [a]	3.21 ± 0.20 [a]	8.08 ± 0.21 [a]
L. edodes	34.35 ± 1.07 [b]	33.59 ± 0.78 [b]	17.98 ± 0.36 [b]	9.30 ± 0.23 [b]	9.25 ± 0.40 [a]
P. ostreatus	35.97 ± 0.92 [b]	40.10 ± 1.29 [c]	6.59 ± 0.53 [c]	9.10 ± 0.82 [b]	13.70 ± 1.10 [b]
Protein (%)					
G. frondosa	42.99 (3.50) [a]	22.83 (3.81) [ab]	1.05 (0.29) [a]	0.67 (0.04) [a]	-
L. edodes	36.55 (0.98) [b]	19.41 (5.62) [a]	1.39 (0.64) [a]	3.03 (0.35) [b]	-
P. ostreatus	36.66 (1.01) [b]	28.75 (2.33) [b]	1.57 (0.16) [a]	2.01 (0.66) [c]	-

Different letters in the same column mean that the values are significantly different. F1 and F2 are the cold and hot aqueous extracted samples, respectively. F3 and F4 are the cold and hot alkaline extracted samples and R refers to the residue.

Alkali treatments are commonly applied to a variety of biomass for polysaccharide extraction, as they are known to cause a disruption of the cell wall facilitating their

release (Ruthes et al., 2016). Alkaline treatments applied to extract β-glucans much differ in the literature with regards to alkali concentration, temperature and time (da Silva Milhorini et al., 2022; Morales, Rutckeviski, et al., 2019). However, a substantial number of articles have dealt with alkaline extracts at room temperature as good options to obtain highly bioactive β-glucans, which would further add on cost-efficiency and justify the conditions in the present study (da Silva Milhorini et al., 2022; Morales, Rutckeviski, et al., 2019). Very interestingly, a previous work dealing with β-glucan extraction from *L. edodes* reported much larger yields in the alkaline fractions from this mushroom, which can be explained by the limited previous aqueous extraction applied (Morales, Smiderle, et al., 2019). This indicates that, in this fungal biomass most of the polysaccharides present can be extracted in water by applying longer treatments at room temperature, thus making the extraction processes greener. The recalcitrance of *G. frondosa* was again patent after the alkaline treatments as the same trend was observed with the two consecutive extraction steps, i.e. displaying the lowest yield of the three mushrooms for the F3 and the greatest yield for F4, indicating that a second alkaline treatment or harsher conditions are needed for an efficient extraction. In contrast and similarly to what was observed in the aqueous extraction steps, the F4 yields for *L. edodes* and *P. ostreatus* were very low and, thus, the process for these species could be, again, simplified.

3.3. Polyphenol content and antioxidant capacity of the different fractions

Amongst the biologically active compounds present in mushrooms, polyphenols have been widely recognized as very beneficial compounds with antioxidant and chemopreventive properties (Smolskait et al., 2015) and, thus, evaluating their content in the various fractions generated is of practical interest for valorisation purposes. Table 2 compiles the results of polyphenol content (expressed as mg of gallic acid –GA- per gram of sample) and antioxidant capacity (μmol Trolox equivalents per gram of sample) of the different fractions obtained. Most of the

polyphenols were concentrated in the aqueous fractions, without significant differences between the first and second extractions (i.e. F1 and F2), and representing around 7% of the dry extracts. This result was not surprising as the most abundant polyphenols in mushrooms are phenolic acids with good water solubility. A strong correlation has been found between the amount of polyphenols and the antioxidant activity of mushroom species, ascribed to the scavenging capacity of their hydroxyl groups (Smolskait et al., 2015). It is interesting to note that the phenolic content of the aqueous fractions was, for the three mushrooms, greater than 30 mg GA per gram of extract, being significantly greater than that from ethanolic extracts obtained from a wide range of mushrooms (Smolskait et al., 2015). This result is in agreement with a previous study dealing with the antioxidant properties of *Pleurotus citrinopileatus* extracts, where it was found that the total phenol content of the cold water extract was greater than that from an ethanolic extract and similar to the extract obtained using hot water (Lee et al., 2007). This has practical implications, showing that green and simple extractions can be applied to these mushroom species and their discards to obtain extracts with high antioxidant capacity. In fact, the antioxidant capacity values from the mushrooms aqueous extracts (all greater than 250 µmol TE per gram of extract) were very similar to the values from a range of medicinal Indian plants (with an average antioxidant capacity value of 270 µmol TE/g extract) and higher than that of some fruits with great antioxidant capacity, such as cranberries (Shahidi and Ambigaipalan, 2015). While being a simple method for quantification of antioxidant capacity, ABTS is not found naturally, so there is possible criticism that the assay is not directly relevant to any biological function (Opitz et al., 2014). Significantly lower phenolic contents were observed in the alkaline-extracted fractions (F3 and F4). It is well-known that the reactivity of polyphenols is enhanced under alkaline conditions resulting in degradation (Honda et al., 2019). An intriguing result was, however, obtained for *L. edodes*, as its F3 fraction had a much greater phenolic content and antioxidant capacity than the other alkaline

fractions. F4 from *L. edodes* also displayed a reasonable antioxidant capacity. The differences observed may be related to the different chemical structure of polyphenol as well as to the type of interaction with other cell wall components present in the three mushroom species, all of which deserves further study. *L. edodes*, for instance, has been reported to contain, amongst others, some phenolic acids, such as ferulic and cinnamic acid and flavanols like catechin which have been reported to resist major pH-induced degradation (Nam et al., 2021). Another possible explanation for the increased antioxidant capacity of F3 and F4 extracts from *L. edodes* may come from other molecules like mannogalactans (as it will be shown below), to which antioxidant properties have also been attributed (Ruthes et al., 2016).

The residual fractions (R) showed significantly lower antioxidant capacity and phenolic content. Nevertheless, the opposite tendencies in e.g. *L. edodes* and *G. frondosa*, with greater and lower polyphenol and antioxidant capacity in alkaline or residual fractions, respectively, again point out major differences in the types of polyphenols and their interactions depending on the species.

3.4. Carbohydrate compositional analysis and distribution in the different fractions

The absolute estimated amounts of the different monosaccharides present in the fractions obtained from the three mushroom species are shown in Figure 1. Figure 1a depicts the concentration of all detected monosaccharide units, excluding chitin contribution. For better visualization, Figures 1b and 1c display the chitin and the rest of sugars (excluding glucose) in all sample fractions, respectively (sections 3.4.2 and 3.4.3). All these data can again be found in detail on Table S1. Further insight into these results are given in the following subsections.

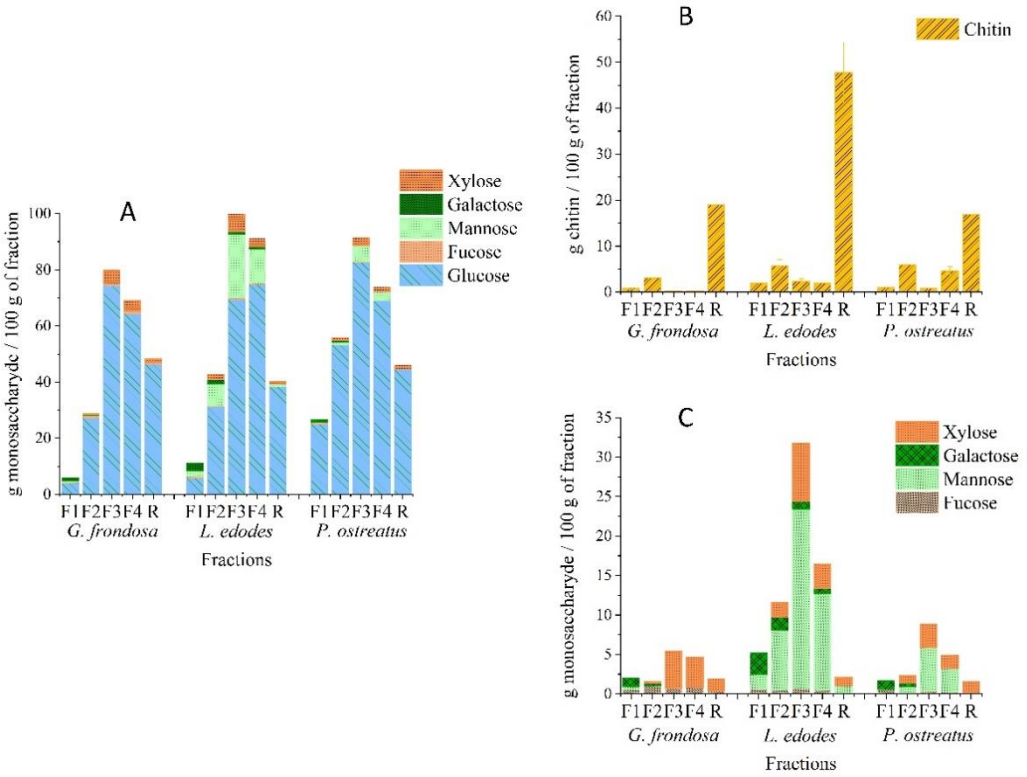


Figure 1. Main monosaccharides of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*, expressed as percentage by fractions weight. A) Total monosaccharides excluding chitin; B) Chitin (N-acetyl glucosamine) content; and C) Total monosaccharides content excluding glucose and chitin.

3.4.1. Glucans

Not surprisingly, glucose was the most abundant monosaccharide, as the main carbohydrates present in mushrooms are glucans (Figure 2). The much higher β-glucan content compared to the α-glucan contribution in mushrooms justifies higher glucan purity in alkaline fractions (F3 and F4), as it is generally known that β-glucans are preferentially extracted in alkaline conditions. This glucan purity ranges between 69-83% for the first alkaline treatment (F3). However, a significant amount of glucose was also present in the residues from the three

mushrooms, pointing out to a certain fraction of strongly bound glucans present in the three species. Although in lesser relative amounts, glucans were also present in the more abundant aqueous fractions, with significant differences among samples. As opposed to β-glucans, α-glucans are frequently non-structural carbohydrates and their content can widely vary depending on the species and developmental stage, normally being below 10% (Avni et al., 2017). While β-glucans are preferentially extracted using alkali treatments, α-glucans, can usually be dissolved in cold or hot water depending on their degree of branching, some even needing alkaline treatments.

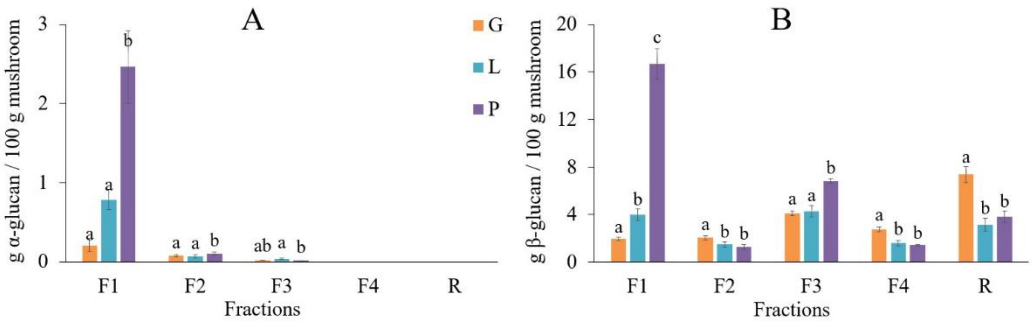


Figure 2. Glucan content from aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) fractions obtained from *G. frondosa* (G), *L. edodes* (L) and *P. ostreatus* (P), expressed as a percentage of α and β-glucans (A and B respectively) by weight of the initial samples. Different letters in the same fractions mean that the values are significantly different.

To have further insight into the cell wall compositional differences and the inherent recalcitrance of the different glucan structures, the α-glucan and β-glucan distribution along the different fractions was also evaluated and is depicted in Figure 2. As expected, the overall α-glucan content accounting for 3.31%, 0.28% and 7.57%, for *G. frondosa, L. edodes* and *P. ostreatus,* respectively, was much lower than the β-glucan content (Fig. 2A). Most of these minor quantities of α-glucans were concentrated in the aqueous F1 and F2 fractions in all samples. *P.*

ostreatus displayed the greatest amount of α-glucans, probably with a low branching degree and, thus, easily extracted within the aqueous steps. In contrast, and despite their lower overall α-glucan content, higher quantities were present in the first alkaline fraction for *L. edodes* and *G. frondosa*, hinting towards a higher recalcitrance involving also α-glucan structures.

P. ostreatus again showed a much higher amount of β-glucans than the other two species, while no significant differences were found in the overall β-glucan content of *L. edodes* and *G. frondosa* (Table 1). However, significant differences were found in β-glucan distribution along the different fractions, depending on the species. The β-glucan in *P. ostreatus* was found to be highly soluble or accessible, with about 40-45% of the total β-glucan present in the cold water extract (F1). Furthermore, and despite its higher overall glucan content, it showed the least β-glucan concentration in the residue. On the contrary, the lower β-glucan content in *G. frondosa* showed a remarkable recalcitrance, with >30% of all β-glucan unextracted and present in the residue (R) even after several consecutive aqueous and 2 alkaline extractions. The relatively high yields of each second set of extractions (F2 and F4) compared to other samples further indicates a higher recalcitrance of both water and alkali soluble β-glucan fractions.

3.4.2. Chitin

In order to better understand the different accessibility of β-glucan and evaluate to what extent β-glucan-chitin interactions interfere with the release of β-glucans in the three mushroom species, the chitin content in all fractions was assessed (Figure 1b). Common hydrolysis methods, such as TFA hydrolysis or Saeman hydrolysis, are either not aggressive enough to disrupt the crystalline chitin network or promote degradation of N-acetyl glucosamine, respectively. There is also discrepancy among methods used for chitin determination. Based on previous literature and preliminary optimization assays on fungal samples at different

conditions (see Figure S3 in the Supplementary material), a hydrolysis treatment with 6M HCl at 100 ºC for 7 hours was selected.

As expected, chitin content was predominantly found and enriched in the solid residual fractions for all species. Interestingly, chitin was much more enriched (around 50%) in the less abundant residual fraction of *L. edodes*, evidencing a lower recalcitrance of this species towards extraction of protein and β-glucan components. This evidences that higher chitin content is not correlated with higher recalcitrance. In fact, the higher recalcitrance in *G. frondosa* cannot be ascribed to chitin content alone, but could be better explained either by stronger β-glucan-chitin bonds or by a greater structural complexity of the β-glucans present. Although present in minor quantities in all fractions, chitin content was also relatively increased in the second round of each of the aqueous or alkali treatments (F2 and F4, especially for *P. ostreatus*), compared to the previous one, thus highlighting the harsher conditions needed for chitin extraction.

3.4.3. Other polysaccharides

The main monosaccharides detected, apart from the glucose or N-acetylglucosamine units from glucans or chitin, were xylose, mannose, galactose and minor amounts of fucose. These sugar units are derived from several polysaccharides known to be present in mushrooms, such as xyloglucans, xylomannans, mannogalactans or fucomannogalactans (Ruthes et al., 2016). The results from Figure 1c point out to differences, not only in composition, but also in recalcitrance of the three mushroom species. The significantly higher galactose, xylose and, especially, mannose contents in *L. edodes* points towards a richer content in mannogalactans and xylomannans, compared to the other species. *G. frondosa*, on the contrary, displays the lowest content in non-glucan polysaccharides, with much lower mannose content, predominance of xylose and the highest fucose contents among the three mushrooms, all of which point towards specific xyloglucan structures present in this species. These

polysaccharides were predominantly enriched in the alkali fractions. The higher relative abundance of galactose in the F1 fractions points towards a higher solubility of galactomannans with higher galactose contribution. On the other hand, xylose is present in all residues and hardly represented in F1, evidencing a higher recalcitrance. As also observed from the table, the xylose-containing polysaccharides were more easily extracted in *L. edodes* and *P. ostreatus*, thus confirming the greater recalcitrance of *G. frondosa* cell walls.

3.5. Protein content of the fractions

In mushrooms there are two nitrogen sources: protein nitrogen and chitin nitrogen. The protein content was calculated as the difference between the total nitrogen from the elemental analysis and D-glucosamine nitrogen, applying the corrective factor of proteins for foods (6.25), and the results are compiled in Table 2. For all tested species, protein was predominantly concentrated in the cold aqueous fraction (F1), with a lower relative content in the hot water extract compared to other carbohydrate components. Only minor amounts of protein content were found in the alkaline extracts (F3 and F4) and no significant quantities were found in the residues. This points out to an easily extractable water soluble protein, indicating that, in these fungal species, protein is not tightly bound to other carbohydrate components, thus allowing for a cost-affordable extraction just using cold water. Interesting differences were, nevertheless, found among the relative amounts of protein in the fractions depending on the tested species. The highest relative protein content was found in the more recalcitrant *G. frondosa*, which suggests that protein does not play an important role in the structural complexity and recalcitrance of the β-glucan components in this species. On the contrary, in *P. ostreatus*, with much greater soluble β-glucan contents, a higher relative protein content was found in the hot water extract, thus pointing out to differences in the protein composition from the different mushrooms which deserves further investigation.

3.6. Fourier-transform infrared (FT-IR) spectroscopy

Figure 3 shows the FT-IR spectra of the three mushrooms and their corresponding fractions. Lentinan and chitin controls were also included for comparison purposes. The FTIR spectra correlate very well with the previous compositional characterization. Lentinan, as a control of a relatively pure β-glucan, showed characteristic vibrational bands centred at 3450 cm^{-1} (O-H stretching), the vibrational bands corresponding to C-H stretching (2930 and 2890 cm^{-1}), and the region between 800 and 1200 cm^{-1}, typical of carbohydrates and containing highly coupled modes arising from C-C, C-O, C-H stretching and COH bending modes (Ji et al., 2019). The broad vibrational band in this region contains maximum absorbances at ~1148, 1075 and 1015 cm^{-1}, being the one around 1075 cm^{-1} mainly ascribed to β-glucan. The weaker band around 894 cm^{-1} is specific for β-glycosidic bonds (Synytsya et al., 2009). The FTIR spectrum from the chitin control, clearly shows the greater molecular order that this biopolymer has, evident from the well-defined and sharper spectral bands, displaying the N-H stretching band at 3270 cm^{-1} overlapped with the O-H stretching band. Other characteristic vibrational bands from chitin are the amide I (1660 and 1620 cm^{-1}), amide II (1560 cm^{-1}) and amide III (1306 cm^{-1}) bands and other vibrational bands from the carbohydrate backbone mainly ascribed to C-O vibrations within the N-acetyl-D-glucosamine units (Ifuku et al., 2011). The spectra from the three mushroom species are similar, with main contributions arising from the carbohydrates and proteins present, although certain differences are observed related to different molecular structure in their cell walls. The greater β-glucan content of *P. ostreatus* leads to the IR spectrum of this mushroom having a higher similarity with lentinan than those from the other two mushroom species. The spectra from the F1 fractions reflected that most of the proteins were extracted in this first step, with amide I and II bands clearly observed in the three mushrooms, but having variable relative intensity depending on the species. For instance, *G. frondosa*, being the more recalcitrant mushroom, shows the greatest intensity in these bands relative to

the carbohydrate region, in which a small vibrational band, probably coming from low molecular weight compounds and simple sugars, is observed. In contrast, the F1 infrared spectrum from *P. ostreatus*, clearly reflects that a much greater amount of carbohydrates was obtained in this case (as seen from the 800-1200 cm^{-1} region and the relative intensity between this region and the amide I and II regions). The F1 fraction of *L. edodes* shows typical absorption bands from chitin, thus confirming that in this specific mushroom some chitin is extracted just using water at room temperature. It is known that chitin from mushrooms is γ-chitin (which appears to be a combination of the α-, β- allomorphs) (Kumirska et al., 2010) and the results obtained point out different chitin structures present in the three mushroom species, patent from the amide I shape. The F2 fractions, although similar to the F1, clearly indicate a greater prevalence of carbohydrates, as their extraction was promoted by increasing the temperature. The spectra of the F3 and F4 fractions, obtained from the alkaline extraction steps, confirm that these are mainly composed of β-glucan, being very similar to that of lentinan, and displaying the bands typically ascribed to β-glucans around 894, 990, 1040, 1080, 1100, 1162, 1317 and 1376 cm^{-1} (Synytsya et al., 2009). The spectra from the residues as well display vibrational bands characteristic from β-glucans but also from chitin, in line with the compositional results.

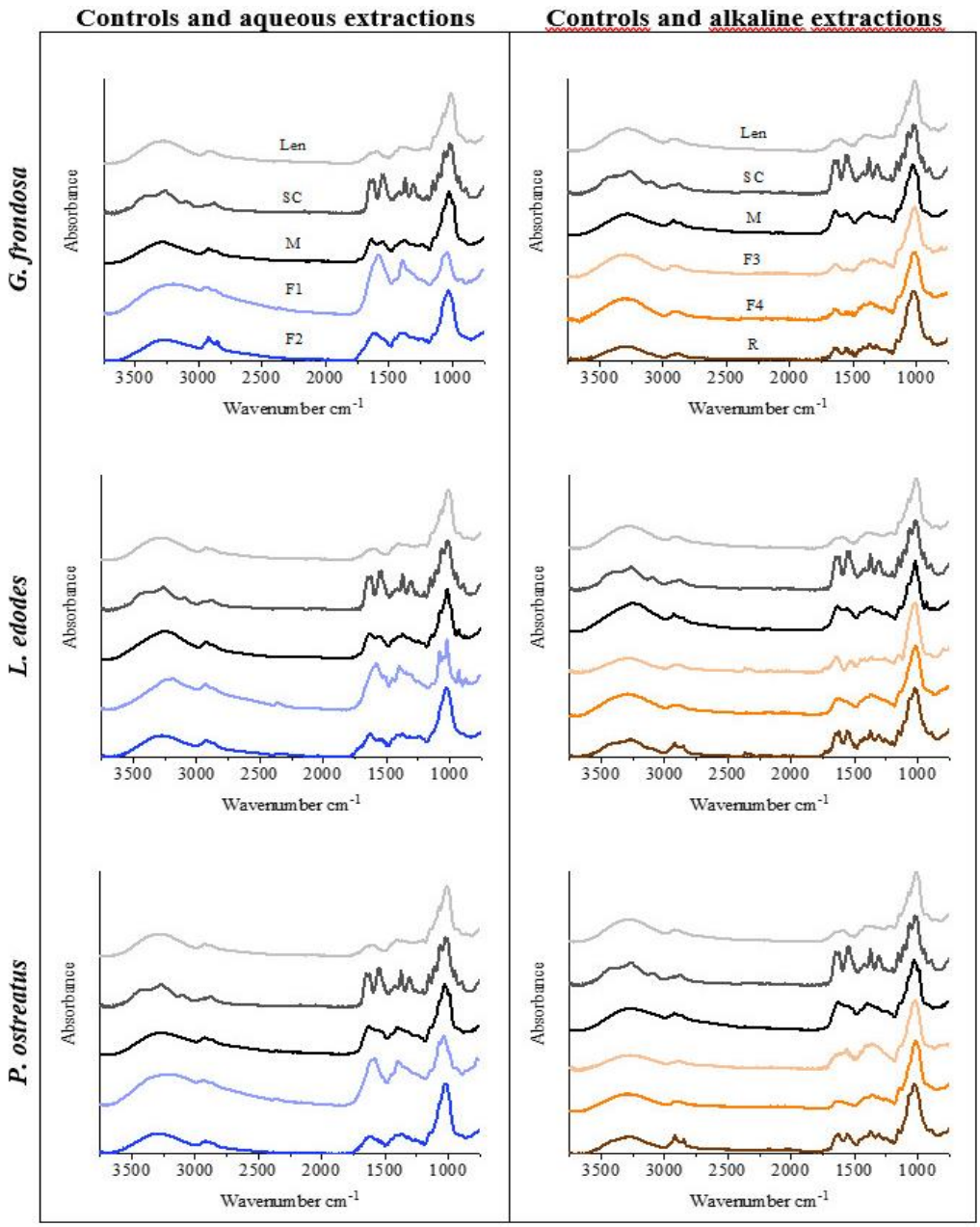


Figure 3. FT-IR spectra of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from *G. frondosa*, *L. edodes* and *P. ostreatus*. Each graph also includes the FT-IR spectra of the whole mushroom biomass (M), shell chitin (SC) and lentinan (Len), as controls.

3.7. Thermogravimetric analysis (TGA)

Figure 4 shows the TGA first derivative (DTGA) graphs from the three mushroom (M) species and their corresponding fractions. As with the FTIR, the DTGA curves from chitin and lentinan controls are also included. As observed from Figure 4, the mushroom biomass is characterized by four thermal transitions. The first one is a shoulder between 140 and 175 ºC, whose intensity and main degradation temperature varies with the species, being weaker for *G. frondosa*, much more intense for *P. ostreatus* and taking place at a greater temperature for *L. edodes*. This shoulder is probably related to the loss of tightly bound water and to the degradation of low molecular weight compounds present in the mushrooms' cell walls. The second transition, around 240 ºC has been ascribed to the degradation of polysaccharide side chains (Girometta et al., 2020) and degradation of some hemicelluloses like xylan or glucomannan (Werner et al., 2014). The third degradation peak, with a maximum between 295 and 300 ºC is usually ascribed to the depolymerization of glucan chains and also associated with the cleavage of the C-C and C-O bonds in the ring structure of the sugars (Aburas et al., 2020). In fact, different polysaccharide fractions obtained from *L. edodes* have been reported to thermally degrade between 200 and 400 ºC, being difficult to unambiguously ascribe the maximum degradation temperatures to specific polysaccharide structures. In fact, the DTGA of lentinan, also shows a broad degradation curve with two maximums at 256 ºC and 298 ºC. The fourth transition above 400 ºC could be ascribed to chitin and chitin-glucan complexes degradation (Girometta et al., 2020).

The DTGAs of the aqueous fractions (F1 and F2), substantially vary depending on the mushroom species and in line with the different composition of the fractions described before. The more recalcitrant nature of *G. frondosa* is patent in the DGTA of its F1 fraction, with very little contribution from carbohydrates (small shoulder at 300 ºC) and two more pronounced transitions at ~130 ºC and 485 ºC probably corresponding to proteins and minerals, respectively. It is interesting to

note that while F1 from *P. ostreatus* contains significantly more carbohydrates than the F1 fractions from the other two mushrooms, this is not reflected by a greater transition around 300 ºC, being the contribution of the first thermal degradation transition the most significant one.

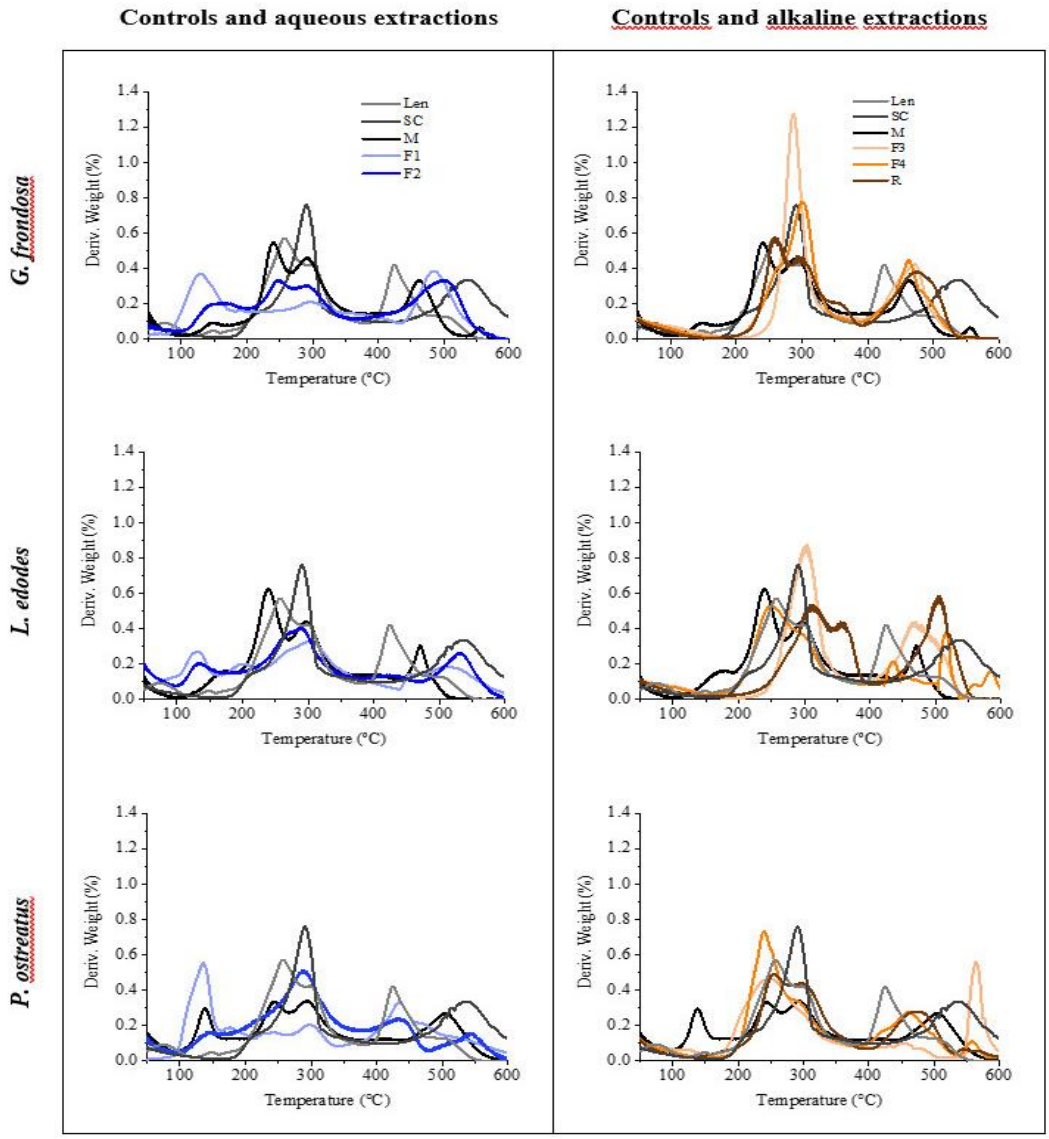


Figure 4. Derivative thermogravimetric (DTG) curves of aqueous (F1 and F2) and alkaline (F3 and F4) fractions together with the residue (R) from G. frondosa, L. edodes and P. ostreatus. Each graph also includes the DTG curves of the whole mushroom biomass (M), shell chitin (SC) and lentinan (Len) as controls.

The alkaline fractions (F3 and F4), which have been seen to mainly consist on polysaccharides confirm the very different structure of the β-glucans extracted from the three mushroom biomass. While F3 from the more recalcitrant *G. frondosa* shows the sharper carbohydrate transition (maximum at 288 ºC), thus indicating more homogeneous structures in this fraction, the DTGA from F3 of *P. ostreatus* shows a broader band with a maximum around 250 ºC, indicating much more heterogeneous polysaccharide structures with lower thermal stability. A partial degradation upon a second alkaline treatment is observed in F4 fractions from *L. edodes* and *P. ostreatus*, reflected in a decrease in the maximum degradation temperature from the β-glucans (from 302 to 258 ºC and from 250 to 240 ºC, respectively), while two populations are observed for *G. frondosa*, the second one degrading at a greater temperature than F3, thus confirming the recalcitrant nature of the β-glucan present in this mushroom. Finally, the residues left after the sequential extraction steps, show a DTGA profile similar to that of the mushroom, but with the maximum degradation temperatures of each step displaced towards higher temperatures, indicating the presence of residual β-glucan and chitin of greater thermal stability.

4. Conclusions

The sequential fractionation process involving several consecutive cold or hot aqueous and alkaline treatments applied to *P. ostreatus*, *L. edodes* and *G. frondosa* revealed substantial differences in recalcitrance of these mushroom species reflected in the distribution of the different components in the various extracted fractions depending on the species. Highest yields corresponded to the aqueous treatments at room temperature (F1) for all species, with highest and lowest yields for *P. ostreatus* (82%) and *G. frondosa* (~56%), respectively. This fraction was the richest in sugars, proteins and polyphenols and also showed the highest antioxidant capacity. The lower recalcitrance of the cell wall architecture of *P. ostreatus* was patent in the higher relative abundance of β-glucans in this fraction (~16 g β-

glucan/100 g mushroom) compared to the alkaline or residual fractions, evidencing cost-affordable valorisation possibilities for both antioxidants and glucans. *L. edodes* showed an intermediate recalcitrance, with distinct β-glucan populations distributed along aqueous or alkaline extracts, higher abundance of non-glucan polysaccharides and higher chitin purity in the residue. *G. frondosa* showed, in contrast, a remarkable recalcitrance, with highest residual content and most β-glucans remaining in the residue (~8 g β-glucan/100 g mushroom). This overall recalcitrance was further patent in the distribution of α-glucans and the higher relative yields of each second set of aqueous or alkaline extractions (F2 and F4). Furthermore, the relatively lower content on non-glucan carbohydrates was represented by xylose or fucose containing polysaccharides, pointing out to more recalcitrant xyloglucan structures compared to the higher mannose and galactose contents in *L. edodes*. These differences in the heterogeneity and recalcitrance were further evidenced in the thermal degradation patterns. These results reveal great differences in the cell wall architecture of the different mushrooms, which must be taken into account when designing appropriate fractionation or valorisation strategies. Future works could be directed towards the elimination of extraction steps depending on the species and a deeper structural analysis of these specific populations in order to unravel specific structure-bioactivity relationships.

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Supplementary Material

Fungal biomass materials

Figure S1. Photos of *G. frondosa, L. edodes and P. ostreatus.*

As it can be seen from Figure S1, the three mushrooms are quite different from each other. The most distinct one was *L. edodes* with a hemispherical cap and a brownish colour. On the other hand, *G. frondosa* and *P. ostreatus* were characterised by a cap with wavy or rolled margins. In the case of *G. frondosa*, the flesh was thinner, whereas *P. ostreatus* was characterised by marked lamellae under the cap and whiter, thicker flesh.

Scheme of extraction method

A fractionation protocol was applied to the three mushroom species studied (*G. frondosa, L. edodes* and *P. ostreatus*), applying aqueous and alkaline extractions as displayed in Figure S2.

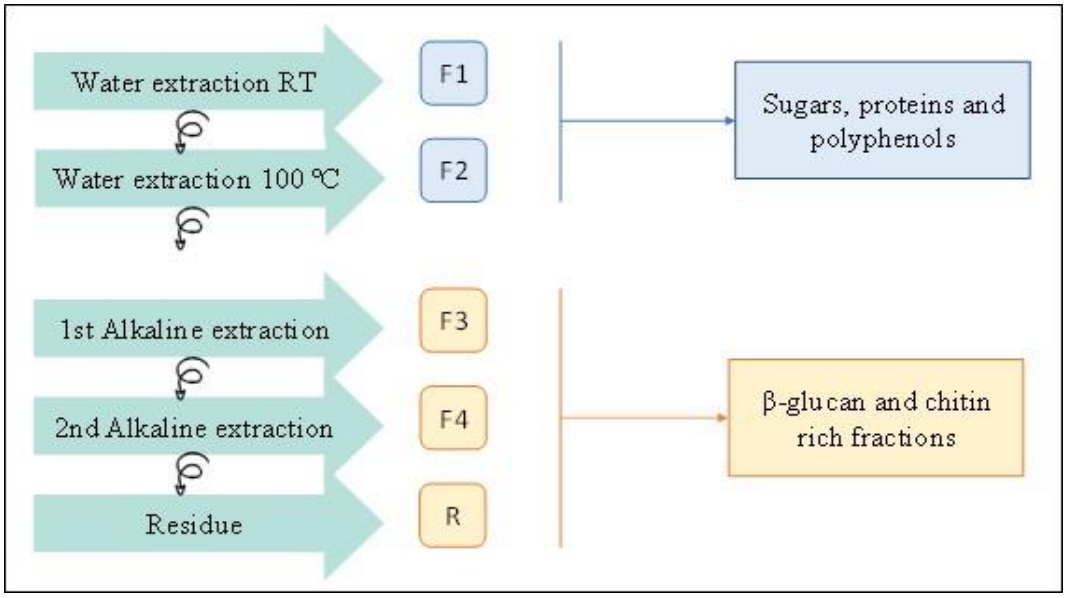


Figure S2. Scheme of extraction method. F1 and F2, are the fractions obtained from aqueous extractions, F3 and F4 are the fractions obtained from the alkaline extractions and R is the resulting residue.

Preliminary trials for chitin content determination

G. frondosa, being the most recalcitrant mushroom was selected for these trials. Initially, different hydrolysis times (1, 3, 7 and 24 hours) were evaluated in two controls: Shell chitin and a D-glucosamine standard. As observed in Figure S3-A, while in the D-glucosamine standard 7 hours were seen to partially degrade the material, thus providing lower glucosamine content, for shell chitin, no significant differences were observed between 3 and 7 hours of hydrolysis, while 24 hours resulted in degradation. Therefore, these two hydrolysis times (3 and 7 hours) were evaluated for *G. frondosa* and the results are shown in Figure S3-B. As observed from the graph, while for the mushroom and different fractions, no significant differences were seen in the glucosamine content, if significantly increased at 7 hours when applied to the residue and, thus, this hydrolysis time was selected for chitin quantification.

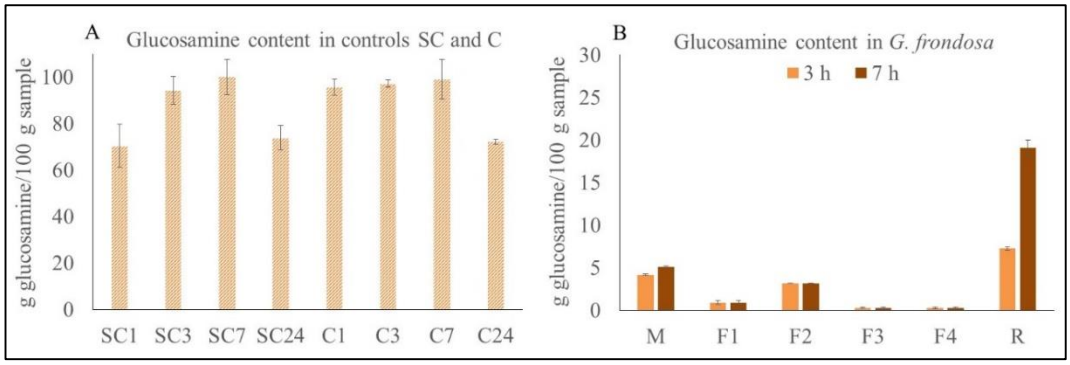


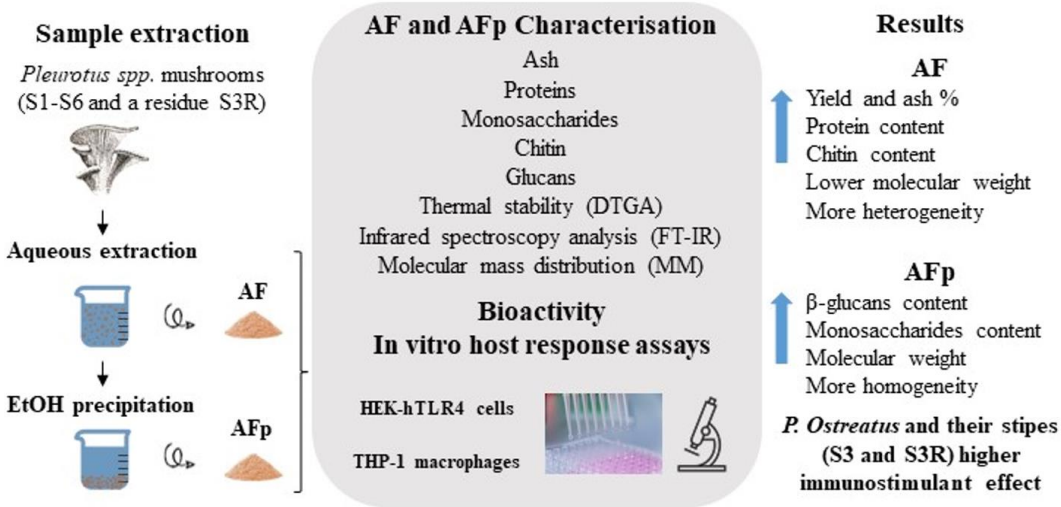
Figure S3. A) D-glucosamine content from a hydrolysis assay with 6M HCl at 100°C at different times (1, 3, 7 and 24 hours) on control samples of shell chitin (SC) and D-glucosamine standard (C). B) D-glucosamine content from a hydrolysis assay with 6M HCl at 100°C at different optimized times (3, 7 hours) on samples from *G. frondosa* mushrooms (M) and fractions (F1-R).

Detailed monosaccharide compositional analysis

Table S1. Main monosaccharides present in the different fractions, excluding N-acetylglucosamine from chitin.

	Mushrooms	F1	F2	F3	F4	R
Fucose	***G***	0.54 (0.03)	0.99 (0.06)	0.72 (0.05)	0.76 (0.07)	0.30 (0.05)
	L	0.61 (0.10)	0.45 (0.05)	0.63 (0.12)	0.46 (0.14)	< 0.1
	P	0.59 (0.15)	< 0.1	0.36 (0.01)	0.25 (0.01)	< 0.1
Galactose	***G***	1.10 (0.10)	0.37 (0.08)	< 0.1	< 0.1	< 0.1
	L	2.75 (0.25)	1.62 (0.18)	0.93 (0.19)	0.67 (0.14)	< 0.1
	P	1.09 (0.20)	0.48 (0.07)	< 0.1	< 0.1	< 0.1
Glucose	***G***	3.84 (0.27)	27.16 (2.15)	74.38 (3.95)	64.39 (4.77)	46.43 (4.25)
	L	5.85 (0.56)	31.14 (4.35)	69.22 (7.56)	74.76 (11.27)	38.28 (6.37)
	P	25.04 (1.68)	53.30 (7.45)	82.72 (2.48)	68.92 (2.58)	44.46 (5.43)
Mannose	***G***	0.38 (0.02)	< 0.1	< 0.1	< 0.1	< 0.1
	L	1.85 (0.16)	7.66 (1.12)	22.78 (3.74)	12.21 (2.12)	0.96 (0.08)
	P	< 0.1	0.91 (0.13)	5.51 (1.50)	2.96 (0.30)	0.16 (0.04)
Xylose	***G***	< 0.1	0.27 (0.09)	4.74 (0.49)	3.95 (0.37)	1.56 (0.21)
	L	< 0.1	1.89 (0.12)	7.46 (0.56)	3.13 (0.22)	1.16 (0.01)
	P	< 0.1	1.00 (0.22)	2.94 (0.11)	1.74 (0.34)	1.40 (0.23)

1.2. Composition, structural properties and immunomodulatory activity of several aqueous *Pleurotus* β-glucan-rich extracts.



This section is an adapted version of the following published research article:

Zaida Pérez-Bassart, Christine Bäuerl, Maria Jose Fabra, Antonio Martínez-Abad, Maria Carmen Collado, Amparo López-Rubio, 2023.

"Composition, structural properties and immunomodulatory activity of several aqueous *Pleurotus* β-glucan-rich extracts"

International Journal of Biological Macromolecules, volume 253, 127255.

Abstract

In this work, aqueous extracts from six different *Pleurotus* species were obtained and their yield, gross composition, β-glucan content, monosaccharide profile, thermal stability, molecular weight distribution, and FT-IR were analyzed before and after purification through ethanol precipitation of the carbohydrate-rich fractions. The bioactivity (anti-inflammatory and immunomodulatory activity) of the various fractions obtained was also analyzed in three different cell cultures and compared with a lentinan control. The trend observed after purification of the aqueous fractions was an increase in the concentration of polysaccharides (especially β-glucans), a decrease in ash, glucosamine and protein content and the elimination of low molecular weight (Mw) compounds, thus leaving in the purified samples high Mw populations with increased thermal stability. Interestingly, all these purified fractions displayed immunomodulatory capacity when tested in THP-1 macrophages and most of them also showed significant activity in HEK-hTLR4 cells, highlighting the bioactivity observed for *Pleurotus ostreatus* (both the extracts obtained from the whole mushroom and from the stipes). This specific species was richer in heteropolysaccharides, having moderate β-glucan content and being enriched upon purification in a high Mw fraction with good thermal stability.

1. Introduction

Edible mushrooms and their extracts have been used both in food and traditional medicine since ancient times, not only due to their nutrient composition, but also because of their beneficial biological activities, including immunomodulatory properties, thus being included in nutraceuticals and pharmaceuticals [1,2]. Mushrooms with known immunomodulatory capacity contain diverse organic compounds (including terpenes and terpenoids, proteins like lectins and various polysaccharides) responsible for this activity with very diverse molecular weight and structure [3]. Immunostimulatory polysaccharides like glucans, mannans,

arabinogalactans, fucoidans, galactans and xylans can be found in the cell walls of mushrooms and, specifically, β-glucans have attracted a great deal of research interest, due to their proved bioactive properties [4]. Structurally, fungal β-glucans consist of polysaccharide chains of β-(1,3) or β-(1,4) linked glucose subunits, with β-(1,6)-linked branches [5]. In fact, β-glucans are considered a key pathogen-associated molecular pattern (PAMP), recognized upon fungal infection and responsible of triggering the immune response [6]. The modulation of the immune system by β-glucans is rather complex, depending on many factors not yet fully understood. Immunostimulation has been described to be mediated through the binding of polysaccharides like β-glucan to several receptors such as Dectin-1, complement receptor 3 (CR3), selected scavenger receptors, and lactosylceramide (LacCer), triggering signal transduction in several immune cells [7]. The activity of these β-glucan receptors seems to be highly dependent on the cell types and, for instance, recognition of β-glucan by Dectin-1 on macrophages activates the downstream signaling pathway, activating phagocytosis, ROS generation, microbial killing, and cytokine production [8]. However, it is important to note that the immunomodulatory effects of glucans depend on their structural characteristics, including branching degree, solubility, molecular weight, polymer charge, and conformation in solution [9]. It is still unclear how structural differences of glucans might affect their biological functions. Moreover, whether these biological effects also depend on the presence of additional compounds is unclear, due to many studies being carried out using mushroom extracts and not purified β-glucans. For instance, in recent years, fungal lectins have also attracted considerable attention due to their anti-tumor, anti-proliferative and immunomodulatory activities. Fungi express high levels of lectins as storage proteins, which also bind to various sugar structures with a high degree of selectivity and stereo specificity [10]. Thus, mushroom lectins may play an important role in the interactions with bioactive sugars from mushroom biomass. Extraction of polysaccharides and, specifically of β-glucans, from fungal biomass

has been widely explored, mainly using aqueous or alkaline treatments, although hot water extraction is one of the most widely used methods [11]. Aqueous extractions, apart from being greener and simple, allow obtaining water-soluble bioactive extracts, which facilitate their testing in cell cultures for the evaluation of their immunomodulatory or anti-inflammatory capacity. Numerous assays have been done with aqueous polysaccharide-rich fractions and β-glucan-rich fractions of different species of edible mushrooms, which have demonstrated their immunomodulatory activity. For instance, the induction of an innate immune response against breast and lung cancer cells from β-glucan isolated from the aqueous fraction of *Pleurotus ostreatus* was reported [12], while an heteropolysaccharide obtained from *Lentinula edodes* showed immuno-stimulating activities involving Toll-like receptor 2 (TLR2) [13]. Some studies have also been carried out to investigate the immunomodulatory activity of aqueous fractions from mushrooms of the *Pleurotus* genus [14–16], many of them focused on *Pleurotus ostreatus*, with a high water-soluble polysaccharide content [16]. *P. ostreatus* is one of the most widely consumed mushrooms worldwide and its cultivation and further processing generates great volumes of mushroom residues (part of the stipes), which could be valorized as a source of bioactive compounds. Interestingly, a previous study showed that *Pleurotus ostreatus* had a more accessible type of β-glucan than other widely consumed mushrooms [17]. It is hypothesized that different β-glucan complex structures are present in the various *Pleurotus* species considered and that this will have implications in their immunostimulatory activity. Therefore, the aim of the present study was to investigate the compositional and structural differences of aqueous extracts of various *Pleurotus* spp. and from the residue of *P. ostreatus*, also testing their biological activity through various cell assays.

2. Materials and methods

2.1. Reagents and materials

Different mushrooms biomass used in the assays were kindly donated by Centro Tecnológico de Investigación del Champiñón –Asochamp- (Rioja, Spain). Specifically, the *Pleurotus* used were: *Pleurotus citrinopileatus* (S1), *Pleurotus ferulae* (S2), *Pleurotus ostreatus* (S3), *Pleurotus ostreatus* residue o discard (S3R), *Pleurotus eryngii* (S4), *Pleurotus djamor* (S5) and *Pleurotus pulmonarius* (S6). Reagent EtOH (96 %) was purchased from PanReac AppliChem. Lentinan (min. 30 %) was acquired from Carbosynth. Shrimp shells Chitin, Folin-Ciocalteu reagent, sodium carbonate (≥99.0 %), gallic acid (97.5–102.5 % titration), potassium persulfate (≥99.0 %), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphate buffered saline (PBS), 6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox), Trifluoroacetic acid (TFA), fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid, glucuronic acid, *N*-Acetyl-D-glucosamine and D-(+)-Glucosamine hydrochloride were all purchased from Sigma-Aldrich.

2.2. Aqueous extractions

To obtain the aqueous bioactive fractions from each mushroom, 1 g of freeze-dried and sieved (0.5 mm) raw material dispersed in 12 mL of water was subjected to boiling temperature for 7 h with stirring on a vortex mixer every hour. Each sample was then centrifuged and then freeze-dried to obtain the so-called AF fractions.

In order to obtain purer β-glucan fractions, the supernatant obtained from the hot aqueous extraction was subjected to precipitation with 3 times its volume in 96 % ethanol for 1 h under mechanical agitation, being subsequently centrifuged. The obtained precipitate was freeze-dried, thus obtaining the so-called FAp fractions. All extractions were made in triplicate.

2.3. Fractions characterization

2.3.1. Ashes

Ashes were determined by TGA, with TA Instruments TGA550 equipped with a Pt/Rh furnace. The samples were heated up in platinum capsules to 700 ◦C under air atmosphere. The results were expressed as a percentage of the remaining weight.

2.3.2. Antioxidant capacity

The determination of the antioxidant capacity was carried out using the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) method [18]. For this purpose, 0.192 g ABTS and 0.033 g potassium persulfate were dissolved in 50 mL PBS (phosphate buffered saline) at pH 7.4, and this solution was left to stir overnight. Prior to determination, the $ABTS^{-+}$ (ABTS monocations) was diluted in PBS to an initial absorbance of 0.70 ± 0.02 at 734 nm. For the determination, 20 µL of each sample was used and this volume of each sample was completed with 230 µL of ABTS+ solution in microplate, incubated for 6 min at room temperature, in darkness, and then the reading of the absorbance at 734 nm was performed in a reader adapted for reading in microplates and specific software of the CLARIOstar spectroscopy equipment (BMG LABTECH). The calibration chamber was performed with 6-hydroxy- 2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox). The antioxidant capacity of the samples was expressed as mg Trolox equivalents (TE)/g sample or fraction. All determinations were performed in triplicate.

2.3.3. Total phenolic compounds

The total phenolic content of fractions was determined by the Folin- Ciocalteu method modified by Singleton [18]. For this purpose, 1000 µL of 1/10 diluted Folin-Ciocalteau reagent was mixed with 200 µL of samples and the mixture was allowed to incubate for 1–8 min. Then 800 µL of sodium carbonate (75 mg/mL)

was added and the samples were incubated for 30 min in a bath at 40 ◦C. Finally, the absorbance of the samples was measured at 760 nm. A calibration curve was performed using gallic acid as a standard. The total concentration of polyphenols was expressed as mg gallic acid/g sample.

2.3.4. Glucosamine and protein content

Since both the glucosamine present in chitin and, to a minor extent, in glycoproteins and other heteropolysaccharides, and also the protein contribute to the nitrogen content in mushrooms, the glucosamine content was quantified on the one hand and the total nitrogen content on the other hand, thus obtaining the protein content of the samples by subtracting the carbohydrate nitrogen content to the total nitrogen content and applying the correction factor for proteins in foods of 6.25. To determine the aminosugars content, an acid hydrolysis was carried out followed by glucosamine quantification by HPAEC-PAD as previously described in [17], and total nitrogen content was determined using an elemental nitrogen analyzer (rapid N-surplus analyzer) based on the modified Dumas method.

2.3.5. Monosaccharide characterization

The monosaccharide composition in AF and AFp fractions, excluding glucosamine, was carried out by trifluoroacetic acid (TFA) hydrolysis as reported in [17]. Briefly, 2 M of trifluoroacetic acid (TFA) at 120 ◦C for 3 h were used, allowed to dry at 40 ◦C for complete evaporation of the TFA and re-suspended in deionized water. The hydrolysed material was analyzed by ion exchange chromatography (IC-HPLC, Dionex) using a CarboPac™ PA1 column using known concentrations of mixtures of fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid for calibration. All experiments were carried out in triplicate.

2.4. α-glucans and β-glucans content

Briefly, α-glucan contents were estimated by an enzymatic hydrolysis with amyloglucosidase plus invertase and using a glucose standard solution and glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-aminoantipyrine), following the protocol determined by the kit "Mushroom and Yeast β-glucan Assay Procedure" K-YBGL 09/ 2009 kit (Megazyme Int.) as in [17]. The absorbance of all solutions was analyzed spectrophotometrically at 510 nm against the reagent blank, expressing the values of glucan contents as g of glucose equivalents per 100 g of dry matter. Finally, the β-glucan content was calculated by subtracting the α-glucan glucose from the total glucan glucose content previously determined by acid hydrolysis. All the determinations were made in triplicate.

2.5. Fourier-transform infrared (FT-IR)

The equipment used for the Infrared analysis was a Jasco 4100 FT-IR spectrometer. The assay was performed at 21 ◦C and the spectra were taken at 4 cm^{-1} resolutions in a wavelength range of 600–4000 cm^{-1} and a minimum of 32 scans. Origin pro 2019 software was used for processing the results.

2.6. Thermogravimetric analysis (TGA)

Thermogravimetric curves (TG) were made with TA Instruments TGA550 equipped with a Pt/Rh furnace with an autosampler in a maximum temperature of 1000 ◦C. The samples were heated (10 to 700 ◦C) with a heating rate of 10 ◦C/min under air atmosphere in platinum capsules. Derivative TG curves (DTG) to express the weight loss rate as a function of temperature were made using Origin pro 2019 software.

2.7. High-Performance Size Exclusion Chromatography (HPSEC)

Different samples of AF and AFp fractions were diluted with NaCl 1 M solution and filtered through 0.22 μm pore syringe filters. The molecular weight of the

samples was estimated by HPSEC using Waters ACQ Arc Sys Core 1–30 cm CH (Waters, USA). HPLC system was equipped with a Waters 2998 PAD module, a Waters 2414 refractive index detector (Waters, USA), and 2475 FLR module (Waters, USA). The samples were injected into columns in series (PolySep-GFC-P 4000 and 2000 (300 mm × 7.8 mm) Phenomenex Inc., CA, USA) equilibrated at 40 ◦C. The mobile phase was 1 M NaCl aqueous solution at a flowrate of 0.5 mL/min. The sample concentration was about 3 mg/mL and an injection volume of 20 μL was used. Pullulan solutions (0.34 kDa to 800 kDa) were used as standards (PSS polymer standards service GmbH, Mainz, Germany). The data were processed using Origin pro 2019 software (OriginLab Corporation, Northampton, MA, USA).

2.8. *In-vitro* experiments

2.8.1. NF-κB activity in HT-29-reporter cells

To test the effect of β-glucans on the intestinal epithelial cell line HT- 29, a reporter gene assay in a stably transfected HT-29 clone #16 was used to monitor for the activation of NF-κB [19], a master regulator involved in inflammatory processes [20]. After activation of NF-κB, HT- 29 reporter cells secrete embryonic alkaline phosphatase (SEAP). Cells were routinely grown in DMEM High Glucose (BioWest) supplemented with 10 % fetal bovine serum (FBS, Capricorn), 2 mM L-glutamine (BioWest), 200 μg/mL zeocin (invivogen), 100 U/mL penicillin and 100 μg/mL streptomycin (BioWest) at 37 ◦C, 5 % CO_2, under a humidified atmosphere. NF-κB activation in response to β-glucans was analyzed as previously described [21]. Briefly, cells were seeded at 60,000 cells/well of a 96-well plate and grown for 24 h until exposure to 0.25, 2.5 and 25 μg/mL of β-glucans for further 24 h. Lentinan, (β-glucan from *Lentinus edodes*) was included at the same concentration of the samples, as a positive control. Supernatants were evaluated for presence of SEAP using *p*-nitrophenyl phosphate as substrate, which was

converted to p-nitrophenol and the resulting optical density was measured in a microplate reader at 405 nm (Clariostar, BMG Labtech). TNF-α (Immunotools) was used as inflammatory inductor at 10 ng/mL and the effect of the extracts was tested in presence or absence of TNF-α. Cells of each well were lysed in PBS, 0.1 % Triton, 1 mM phenylmethylsulphonylfluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid (EDTA) and protein content was determined using the Bradford Protein Assay (BioRad). SEAP activity is expressed as SEAP activity = (A405 nm test - A405 nm initial) × the total assay volume (mL)/mM extinction coefficient of p-nitrophenol (18.5) × the cell culture supernatant employed (mL) × time (min) and normalized to the protein content of each well.

2.8.2. Immunomodulation in THP-1 macrophages

THP-1 cells were routinely cultured in RPMI 1640 medium supplemented with 10 % FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin [all cell culture reagents BioWest, LabClinics]) at 37 ◦C, 5 % CO_2, under a humidified atmosphere. To differentiate THP-1 monocytes to macrophages, cells were seeded in 24-well plates at a density of 200,000 cells/well and incubated with 100 nM of PMA (Phorbol 12-myristate 13-acetate, Sigma) for 72 h. Then, cell culture medium was replaced with culture medium without FBS for 24 h before incubation with 50 μg/mL of different AF and AFp fractions of β-glucan for further 18 h. In order to test the effect of extracts under pro-inflammatory conditions, cells were simultaneously stimulated with 100 ng/mL of purified phenol-extracted LPS from *E. coli* 055:B5 (Sigma) during 18 h. Supernatants were collected and analyzed for IL-8 secretion.

2.8.3. Determination of IL-8

IL-8 was determined by ELISA (Invitrogen, Cat. No. 88-8086-86) following the instructions of the manufacturer. Samples were diluted accordingly from 1:4 to 1:40.000 in order to be detected in the standard curve range (2–250 pg/mL).

2.8.4. HEK-hTLR4 Assay

The HEK-Blue™hTLR4 cell line (InvivoGen) is a transfected reporter cell line designed to study TLR4 stimulation and expresses the secreted embryonic alkaline phosphatase (SEAP) gene under the control of NF-κB and AP-1 as a reporter gene. Cells were routinely maintained in DMEM, 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (all BioWest, LabClinics), 100 µg/mL Normocin (Invivogen) and 1× HEK-Blue selection reagent (Invivogen). HEK-hTLR4 cells were seeded at 25,000 cells/well in 96-well plates and SEAP activity was measured as described for HT-29 reporter cells. In order to test the effect of extracts under pro-inflammatory conditions, cells were simultaneously tested in the presence and absence of purified phenol-extracted LPS (10 ng/mL) from *E. coli* 055:B5 (Sigma) during 18 h.

2.8.5. Cell viability assays

After β-glucan treatments the cell viability was assessed by reduction of resazurin sodium salt (Sigma). Briefly, at the end of β-glucan incubations, cell medium was withdrawn and replaced with resazurin solution at 10 µg/ml dissolved in the respective cell culture medium and incubated for 2–4 h at 37 ◦C, 5 % CO2. Viable cells convert the non-fluorescent resazurin to the fluorescent resorufin, and the resulting fluorescence was measured at 540 nm excitation wavelength and 590 nm emission wavelength in a 96-well plate reader (CLARIOStar, BMG Labtech). Arbitrary fluorescence units of test wells (β-glucan treatments) were compared to the respective control wells.

2.9. Statistical analysis

Data analysis of results was carried out using Statgraphics. One-way analysis of variance (ANOVA) was done to determine the significant differences between samples, with significance level of $P < 0.05$.

3. Results and discussion

While the immunomodulatory activity of mushrooms has been long recognized, whether this activity is related to the presence of specific β-glucan structures or other components from the fungal biomass is largely unknown. In this work, the aim was to get a deeper understanding on the relationship between composition, structural characteristics and bioactivity of aqueous extracts from various *Pleurotus* species. In order to investigate the potential role of β-glucans in the immunomodulatory activity of the extracts, two types of extracts were obtained from each of the species: an aqueous extract fraction (AF) and an enriched β-glucan fraction (AFp), obtained through ethanol precipitation of the polysaccharides present in the previously mentioned aqueous extracts. All these fractions were fully characterized in terms of yield, composition, structural and physicochemical properties and bioactivity as explained below.

3.1. Extraction yields and fractions characterization

Very good yields were obtained for the aqueous fractions (AF) of the various *Pleurotus* species (ranging between ~27 and 42 %), being within the range previously reported for hot water (~40 %) [22] and hot water with supercritical carbon dioxide (~30 %) extractions [23] from different *Pleurotus* species. In contrast, a significant lower aqueous extraction yield was obtained from *P. ostreatus* residue (stipe) (~17 %), probably ascribed to its more recalcitrant nature (see Table 1). In fact, mushroom stipes have a structural function within the fruiting body of the mushrooms and are known to have a recalcitrant chitin microfibrils structure supported by the cross-linking between chitins and β-1,3-

glucans on both the inner and outer cell wall surfaces [24]. The high yields observed in the various AF were a consequence of the amount of soluble compounds present in the mushrooms, including proteins, simple sugars and soluble carbohydrates, salts, polyphenols and other low molecular weight compounds. *P. citrinopileatus* (S1), *P. ferulae* (S2) and *P. eryngii* (S4), had significantly greater yields (~40 %), than the other three *Pleurotus* species which had around 30 % yields. After ethanol precipitation and, thus, purification of the polysaccharide fraction of the aqueous extracts (AFp), a significant drop in the yield was observed, specially for the *P. djamor* species (S5), being the extracted polysaccharide fraction lower than 5 % of the initial biomass. In contrast, the residue from *P. ostreatus* (S3R), had the greatest yield, implying that most of the initial aqueous extract mass (around 80 %) were polysaccharides. Table 1 also includes the ash content of the different fractions which, as expected, decreased with purification. All mushrooms had a salt content within the usual range [25–27], with the exception of the residue (S3R), whose percentage was significantly higher, probably due to its closer proximity and contact with the substrate.

Table 1. Extract yields and ash content of the fractions obtained. Yields are expressed as a percentage by weight of the initial biomass material, and ashes as a percentage in weight from each fraction.

Mushroom	Yield (%)		Ash (%)	
	AF	AFp	AF	AFp
S1	38.02 (1.02) ^{aA}	8.96 (1.21) ^{aB}	10.42 (0.52) ^{aA}	7.92 (0.43) ^{aB}
S2	42.18 (3.49) ^{aA}	13.59 (0.07) ^{cdB}	7.51 (0.12) ^{bA}	5.20 (0.62) ^{bcB}
S3	27.45 (1.90) ^{cA}	10.45 (0.29) ^{abB}	9.70 (0.64) ^{aA}	3.80 (0.24) ^{bB}
S3R	17.36 (1.56) ^{bA}	13.98 (0.15) ^{dB}	22.37 (0.09) ^{cA}	7.46 (0.14) ^{aB}
S4	39.37 (1.96) ^{aA}	11.94 (0.14) ^{bcB}	6.18 (0.27) ^{dA}	3.84 (0.04) ^{bcB}
S5	28.69 (2.14) ^{cA}	4.47 (0.14) ^{eB}	12.20 (0.11) ^{eA}	8.62 (0.89) ^{aB}
S6	27.15 (3.07) ^{cA}	10.49 (1.36) ^{abB}	9.83 (0.38) ^{aA}	5.43 (0.41) ^{cB}

Different letters in lower case mean that the values are significantly different in the same column and different letters in upper case mean that the values are significantly different in the same row separately for yield and ash content.

3.1.1. Gross composition of the Pleurotus fractions

Amongst the different biologically active compounds present in mushrooms, polyphenols are interesting due to their antioxidant properties. The total phenolic content was determined by the modified Folin- Ciocalteu method and is included in Table S1 of the Supplementary Material. However, the results should be taken cautiously, as it is well-known that this methodology is highly dependent on the matrix composition and some compounds in the analyzed sample, such as reducing sugars, may hamper the accuracy of the assay [28]. Table S1 also includes the antioxidant capacity of the extracts (expressed as μmol of Trolox equivalents per gram of sample). It is interesting to note that extract purification led to a decrease in the antioxidant activity of the samples, probably due to the elimination of low Mw compounds with antioxidant capacity during ethanol precipitation. It should be also highlighted that all AF fractions had significantly greater antioxidant capacity and higher polyphenol content than other aqueous plant extracts [29], thus making them interesting green extracts with bioactive properties. While polyphenols and other antioxidant substances could play a role in the immunomodulatory activity of mushroom extracts, a study using cellular assays [30] showed that their contribution to this activity would be marginal, as, in the THP-1 macrophage assays, the purified fractions (containing a lower percentage of these compounds) generally showed a greater activity. Edible mushrooms are also a good source of proteins, generally ranging between 19 and 35 %, and having a complete profile of essential amino acids [31]. The proteins present in mushroom biomass may be more or less accessible depending on their arrangement within the cell walls and possible interaction with other compounds such as β-glucans or other polysaccharides. Fig. 1 displays the protein content of the various fractions,

showing that AF extracts of *P. citrinopileatus* (S1), *P. djamor* (S5) and *P. pulmonarius* (S6) were the ones with greater protein contents (between ~30 and 40 %). *P. ferulae* (S2) and *P. ostreatus* (S3) had lower, but still relatively high protein contents (around ~20 %), while the aqueous extract of *P. eryngii* (S4) only contained ~10 % protein. It is worth noting that the residues of *P. ostreatus* (S3R) showed lower protein content. The protein content of mushrooms can depend on several factors such as seasonality, the part of the fruiting body and the protein content of the growth substrate. It has been reported, for example, that the cap part of *P. ostreatus* has a higher protein content than the stipe part [32], which is in agreement with the results obtained in our previous study [17]. In general, after purification, a decrease in protein and glucosamine content was observed in all species (see Fig. 1 and Fig. 2) and, interestingly, AFp of *P. ostreatus* (S3) had similar protein content than AFp from S1 and S6, indicating that a certain protein fraction precipitated together with the polysaccharides, either due to their bonding or as a consequence of denaturalization and subsequent precipitation when high ethanol concentration was used. The much lower percentage of glucosamine compared to protein in the other AF and AFp fractions is because alkali soluble fractions contain these aminosugar as part of heretopolysaccharides and glycoproteins only; chitin is insoluble and stable at the extraction conditions [33]. From the previous results it can be inferred that purification of the different extracts obtained from the *Pleurotus spp*, led to a decrease in protein, polyphenol, glucosamine and ash contents, thus increasing the amount of polysaccharides in AFp extracts (Fig. 2), specifically in β-glucans (Fig. 3), as it was the aim pursued in this study.

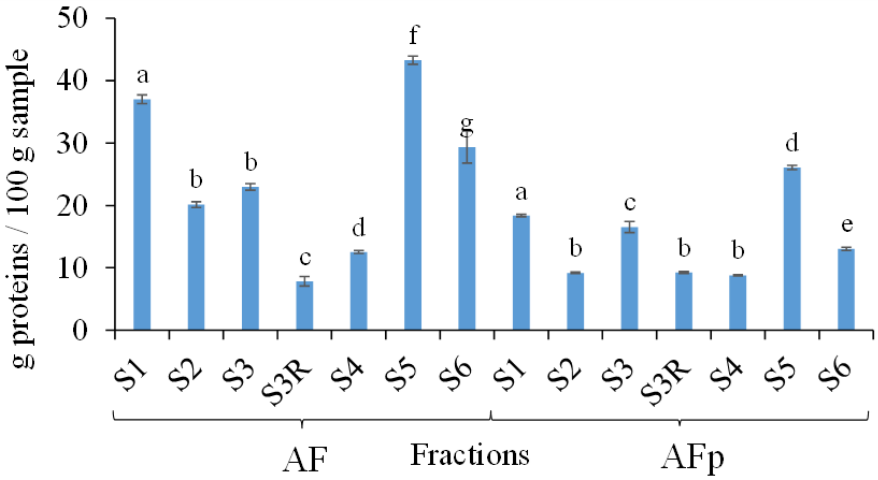


Figure 1. Protein content in AF and AFp fractions. Different letters in the same fractions of different *Pleurotus* mean that the values are significantly different. The experiments were carried out in triplicate for each different sample.

3.1.2. Monosaccharide analysis of the different fractions

The composition of some mushroom cell wall polysaccharides consists of around 80–90 % of glucose (as α- and β-glucans), 3–7 % of mannose, 2–6 % of galactose, 0.5–1 % of xylose, 0–1 % of arabinose and 2–3 % of uronic acids [34]. This corresponds to the majority monosaccharides present in the *Pleurotus* spp. extracts, except for fucose (present in most of the AFp extracts) and rhamnose (present in AFp extracts from S3 and S3R) which were also found (although in very small amounts). As observed in Fig. 3, albeit all being extracts from *Pleurotus* spp., the unpurified aqueous fractions greatly varied in the amount of glucans present, which ranged from ~20 % in *P. citrinopileatus* (S1) to >80 % in *P. eryngii* (S4). The percentage of α- and β-glucans also varied between species, having the extracts of S1, S2, S4 and S6 a greater α-glucan content, while S3 and S5 being richer in β-glucans. It is also interesting to note that the increase in glucan content of the fractions upon purification was greatly dependent on the species and while only a slight increase was observed after ethanol precipitation from the *P. ostreatus* sample (S3), a huge glucan enrichment was observed for *P.*

citrinopileatus (increasing its glucan content from ~20 % to ~80 %). In contrast, the greatest increase in percentage of other monosaccharides after purification was observed for S3 and both the AFp from *P. ostreatus* and its residue showed the greatest diversity in monosaccharide composition (see Fig. 2). Big differences were also observed related to the presence of other monosaccharides in the various *Pleurotus* spp. fractions. As seen in Fig. 2, the main monosaccharides (apart from glucose) present in all the fractions were galactose and mannose. These monosaccharides have been found to form part of complex structures in the genus *Pleurotus*, like a 3-*O*-methylated α-galactan, and an α-(1 → 4)-mannogalactan [35]. These results highlight the differences in composition and structural characteristics of the polysaccharides present in the various *Pleurotus* species, which are expected to result in different functional properties of the extracts as it will be shown below.

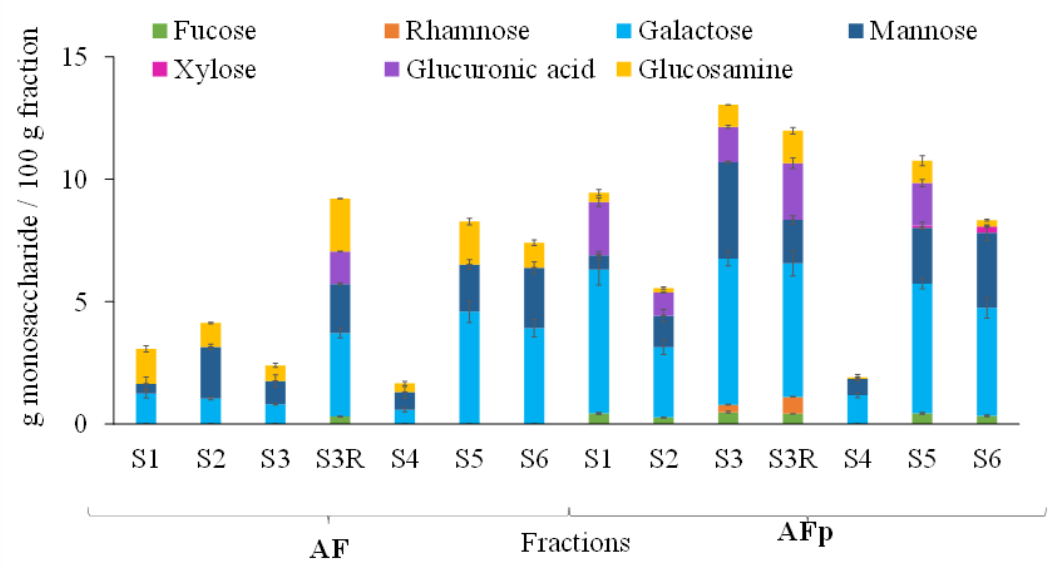


Figure 2. Percentage of the main monosaccharides found in mushrooms (fucose, rhamnose, galactose, mannose, xylose, glucuronic acid, and glucosamine), excluding glucose, from aqueous fractions (AF) and purified aqueous fractions (AFp). All the experiments were carried out in triplicate for each different sample.

Summarizing, for the AF fractions, S1 had the lowest β-glucan content, closely followed by S5, while the highest content was found in S4. This is very interesting because S4 also had one of the highest yields, thus giving rise to fractions very rich in soluble β-glucans with a very simple treatment. In general, it could be stated that the extracts with lower β-glucan content in the AF fraction were richer in proteins (S1, S5 and S6). After the purification treatment by ethanol precipitation, the greatest enrichment was found in S1, consistent with the relative decrease in other compounds (proteins, polyphenols and ashes). The most purified AFp fractions in view of their lower protein and polyphenol content were S2 and S4, yielding extracts of around 80 % of soluble β-glucan. On the other hand, the S3 fraction was practically unchanged in terms of β-glucan yield upon purification, while its percentage of other monosaccharides such as GlcA, mannose or galactose significantly increased, thus suggesting that either these monosaccharides are part of complex structures within the matrix or that there are more heteropolysaccharides of high Mw, which become enriched with the purification process. Regarding the α-glucan content, it should be noted that these polysaccharides are practically absent in the aqueous fractions of S3 and S3R, both in AF and AFp.

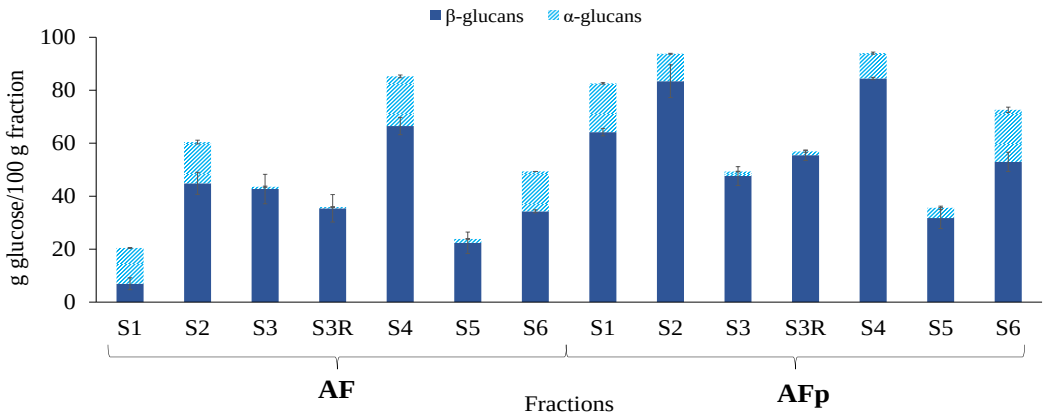


Figure 3. Total glucans content expressed as g of glucose/100 g of each fraction. The β-glucans percentages are represented in blue and the α-glucans using a dashed pattern. All determinations were carried out in triplicate.

3.1.3. Thermal stability analysis

The thermal stability and infrared spectra of the extracts can also be useful to understand their compositional and structural differences.

Fig. 4 shows the TGA first derivative (DTGA) graphs (Fig. 4A) and FT-IR spectra (Fig. 4B) from AF and AFp fractions. As observed from the TGA plots, more defined thermal degradation profiles were observed for the purified fractions, confirming the more heterogeneous composition of the unpurified AF extracts. Most of the samples show a small shoulder in the AF fractions between 100 and 150 ◦C, probably related to the loss of tightly bound water and the degradation of low molecular weight compounds, and a second shoulder between 200 and 350 ◦C with a maximum at 300 ◦C, associated with the cleavage of the C–C and C–O bonds in the ring structure of the sugars and the depolymerization of glucan chains [36]. In agreement with the glucan content data (see Fig. 3), the intensity of the shoulder centred at 300 ◦C was the greatest for the AF fraction of *P. eryngii* (S4) with >80 % glucan content. The thermal degradation profiles from the AF extractions, except for S3R, showed another broad shoulder between 400 and 500 ◦C, which has been ascribed to the degradation of chitin-glucan complexes [36,37], being rather intense the AF sample of *P. pulmonarius* (S6).

Upon purification and polysaccharide enrichment of the samples (AFp), the thermal degradation profiles significantly changed and most of the samples showed a very significant increase in the degradation peak centred around 300 ◦C (corresponding to glucan degradation), although as it could be observed from the derivative TGA profiles, both the position and shape of the degradation peak was different for all the samples, thus indicating the various types of glucan or polysaccharide molecules extracted from the different *Pleurotus* species. Interestingly, AFp from S4, S2 and S1, which were fractions with the greatest glucan contents, displayed a similarly shaped degradation peak centred around 270 ◦C. In contrast, AFp from S3 and S3R, showed a double degradation peak with two maxima between 200 and 300 ◦C. It should be highlighted that AFp from *P.*

ostreatus and its residue had greater contents of other monosaccharides and glucosamine, probably arising from glycoproteins and other complex polysaccharides, and very low α-glucan content, fact which apparently had a great influence in β-glucan structure and, thus, in its thermal degradation profile.

It is also interesting to note that AFp samples from S5 and S6 showed a degradation profile which could correspond to glycoproteins according to [38], with a first sharp peak around 240 ◦C followed by a broader peak between 250 and 300 ◦C which are attributed to dehydration and intense degradation of the amino groups with the release of H2O, NH3 and the decomposition of other components with the release of CO and CO2, respectively. In the case of S5, the most intense degradation peak was the one at 240 ◦C, probably related to the degradation of amino groups, as this sample had more protein and glucosamine contents than the others. Finally, another feature to highlight from the DTGA profiles from S3 and S3R is the relatively high intensity of the degradation peaks between 400 and 450 ◦C, that have been previously described to be a consequence of chitin-glucan complexes degradation [36], but that have been also related to backbone polysaccharides, as the thermal degradation of these has been reported to be higher than that of polysaccharide side chains [39].

Thus, apparently *P. ostreatus* samples may contain a higher percentage of large backbone polysaccharides than the other *Pleurotus* species, which may have implications in the immunomodulatory properties, as previous studies using cell assays have demonstrated the improved immunostimulatory activity of large molecular weight linear β-glucans [40].

3.1.4. Infrared spectroscopy analysis (FT-IR)

Fig. 4 also shows the FT-IR spectra of the aqueous unpurified (AF) and purified (AFp) fractions obtained from the different *Pleurotus* spp., which correlate with the above compositional characterization. The FTIR spectrum shows a smooth N–H stretching band between 3000 and 3500 cm^{-1} that overlaps with the O–H stretching band. The C–H stretching band centred around 2835–2940 cm^{-1} is also apparent in all the samples [41].

Other vibrational bands characteristic of N–H bonds, which may come from glucosamine units in glycoproteins/chitin and/or proteins are also present, such as the amide I (1660 and 1620 cm-1), amide II (1560 cm-1) and amide III (1306 cm-1) bands and other vibrational bands from the carbohydrate backbone mainly attributed to C– – O stretching [42,43]. In some of the AF samples with higher glucosamine content such as S1, S5 and S6 (see Fig. 4), the amide II band at 1558 cm-1 was clearly seen. Within the broad amide I band, the small vibrational band at 1660 cm-1 observed in S3R (AF) could be a consequence of the bonding between the amino group and the carbonyl group within the same chain. As with the DTGA profiles, sample purification was also apparent in the FTIR spectra. Specifically, a relative increase in the intensity of the carbohydrate-related vibrational bands in the 950–1150 cm^{-1} range was clearly observed.

Interestingly, in the FTIR spectra from the AFp samples, the band related to the bonding between amino and carbonyl groups has a lower intensity in S3R than in S3, consistent with the lower protein content of the former. For the purified fractions the peaks were more sharply defined with the exception of S3 and S5 with lower percentage of β-glucans, showing more heterogeneous peaks than the other purified fractions.

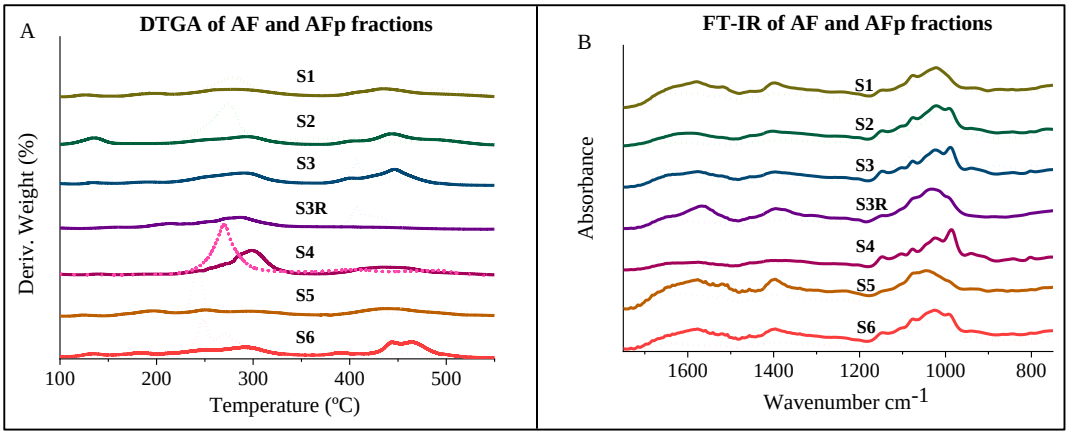


Figure 4. 1st derivatives thermogravimetric (DTGA) curves (A) and FT-IR spectra (B) of aqueous fractions (AF) and purified aqueous fractions (AFp) from different *Pleurotus*. Solid line graphs correspond to AF fractions and dot graphs correspond to purified AF fractions. The main vibrational bands have been specified in the FTIR spectra (B).

3.1.5. Molecular mass distribution

The size and distribution of molar mass (MM) of the different fractions were also analyzed (Fig. 5; Fig. S1 in the Supplementary Material). The first thing to highlight is that a great percentage of the lower molecular weight compounds present in the extracts were eliminated during the purification step, showing a great intensity reduction in the peaks corresponding to molecules in the range from 342 Da to 6 kDa. It is worth highlighting the differences obtained between species. For instance, when purifying the extract from *P. citrinopileatus* (S1), a huge decrease in the percentage of molecules lower than 6 KDa was observed, increasing up to 95.16 % the compounds with Mw >6 KDa. In contrast, greater percentages of compounds lower than 6 KDa (>50 %) remained after purification of the extracts obtained from *P. ostreatus* (S3) and *P. djamor* (S5) (see Table S2 from Supplementary Material). Interestingly, two distinct MM distribution populations can be discerned; one around 10-25 kDa and another broad peak around 400 KDa (Fig. 5; Table S2 in suppl. material). While the smaller peak is

present in most samples, the latter was observed in AFp samples from S1, S2, S4 and S6; whereas, a small shoulder corresponding to higher molecular weight compounds (>800 kDa) was observed for those coming from S3, S3R and S5 samples, which may correspond to some heteropolysaccharides, high Mw glycoproteins or glucan-chitin complexes isolated from these *Pleurotus* species [44]. High molecular weight β-glucans have been associated with higher immunoregulatory activity [40]. These results are consistent with the previous characterization results, which indicate that purification leads to the removal of low molecular weight compounds, having the purified fractions more thermally stable molecules, as indicated by the TGA assays, which should correspond to higher molecular weight polysaccharide chains and complexes.

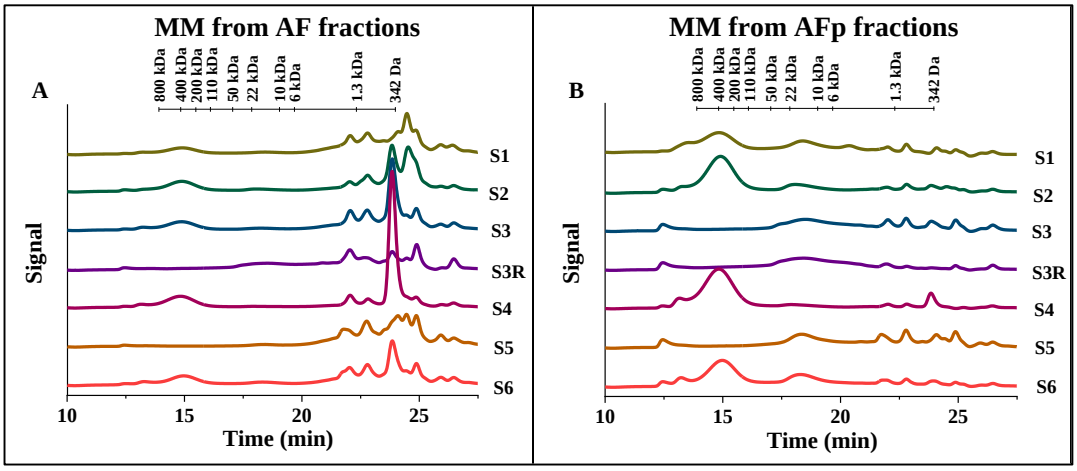


Figure 5. Molar mass (MM) distribution of: (A) the different unpurified (AF) aqueous fractions from *Pleurotus*, and (B) the ones from the purified (FAp) fractions. Chromatograms from the reference pullulan standards can be found in Fig. S1 from the Supplementary Material.

3.2. In vitro host response assays

In order to characterize the immunomodulatory activity of the extracts and ascertain whether it was due to the presence of β-glucans, three different cell culture lines were used. Initially, the extracts were tested on HT-29 cells as they

have been frequently used to study the intestinal immune response to bacterial infection, and survival, adhesion or invasion [45]. After induction of pro-inflammatory conditions using the cytokine TNF-α, none of the assayed extracts including Lentinan, a β-glucan with reported anti-inflammatory and immunomodulatory activity [46], showed a significant effect. The results from this assay can be found in the Supplementary Material (Fig. S2). The Lentinan sample was used as a reported positive control, at the same concentration as the different mushroom fraction samples for each assay. It is worth highlighting that, at the concentrations tested, none of the extracts had toxic effects on the cells, as evaluated through cell viability assays.

3.2.1. Assays in HEK-hTLR4 cells

Immunomodulatory effects of β-glucans have been described to be determined by combined effects of different pattern recognition receptors (PRRs) such as C-type lectin receptor Dectin-1, complement receptor 3 (CR3) and Toll-like receptors [9,47]. Dectin-1 is still considered the most relevant receptor for β-glucan signaling, however, TLR4 was recently described to synergistically enhance effects of β-glucan on immune cells [9,48]. Therefore, a human TLR4 reporter cell line was used to test for NF-κB activation mediated by TLR4 in both, the presence and absence (basal condition) of the pro-inflammatory TLR4 ligand LPS. As shown in Fig. 6, none of the extracts could modulate LPS-induced NF-κB activation, except AF extracts S1 and S6 that showed a significant anti-inflammatory effect, thus highlighting the potential of this specific fungal fraction as a natural anti-inflammatory extract obtained through a simple and green method. This seems to point out that the β-glucans present in the extracts were not the responsible of this anti-inflammatory effect, as the purified extracts did not show a significant activity.

Without the pro-inflammatory LPS, β-glucan AF and AFp exerted varying effects on TLR4 with strong and significant activation of AF and AFp extracts S3 and

S3R. AFp extracts of S4 and S6 also activated significantly TLR4, although to reporter gene activity levels inferior than for S3 and S3R (74.7 mU/mg/mL for S4 and 76.7 for S6 when compared to 210.7 and 494.8 mU/mg/mL for S3 and S3R respectively). These results are highly interesting and deserve further investigation, as purification of S3 and S3R did not conduct to a very high increase in β-glucan content, while it led to an increase in other heteropolysaccharides, including the presence of rhamnose (not detected in the other samples). Similarly, the DTGA assays showed a specific degradation pattern of certain thermally stable structures (with a peak degradation temperature around 400 ◦C), not observed in the other evaluated extracts.

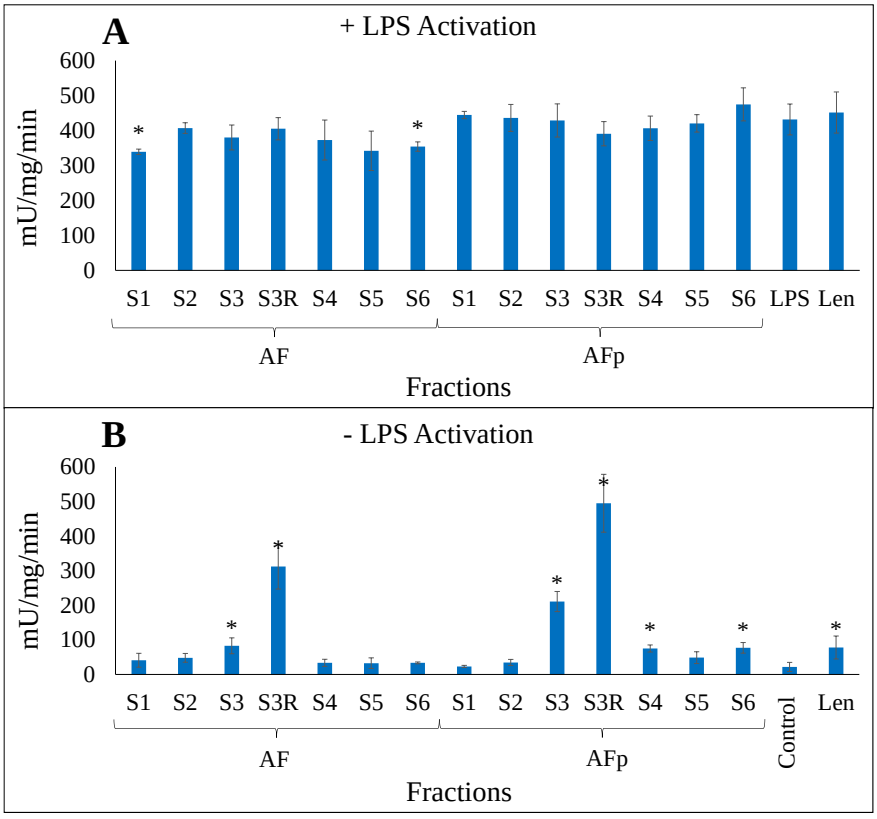


Figure 6. Activation of Toll-Like Receptor 4 (TLR4) in HEK-Blue™ hTLR4 cells, with (A) and without (B) LPS stimulation, from unpurified (AF) and purified (AFp) fractions. The tests were carried out in triplicate for each different sample.

* Significantly different from the LPS (A) and control (B) samples ($P < 0.05$).

3.2.2. Assays with THP-1 macrophages

It has been reported that particulate and soluble β-glucans have an effect on cytokine production in THP-1 differentiated macrophages, through some extracellular receptors such as TLR2, TLR4, TLR5 and Dectin-1 [9]. Previous studies have shown that the immunomodulation of β-glucan extracts, depends on their chemical composition and molecular weight distribution. Their mode of action in THP-1 macrophages has been reported to occur through synergistic action of TLR2 and TLR4, activating Dectin-1 and, thus increasing the pro-inflammatory cytokine IL-8 and TNF-α [9]. In order to assess the immunomodulatory potential of these fractions, the level of interleukin (IL)-8 produced by the exposed THP-1 cells was determined. Incubations were performed with the THP- 1 cell line (with Dectin-1 receptor), increasing the concentration of the extracts to 50 μg/mL based on published literature [49,50], and the production of the cytokine IL-8 was assessed by ELISA.

The results show high cytokine production for the S3 and S3R samples, both in AF and AFp fractions (Fig. 7A), indicating that these fractions have a high immunostimulatory potential, significantly standing out from the rest of the purified fractions. These results, which deserve further research, seem to point out that, either the presence of heteropolysaccharides, glycoproteins, or more complex β-glucan structures are playing a role in the immunomodulatory activity of these fractions. These results agree with the experiments performed in HEK-hTLR4 cells, suggesting a potential role of TLR4 in mediating the effects of S3 and S3R. Interestingly, as previously mentioned, these specific samples S3 and S3R were richer in other monosaccharides, having moderate β-glucan contents, but had a high Mw fraction with very good thermal stability. It is also interesting to note that these samples did not show a high β-glucan enrichment upon purification, so other compounds in the extracts could play a synergistic action, for example with TLR4, or, at least, did not interfere with their bioactivity.

It is also worth mentioning that the differences observed both in the TGA and molecular weight profiles of the unpurified and purified S3 and S3R samples, seem to indicate that purification led to the concentration of specific molecular structures with higher molecular weight and greater thermal stability, which may be the responsible of the bioactivity observed. In future studies it still has to be determined if a synergistic enhancement of dectin-1 signaling through TLR4 exists in response to our extracts as described in the study of [9]. From these assays it is also evident that the immunostimulatory activity could not be ascribed to the low molecular weight compounds from the aqueous AF fractions, since upon their removal through ethanol precipitation, an increase in the activity of S3 was seen while that of S3R was practically unchanged. Interestingly, most of the *Pleurotus* spp. assayed also displayed a certain immunostimulatory activity, as observed in Fig. 7B, where S3 and S3R fractions have been removed from the graph. This Figure shows that, except for S6, purification leads to higher IL-8 production, thus suggesting that the immunostimulatory activity could be largely ascribed to the β-glucan structures present in the fungal extracts. Therefore, to corroborate the activity in a pro-inflammatory environment, the purified fractions were also exposed to LPS (Fig. 7C). Very interestingly, while the S3R fraction significantly increased IL-8 production, thus pointing out to a potent immunostimulatory activity, an anti-inflammatory effect was observed in the case of S2, S4, S5 and specially S1 (AFp). This last fraction was the one in which the highest purification in β-glucans was achieved. This result is of great relevance as it points out that through a simple purification treatment, an extract with remarkable anti-inflammatory capacity is obtained.

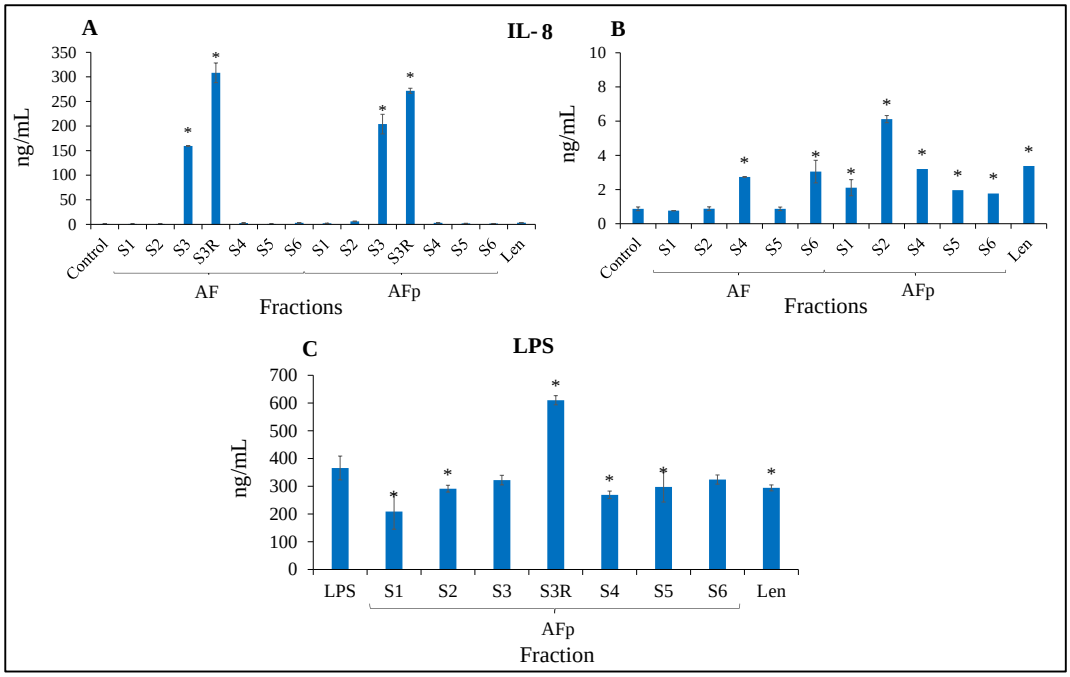


Figure 7. (A) IL-8 quantification for AF and AFp fractions of all samples, expressed in ng/mL. (B) IL-8 quantification for AF and AFp fractions of all samples, except S3 and S3R to better observe the differences with the control, expressed in ng/mL. (C) Assay with the polysaccharides fractions exposed to LPS, expressed in ng/mL. The tests were carried out in triplicate for each different sample.

* Significantly different from the control (A and B) or LPS (C) samples ($P < 0.05$).

4. Conclusions

In this work, various aqueous fractions, unpurified (AF) and purified by ethanolic precipitation (AFp), were obtained from different *Pleurotus* species, which showed different bioactivity against in vitro cellular assays. These fractions have the great advantage of being water-soluble and do not require complicated and expensive processes to be obtained. Upon purification and subsequent yield reduction, enrichment in the polysaccharidic fraction was attained through elimination of low

molecular weight compounds, proteins, glucosamine and ashes. The obtained results suggest that the presence of heteropolysaccharides, glycoproteins or more complex β-glucan structures could be mainly responsible for the immunomodulatory activity of the aqueous fractions obtained from all the different *Pleurotus* species studied. Especially, *P. ostreatus* and the residue of *P. ostreatus* showed an outstanding immunostimulatory capacity compared to the other species. Further work would be done to fractionate the most active extracts to elucidate exactly what type of structures are responsible for their immunostimulatory and anti-inflammatory activity.

Acknowledgements

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Chapter 1.2

Supplementary Material

Phenolic content and antioxidant capacity of the extracts

The total phenolic content was determined by the modified Folin-Ciocalteu method and is included in Table S1. Table S1 also includes the antioxidant capacity of the extracts (expressed as μmol of Trolox equivalents per gram of sample). Both the phenolic content and the antioxidant capacity decreased upon purification, probably due to the elimination of water-soluble polyphenols and low Mw compounds with antioxidant capacity during ethanol precipitation. It should be also highlighted that all AF fractions had significantly greater antioxidant capacity and higher polyphenol content than other aqueous plant extracts [1], thus making them interesting green extracts with bioactive properties.

Table S1. Antioxidant capacity and polyphenols content in AF and AFp fractions. The antioxidant capacity of the samples is expressed as μg of Trolox equivalents (TE)/g fraction, and polyphenols content as mg of gallic acid equivalents (GA)/ g fraction.

Mushroom	AF fraction		AFp fraction	
	μg TE/g	mg GA/g	μg TE/g	mg GA/g
S1	272.24 (5.53) ^{a}	24.65 (2.33) ^{a}	65.97 (0.29) ^{a}	10.52 (0.27) ^{a}
S2	142.34 (3.86) ^{b}	17.71 (0.24) ^{b}	34.43 (0.88) ^{b}	5.36 (0.27) ^{b}
S3	138.55 (1.74) ^{b}	15.35 (0.97) ^{c}	39.36 (2.71) ^{c}	8.73 (0.21) ^{cd}
S3R	118.31 (3.14) ^{c}	12.41 (0.37) ^{d}	40.13 (1.78) ^{c}	9.02 (0.38) ^{c}
S4	80.95 (3.53) ^{d}	6.62 (0.18) ^{e}	36.42 (3.41) ^{b}	4.65 (0.20) ^{b}
S5	276.85 (8.58) ^{a}	23.08 (0.44) ^{a}	74.24 (0.07) ^{d}	18.03 (0.98) ^{e}
S6	218.92 (9.77) ^{e}	16.80 (0.55) ^{bc}	49.19 (2.92) ^{e}	7.97 (0.93) ^{d}

Different letters in the same column mean that the values are significantly different.

Molecular mass distribution of pullulan standards

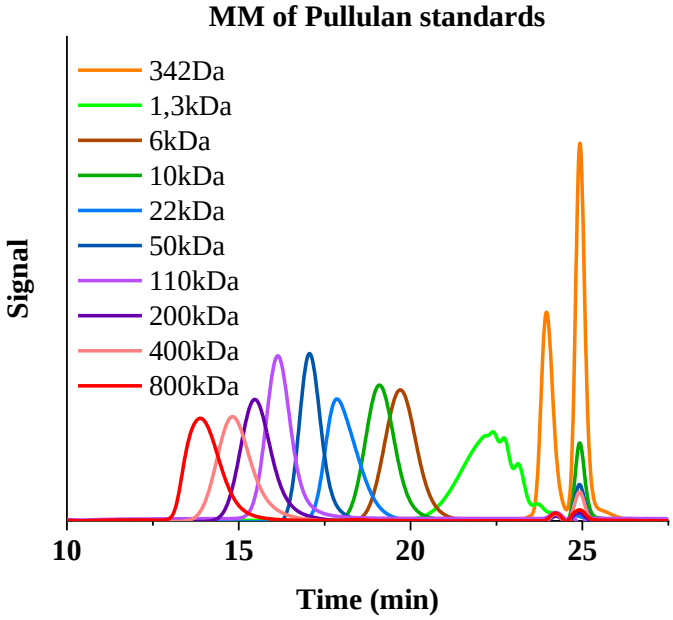

Figure S1. Chromatograms from Molar mass (MM) distribution of the reference Pullulan standards.

Table S2. Percentage areas in the MM distribution for the FA and FAp fractions of the different mushroom species.

Mushroom	AF fraction		AFp fraction	
	Area (%) <= 6KDa	Area (%) > 6KDa	Area (%) <= 6KDa	Area (%) > 6KDa
S1	86.40	13.60	4.84	95.16
S2	84.36	15.64	27.59	72.41
S3	85.90	14.10	50.61	49.39
S3R	77.63	22.37	29.35	70.65
S4	81.83	18.17	13.36	86.64
S5	98.43	1.57	59.34	40.66
S6	83.36	16.64	17.61	82.39

Assays in HT-29 NF-κB reporter cell line

In order to characterize the immunomodulatory activity of the extracts and ascertain whether it was due to the presence of β-glucans, three different cell

culture lines were used. HT-29 cells have been frequently used to study the intestinal immune response to bacterial infection, and survival, adhesion or invasion [2] and, as an intestinal epithelial cell line, it is at the first line of defense in the intestine [3]. In this work, a HT-29 clone carrying a reporter gene to monitor for the activation of the transcription factor NF-κB was used, which is reported to be a regulator of inflammatory processes [4]. The aqueous fractions AF and AFp were assayed at a concentration of 25 µg/mL. After induction of pro-inflammatory conditions using the cytokine TNF-α, none of the assayed extracts including Lentinan, a β-glucan with reported anti-inflammatory and immunomodulatory activity [5], could significantly change TNF-α-induced NF-κB activation and hence, exert an anti-inflammatory/immunomodulatory effect. Lentinan is a β-glucan isolated from the edible shiitake mushroom *Lentinus edodes* and was reported to reduce NF-κB activation in A549 lung cancer cells and exert immunostimulatory effects in THP-1 macrophages [6]. Of note, concentrations of β-glucans including Lentinan used in this work were inferior than in the study of Murphy et. al (25 µg/mL compared to 1–10 mg/mL in the study of Murphy et. al).

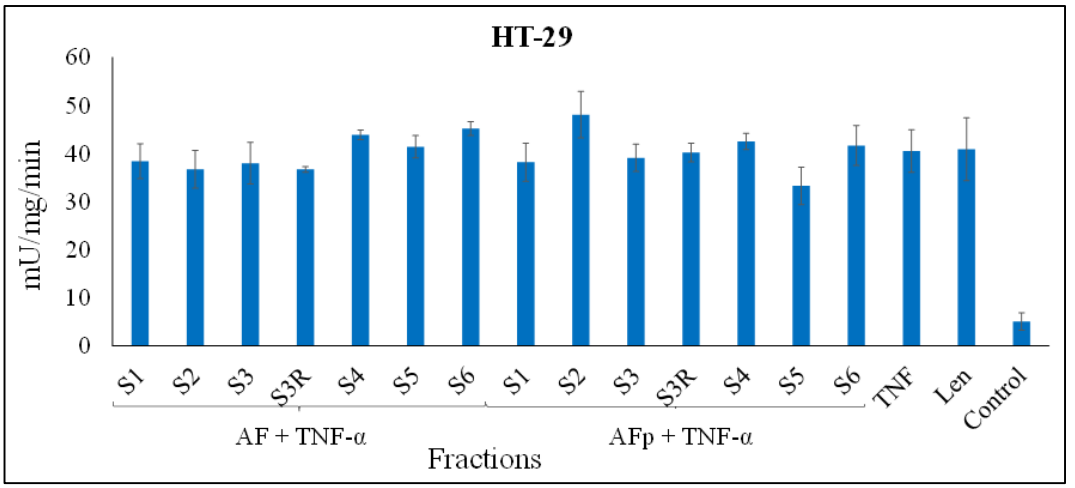

Figure S2. HT-29-cells transfected with NF-κB reporter gene were stimulated with TNF-α and incubated with unpurified and purified extracts. Activity of SEAP reporter gene was determined and adjusted for protein quantity.

*indicates significantly different ($P<0.05$)

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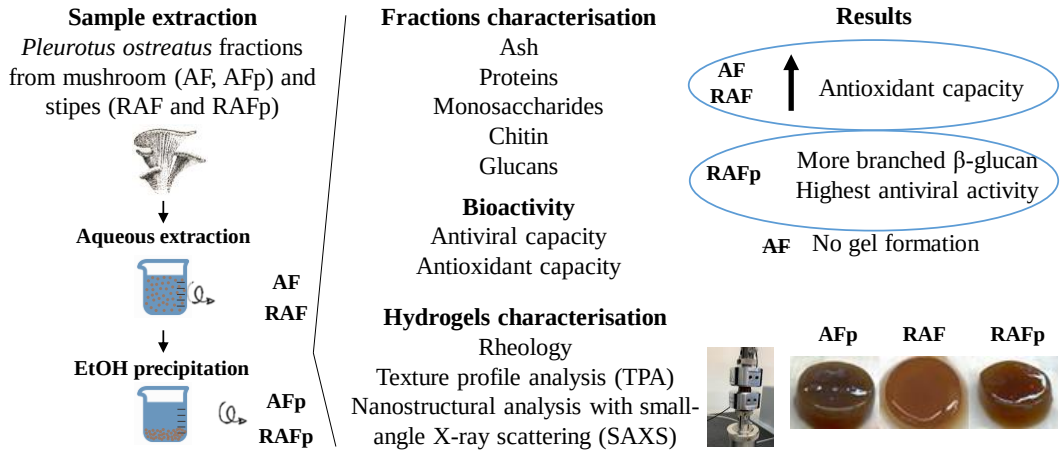
CHAPTER 2

Relationship between structure and functional properties of mushroom biomass as texturizers for food applications

2.1. Antiviral and technological properties of β-glucan-rich aqueous fractions from *Pleurotus ostreatus* waste biomass.

2.2. Emulsifying properties of β-glucan extracts obtained from *Pleurotus ostreatus* mushroom and stipes.

2.1. Antiviral and technological properties of β-glucan-rich aqueous fractions from *Pleurotus ostreatus* waste biomass.



This section is an adapted version of the following published research article:

Zaida Pérez-Bassart, Irene Falcó, Marta Martínez-Sanz, Antonio Martínez-Abad, Gloria Sánchez, Amparo López-Rubio, María José Fabra

"Antiviral and technological properties of β-glucan-rich aqueous fractions from *Pleurotus ostreatus* waste biomass"

Food Hydrocolloids, Volume 146, Part B, 109308.

Abstract

In the present work, aqueous fractions were extracted from the discarded *Pleurotus ostreatus* biomass (AF) and from a waste product (RAF) generated from it in the industry (stipes) with the aim of valorizing these wastes into new food ingredients. Compositional, structural and functional analysis, before and after a purification step (p) to enrich its content in β-glucans, was carried out. Moreover, the rheological properties and the nanostructure of hydrogels obtained from the β-glucan extracts were analyzed. The unpurified fractions stood out for their significantly higher antioxidant capacity (AF, 138.55 ± 1.20 and RAF, 118.31 ± 1.20). As a result of the ethanolic purification process, purified samples had lower protein, ash, polyphenols and α-glucans content than the unpurified ones. Furthermore, extracts from stipes had a greater structural complexity, with more branched β-glucans than their counterparts obtained from the whole biomass, which had a positive impact on the antiviral activity against murine norovirus. Interestingly, purified samples formed stronger hydrogels but the gelation mechanism depended on its composition and structure. AFp was able to form a gelling network with a more tightly packed structure, ascribed to the presence of less branched β-glucans, showing the highest hardness value (2.87 ± 0.27 N). Unpurified RAF fraction showed hydrogels with greater cohesiveness and chewiness values (4.74 ± 0.55 N and 9.08 ± 0.87 N, respectively). In contrast, the presence of small side chains in RAFp could also act as reinforcement agents in hydrogels formed by means of intermolecular associations, with the additional advantages of having greater antiviral activity and coming from agroindustrial residues (stipes).

1. Introduction

The consumption of edible mushrooms is increasing every year due to their nutritional advantages (rich in valuable proteins, minerals, and dietary fibres) (Lu, Lou, Hu, Liu, & Chen, 2020; Maity et al., 2021). However, its increased

production and consumption is causing a large number of by-products (caps, stipes and mushroom that do not comply with commercial standards) and posing an environmental challenge and management cost for the industries that market these products (Antunes et al., 2020; Grimm & Wosten, ¨ 2018; Kumar et al., 2021). *Pleurotus ostreatus* is one of the most consumed and affordable species of cultivated mushrooms globally (Elhusseiny et al., 2021; Mishra, Tomar, Yadav, Vishwakarma, & Singh, 2022). It is an excellent supplier of health-promoting molecules such as vitamins, amino acids, glucans, and essential fatty acids. Specifically, mushroom cell walls are rich in β-glucans, long and short-chain polymers of glucose units with β-1,3 and β-1,6 linkages, that are responsible of the linear and branching structures, respectively (Ehren et al., 2020). Structural, physical, and technological properties of β-glucans vary from different species, cultivars, growing environments, drying conditions and isolation/extractions methods (Bai et al., 2019; Shaheen et al., 2022), with also important changes on their bioactive properties. For instance, β-glucans are used in the food industry as gelling agents and thickeners in the production of low-fat foods with improved texture properties (milk, bread, or yogurt) (Du, Meenu, Liu, & Xu, 2019; Kaur, Sharma, Ji, Xu, & Agyei, 2020). Specifically, hydrogels are three-dimensional, hydrophilic, polymeric networks capable of holding large amounts of water. Moreover, β-glucans also constitute a soluble fibre with a powerful prebiotic effect and stimulate the immune system by having immunomodulatory capacity, antitumor, antioxidant and antiviral activities (Morales et al., 2020; Thatoi, Singdevsachan, & Patra, 2018; Yao, Gong, Li, Hu, & You, 2022)). This antiviral activity correlates with the antioxidant effect and anti-inflammatory cytokines induced by β-glucans (Shi et al., 2022). In addition, high molecular weight β-glucans show greater anticancer properties, although they are more difficult to extract due to the intermolecular interactions they usually form (Bulam, S¸ule Üstün, & Peks¸en, 2018; Miro´nczuk-Chodakowska & Witkowska, 2020). The structural properties of β-glucans, in terms of branching degree and molecular

conformation as well as their association with other cell wall components (such as mannoproteins, chitin or other polysaccharides) will determine their solubility and extractability. Although there are β-glucans that are easily extractable with water, highly branched β-glucans need more aggressive treatments (i.e. highly alkaline solutions, pH 13–14) (Miro´nczuk-Chodakowska & Witkowska, 2020; Morales et al., 2020). However, water-soluble fractions, could be a more eco-sustainable option in the production of new functional ingredients. Furthermore, discarded whole biomass not meeting commercial standards such as size and appearance and, the residues (stipes), that are not commercialized, can be cheap and abundant sources of these compounds. We hypothesized that the structure and composition of β-glucans will depend on the source and extraction conditions and, these aspects will determine their gelling properties and their functionality. Therefore, this work is aimed to elucidate the structure-function relationship of β-glucans extracts obtained from *P. ostreatus*, one of the most cultivated edible mushrooms. Specifically, a complete compositional and functional (antioxidant and antiviral) analysis of water-soluble β-glucan fractions obtained from both discarded whole biomass and stipes was carried out. The gelling capacity of purified and unpurified β-glucan-rich fractions was also explored and, the structure of the hydrogels was characterized using small angle X-ray scattering (SAXS), rheology and texture analysis.

2. Materials and methods

2.1. Materials

Pleurotus ostreatus (*P. ostreatus*) fungal biomass and the residue (stipes) generated from its cultivation in the form of a bunch, the trunk or cob, all grown by Centro Tecnológico de Investigación del Champiñón –Asochamp- (Rioja, Spain), were used as raw material.

The ethanol reagent (96% v/v) used in the purification of the extractions was obtained from PanReac AppliChem. Lentinan (minimum purity 30%) was purchased from Carbynth Biosynth (UK). Other reagents and materials used were shrimp shell chitin, Folin-Ciocalteu reagent, sodium carbonate, phosphate buffered saline (PBS), gallic acid, potassium persulphate, 2,2′ -azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2.5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), trifluoroacetic acid (TFA), fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid, glucuronic acid and deuterated water (D2O) were purchased from Sigma-Aldrich. Finally, the β-glucan assay kit for yeast and mushrooms K-YBGL September 2009 together with yeast β-glucan (about 49% purity) and glucose standard (1 mg/mL), was obtained from Megazyme.

2.2. Aqueous extractions

The biomass used in this study came from the *P. ostreatus* mushroom and its residue (stipes) obtained from its cultivation. The raw material was freeze-dried, ground, and sieved (500 μm) to subsequently carry out the aqueous extractions. For this, 25 g of each sample, *P. ostreatus* mushroom and *P. ostreatus* stipes, were weighed, and 300 mL of distilled water were added to each sample and left under magnetic stirring, performing a reflux extraction at boiling temperature for 7 h as in (P´erez-Bassart, Fabra, Martínez-Abad, & L´opez-Rubio, 2023). The samples were then centrifuged for 10 min at 8000 rpm in an Avanti J-26 XPI centrifuge with a JLA-16,250 rotor. The supernatant obtained was concentrated in a Heidolph rotavapor apparatus and then freeze-dried to obtain the aqueous fractions of the *P. ostreatus* whole biomass, AF, and of the *P. ostreatus* residue, RAF. In order to purify these fractions, an ethanolic precipitation of the aqueous fractions was carried out. The aim was to obtain purer fractions in β-glucans, which are mainly responsible for the gel-forming properties of mushrooms. Thus, the supernatant obtained after aqueous extraction was subjected to precipitation with 3 times its

volume in 96% (v/v) ethanol, keeping the mixture under mechanical agitation for 1 h. Subsequently, the samples were centrifuged at 8000 rpm for 10 min and a precipitate was recovered and left overnight in an extraction hood to remove ethanol residues. The samples were freeze-dried to obtain the purified aqueous fraction of the whole mushroom, AFp, and of the stipes, RAFp.

2.3. Proximate analysis

2.3.1. Carbohydrate content

Monosaccharide composition and chitin content were determined as previously described in (P´erez-Bassart et al., 2023). The chitin content was determined by acid hydrolysis with 6 M HCl at 100 ◦C for 7 h. Monosaccharide composition, including total glucose (from α and β-glucans) and excluding glucosamine (analyzed separately as detailed below), was carried out by acid hydrolysis with TFA (trifluoroacetic acid) followed by HPAEC-PAD on a Dionex ICS-6000 equipped with a CarboPac™ PA1 column. Glucose from α-glucan content was determined after an enzymatic hydrolysis with amyloglucosidase and invertase (Megazyme, Ireland), followed by colorimetric determination of glucose (K-GLUC kit, Megazyme, Ireland). Glucose from β-glucan was determined by the difference between total glucose (TFA hydrolysis) and α-glucan glucose.

2.3.2. Chitin and protein content

Mushrooms have two sources of nitrogen due to their chitin content. Therefore, the protein content was calculated by determining the protein nitrogen by the difference between total nitrogen and nitrogen from chitin, and quantifying it to protein content by using the food correction factor 6.25 as previously described in (P´erez-Bassart et al., 2023). The total nitrogen content was determined using an elemental nitrogen analyzer rapid surplus, based on the modified Dumas method. To determine the chitin content, hydrolysis was carried out in 6 M HCl at 100 ◦C for 7 h, as described in section 2.3.1.

2.3.3. Total phenolic compounds

The total phenolic content of samples was determined using the assay based on the Folin-Ciocalteau method modified by Singleton (Singleton, Orthofer, & Lamuela-Ravent´os, 1999). 1000 μL of 1/10 diluted Folin-Ciocalteau reagent were mixed with 200 μL of sample and allowed to incubate for 5 min. Then, 800 μL of sodium carbonate (75 mg/mL) were added and the samples were incubated in a bath at 40 ◦C for 30 min. The absorbance values were measured at 760 nm. A calibration curve was performed using gallic acid as standard, and the total polyphenol concentration was expressed as mg gallic acid/g sample.

2.3.4. Ashes

For the quantification of ashes, samples of known weight were placed in a muffle furnace at 550 ◦C until complete calcination. The difference in weight at room temperature was recorded and the result was expressed as a percentage of weight lost compared to the initial sample (AOAC, 2022). All experiments were performed in triplicate.

2.4. Structural analysis

Methylation analysis was performed to ascertain the structural features of only purified samples (AFp and RAFp). Partially methylated alditol acetates (PMAA) were produced following the protocol described in (Pettolino, Walsh, Fincher, & Bacic, 2012). The obtained PMAAs were separated and analyzed by gas chromatography using a GC7890B–5977 B GC-MS (Agilent Technologies) with a multipurpose sampler (Gerstel MPS) with a HP-5 capillary column (30 m × 0.25 mm; Agilent Technologies). The temperature program was set from 150 to 210 ◦C at 1 ◦C/min heating rate. The mass spectra of fragments obtained from PMAAs were identified by comparing them with those of reference polysaccharide derivatives. As a complementary analysis, NMR was also performed with samples prepared at a dilution of 5 mg/mL in deuterated water (D2O) and 500 μL of the

samples were added to specific NMR tubes and measured in a Bruker Avance 300 mHz spectrophotometer at 25 ◦C.

3. Functional characterization of β-glucans rich extracts

3.1.1. Antiviral capacity

Murine norovirus (MNV-1), kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA, was assayed and propagated in RAW 264.7 (ATCC CRL-1688) cell line. MNV stock was harvested by infecting cell lines for 2 days, followed by three thaw cycles at 660×*g* for 30 min. Fifty percent of tissue culture infectious dose (TCID50) was determined to enumerate infectious viruses using the Spearmen-Karber method (Pint´o, Diez, & Bosch, 1994). Purified and non-purified extracts were dissolved in PBS 10 mg/mL. Each solution was incubated for 16 h (overnight, ON) at 37 ◦C in an equal volume of MNV (ca. 5 log TCID50/mL), obtaining a final concentration of the extracts of 5 mg/mL. After ON incubation, samples were then ten-fold diluted in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum and the residual infectivity was determined by TCID50. Untreated virus suspensions (without extracts) were analyzed in parallel under the same experimental conditions and used as a positive control. Ten-fold dilutions of treated and untreated virus suspensions were inoculated into confluent cell monolayers in 96-well plates. Each condition was done in triplicate. Virus decay titer was calculated as log10 (Nx/N0), where N0 is the infectious virus titer for untreated samples and Nx is the infectious virus titer for the extracts (Falc´o et al., 2018).

3.1.2. Antioxidant capacity

The antioxidant capacity of the aqueous fractions from the whole mushroom (AF and AFp) and the residue (RAF and RAFp) was determined by the ABTS (2,2′ -azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) method (Re et al., 1999), one

of the most widely used assays on food samples for *in-vitro* studies (Platzer et al., 2021). For this purpose, prior to the calibration line, a solution of 0.192 g ABTS and 0.033 g potassium persulphate was prepared in 50 mL PBS and incubated overnight. Subsequently, ABTS was diluted in PBS to an initial absorbance of 0.700 ± 0.02 at a wavelength of 734 nm. The calibration line was prepared with 6-hydroxy-2,5,7,8-tetramethylchromium-2-carboxylic acid (Trolox) by dissolving 0.0060 g in 25 mL with PBS to a dilution of 1 μmoL/ml. For the determination of the antioxidant capacity of the samples, a microplate was used in which 20 μL of each sample and 230 μL of diluted ABTS were added to each well. The plate was incubated for 6 min at room temperature in the dark and then absorbance was measured at 734 nm using a CLARIOstar instrument (BMG LABTECH). Antioxidant capacity was expressed as μmol of Trolox/gram of extract.

3.2. Development of β-glucans-based hydrogels

Gel-like structures were only obtained from the fractions AFp, RAF and RAFp since AF did not form gels. To this end, a concentration of 3% (w/v) of their respective β-glucan content was used on each sample to better compare their corresponding properties since β-glucan is the main gelling compound in the aqueous fractions. The concentration was stablished according to screening trials as it was found to be the minimum concentration at which most of the samples formed gel-like structures. The freeze-dried fractions were dissolved in distilled water at 80 ◦C for 30 min in a water bath. Once the solutions were tempered, they were transferred to cylindrical containers of 30 mm in diameter and 40 mm in height and stored under refrigeration at 4 ◦C for 24 h, as in (Fontes-Candia et al., 2022).

3.3. Characterization of β-glucans based hydrogels

3.3.1. Rheology

Rheological measurements were performed on a rheometer DHR-3 from TA Instruments (USA) using a Peltier parallel plate of 40 mm diameter and 1000 μm gap. The edge of the samples was covered with a thin layer of paraffin to prevent evaporation of the sample. To determine the linear viscoelastic region, a strain amplitude sweep was performed at 4 ◦C, with a constant frequency of 6.28 rad/s and varying the strain amplitude in a range of 0.01–100%. The viscosity of the samples was measured at 1% β-glucans since, at 3%, the hydrogel-forming samples rapidly gelled even at high temperature. The samples were placed on a preheated plate at 100 ◦C for a temperature ramp where, after an equilibration time of 2 min, the cooling process of the samples started from 100 ◦C to 4 ◦C with a constant rate of 2 ◦C/min, a strain of 0.1% and a frequency of 6.28 rad/s, selected from the viscoelastic region. The samples were then kept at 4 ◦C for 3 min and finally, they were reheated from 4 ◦C to 100 ◦C, at a constant rate of 2 ◦C/ minute, a strain of 0.1% and a frequency of 6.28 rad/s. A frequency sweep was also performed on all samples, except FA, at 4 ◦C, within the frequency sweep region of 0.01–100 rad/s, with a strain of 0.1% and a duration of 2 h.

3.3.2. Texture profile analysis (TPA) of β-glucans based hydrogels

Hardness, cohesiveness, springiness and chewiness were measured on an Instron model 34TM-5. A 40 mm diameter load cell was used, with a height of 20 mm, a clamping gap of 20 mm and a diameter and final height of 10 mm and 100 mm, respectively. The software used was Bluehill Universal version 4.25.

3.3.3. SAXS analysis of β-glucans based hydrogels

Nanostructural analysis with small-angle X-ray scattering experiments (SAXS) was carried out at the ALBA synchrotron light source (www.albasynchrotron.es) on the Non-crystalline diffraction beamline, BL-11. Hydrogels from all the extracts (AF, AFp, RAF and RAFp) were prepared at 3% concentration of β-glucan in water, following the same procedure as described in section 2.6. The samples were placed into 2 mm quartz capillaries (Hilgenberg GmbH, Germany), which were then sealed. The energy of the incident photons was 12.4 KeV or, equivalently, a wavelength, λ, of 1 Å. The SAXS diffraction patterns were collected using a photon counting detector, Pilatus 1 M, with an active area of 168.7 × 179.4 mm2, an effective pixel size of 172 × 172 μm2 and a dynamic range of 20 bits. The sample-detector distance was set to 6570 mm, resulting in a q-range with a maximum value of q = 0.2 Å 1. An exposure time of 10 s was selected based on preliminary trials. The data reduction was processed by the pyFAI python (ESRF) code (Kieffer & Ashiotis, 2014), modified by ALBA beamline staff, to make online azimuthal integrations from a previously calibrated file. Calibration files were created from a silver behenate (AgBh) standard. Intensity profiles were then plotted as a function of q using the IRENA macro suite (Ilavsky & Jemian, 2009) within the Igor software package (Wavemetrics, Lake Oswego, Oregon). The experimental data were fitted using a correlation length model. This model contains a first term, described by a power-law function, which accounts for the scattering from large clusters in the low q region and a second term, consisting of a Lorentzian function, which describes scattering from polymer chains in the high q region:

$$I(q) = \frac{A}{q^n} + \frac{C}{1+(q\xi_L)^m} + bkg \qquad (1)$$

where n is the power-law exponent, A is the power-law coefficient, m is the Lorentzian exponent, C is the Lorentzian coefficient and ξ_L is the correlation length for the polymer chains (which gives an indication of the gel's mesh size).

For the AF hydrogel, an additional term consisting in a Gaussian peak was added to the fitting function:

$$I(q) = \frac{A}{q^n} + \frac{C}{1+(q\xi_L)^m} + I_0 \cdot e^{-\frac{1}{2}\left(\frac{q-q_0}{B}\right)^2} + bkg \qquad (2)$$

The obtained values from the fitting coefficients are those that minimize the value of Chi-squared, which is defined as:

$$\chi^2 = \sum\left(\frac{y-y_i}{\sigma_i}\right)^2 \qquad (3)$$

where y is a fitted value for a given point, y_i is the measured data value for the point and σ_i is an estimate of the standard deviation for y_i. The curve fitting operation is carried out iteratively and for each iteration, the fitting coefficients are refined to minimize χ^2.

3.4. Statistical analyses

Statistical analysis of results was carried out using Statgraphics using one-way analysis of variance (ANOVA) to determine the significant differences between samples, at a significance level of $P < 0.05$.

4. Results and discussion

4.1. Compositional and structural characterization of the β-glucans rich fractions.

Compositional and structural analysis were firstly carried out on unpurified (AF and RAF) and purified (AFp and RAFp) aqueous fractions obtained from *Pleurotus ostreatus* and its residue (stipe). Table 1 summarizes the results from the compositional analysis. The extraction yield for the aqueous fraction from the discarded biomass was around ~27%, whereas a significant lower extraction yield (~17%) was obtained from the stipe, probably ascribed to its more recalcitrant nature. The β-glucan content was 42.7 and 35.4% for AF and RAF, respectively, and significantly increased after purification, reaching values of 47.6 and 55.5% for AFp and RAFp, respectively. It is worth nothing that the increase in β-glucan

content was significantly higher in the purified extract obtained from the stipes (RAFp) than in that obtained from the whole biomass (AFp), evidencing that the composition of unpurified extract from the stipes (RAF) was mostly carbohydrates.

Table 1. Yield and compositional characterization of each fraction. Polyphenols content is expressed as mg of gallic acid equivalents (GA)/g of sample. Yield, chitin, ash, protein, and glucan contents are expressed as g/100 g of dry weight. GalA (galacturonic acid), GlcA (glucuronic acid) and GlcN (*N*-acetyl-glucosamine).

Composition (Dry wt. %)	AF	AFp	RAF	RAFp
Yield	27.45±1.90^{a}	10.45±0.29^{b}	17.36±1.56^{c}	13.98±0.15^{bc}
Ash	9.70±0.64^{a}	3.80±0.24^{a}	22.37±0.09^{b}	7.46±0.10^{a}
Protein	22.97±0.53^{a}	16.53±0.91^{b}	7.84±0.77^{c}	9.26±0.16^{c}
Total polyphenol content (mg GA/g DW)	15.25±0.97^{a}	8.73±0.21^{b}	12.41±0.37^{c}	9.02±0.38^{b}
Carbohydrates (%) *	48.77±8.48^{a}	67.58±5.17^{bc}	49.33±3.85^{ab}	75.04±5.10^{c}
of which (g/100g fraction)				
Fucose**	< 0.1	0.47±0.01^{a}	0.32±0.03^{b}	0.42±0.03^{ab}
Rhamnose**	0.11±0.03^{a}	0.23±0.02^{b}	0.32±0.01^{b}	0.43±0.02^{c}
Arabinose**	0.15±0.03^{a}	0.42±0.03^{b}	0.25±0.04^{a}	0.87±0.01^{c}
Galactose**	1.82±0.09^{a}	6.71±0.42^{c}	3.87±0.09^{b}	6.03±0.07^{c}
Mannose**	1.13±0.07^{a}	5.56±0.44^{b}	2.84±0.11^{c}	3.83±0.08^{d}
Xylose**	0.56±0.03^{a}	0.66±0.06^{a}	0.80±0.04^{a}	0.75±0.18^{a}
GalA**	< 0.1	0.88±0.06^{a}	0.72±0.01^{a}	0.91±0.20^{a}
GlcA**	0.73±0.01^{a}	2.44±0.01^{b}	2.14±0.14^{b}	3.57±0.18^{c}
GlcN (chitin) **	0.64±0.09^{a}	0.90±0.13^{b}	2.16±0.11^{c}	1.32±0.14^{d}
Glucose **	43.57±8.08^{ab}	49.31±3.99^{ab}	35.90±3.27^{a}	56.91±4.19^{b}
of which (g/100 g fraction)				
β-glucans ***	42.70±3.27^{ab}	47.62±4.05^{bc}	35.42±0.56^{a}	55.48±1.42^{c}
α-glucans ***	0.87±0.53^{ab}	1.70±0.12^{a}	0.48±0.02^{b}	1.43±0.28^{ab}

Mean values ± standard deviation. Values with different letters in the same line are significantly different ($p \leq 0.05$).

* As the sum of all detected monosaccharide constituents. ** Monosaccharide composition determined by HPAEC-PAD. ***Measured with mushroom and yeast β-glucan assay kit.

A higher protein and α-glucan content were observed for aqueous fractions obtained from the discarded biomass than their counterparts obtained from stipes, which agrees with the results reported by (Gonz´alez et al., 2020). In contrast, a higher ash and chitin content was detected in those obtained from the stipes, in agreement with the compositional analysis of the raw materials (stipes *vs.* whole biomass, see Supplementary Material S1). As expected, the purification with ethanol had a significant impact on the composition of AFp and RAFp, ascribed to the removal of low molecular weight compounds such as mineral, polyphenols, peptides or even glucans with very low molecular weight (Xue et al., 2019). The higher proportion of water-soluble compounds coming from the whole biomass, which were lost after the purification step, may contribute to the sharp reduction in the AFp yield as compared to RAFp sample. The fungal cell wall is a complex and flexible structure composed of chitin, α- and β-glucans and glycosylated proteins. Proteins are generally associated with polysaccharides resulting in glycoproteins (Miro´nczuk-Chodakowska & Witkowska, 2020; Moreira et al., 2020). It is worth nothing that the protein content in RAFp was similar to its unpurified counterpart (RAF), suggesting that a certain protein fraction precipitated together with the polysaccharides, either due to their bonding (i.e. glycoproteins) or as a consequence of denaturalization and subsequent precipitation with ethanol. Table 1 also shows the concentrations of all detected monosaccharides units. The main monosaccharides detected, apart from glucose units from glucans, were mannose and galactose. These sugar units are derived from mannogalactans and mannoproteins usually found in mushrooms (Ruthes, Smiderle, & Iacomini, 2016). Specifically, mannogalactans are common in species of the *Pleurotus* genus (Baeva et al., 2019). The results revealed significant differences among samples. Extracts from stipes (RAF) were richer in carbohydrates, highlighting the contribution of fucose, xylose and GluA which point towards greater structural

complexity and branched β-glucans present in the stipes. These sugar units were predominantly enriched in purified samples, reaching values around 10–12%, thus evidencing that these heteropolysaccharides are part of medium and high molecular weight compounds or are tightly interacting with the glucans. Once again, purified extracts from the stipes (RAFp) were richer in GluA, fucose and rhamnose units. The linkage analysis of the purified fractions (AFp and RAFp) confirms that the major polysaccharide had a typical branched glucan structure with a (1 → 3)-Glcp backbone and (1 → 6, 1 → 3) side chains (Table 2). Notable differences are evidenced between the glucan structure of the stipe (RAFp) and of the whole fruiting body (AFp), with a substantial increase in branching points and (1 → 6)-Glcp side chains in the stipe. This higher structural complexity of the β-glucans in the stipe, also patent by NMR (Fig. S2), will have an impact on the functional and technological properties, as it will be detailed below.

Table 2. Linkage analysis of purified glucan fractions.

PMAA	Structural unit deduced	Relative abundance (mol %)	
		AFp	RAFp
2,3,4,6-Me$_4$-Glc*p*	Glc*p* (1 →	15.3	22.1
2,4,6-Me$_3$-Glc*p*	→ 3)- Glc*p*-(1 →	41.0	28.0
2,3,6-Me$_3$-Glc*p*	→ 4) Glc*p*-(1 →	4.7	3.7
2,3,4-Me$_3$-Glc*p*	→ 6) Glc*p*-(1 →	11.4	18.0
2,3,4-Me$_3$-Gal*p*	→ 6) Gal*p*-(1 →	2.6	7.1
2,6-Me$_2$-Glc*p*	→ 3,4) Hex*p*-(1 →	2.2	1.0
4,6-Me$_2$-Hex*p*	→ 2,3) Hex*p*-(1 →	3.0	1.6
2,3-Me$_2$-Glc*p*	→ 4,6)-Glc*p*-(1 →	0.8	0.7
2,4-Me$_2$-Glc*p*	→ 3,6)-Glc*p*-(1 →	14.0	20.5
3,4-Me$_2$-Gal*p*	→ 2,6) Gal*p*-(1 →	2.2	5.4

Hex*p*: unassigned hexopyranose linkage due to lack of suitable standards.

4.2. Functional properties of β-glucan-rich fractions

Due to the amount of bioactive compounds that can be extracted by aqueous treatments (Elhusseiny et al., 2021), the functionality of the different fractions obtained was evaluated by determining the antioxidant and antiviral activity, together with the polyphenol content. Table 3 displays the antioxidant capacity (μmol Trolox equivalents per gram of sample) and antiviral activity of the different extracts. When comparing purified and unpurified extracts, it was clearly seen that unpurified samples (AF and RAF samples) showed greatest polyphenol content (see Table 1) and antioxidant capacity, being accentuated in those obtained from the whole biomass. It is known that phenolic acids are the main responsible of the antioxidant activity of mushrooms (Nowacka-Jechalke, Olech & Nowak, 2018). However, β-glucans are also recognized as biologically active carbohydrates which possess antioxidant activity (Barbosa et al., 2020; Shin & Lee, 2014) and thus, could also contribute to the antioxidant activity. The sharp decrease in the polyphenol content and antioxidant activity observed after the purification step (RAFp, AFp), evidenced that less polar compounds such as terpenes and phenols, with known antioxidant activity, could be solubilized in ethanol and release from the samples during the purification process (Ruthes, Smiderle, & Iacomini, 2015). Furthermore, it is probably that non-bounded polar polyphenol compounds will not be able to precipitate with ethanol and rest in the supernatant.

Table 3. Infectivity effect of murine norovirus (MNV) after treatment with unpurified (AF and RAF) and purified fractions (AFp y RAFp) at a concentration of 5 mg/ mL; and antioxidant capacity (TEAC: trolox equivalent antioxidant capacity) of each fraction.

Sample	MNV		TEAC (µmol TE/g sample)
	Log $TCID_{50}$/mL	Reduction	
Control	5.20 ± 0.12[a]		
AF	5.45 ± 0.25[a]	−0.25	138.55 ± 1.20[a]
RAF	4.87 ± 0.33[a]	0.33	118.31 ± 1.20[b]
Control	4.45 ± 0.12[a]		
AFp	3.62 ± 0.07[a]	0.83	39.36 ± 0.97[c]
RAFp	<1.15[b]	>3.30	40.13 ± 0.97[c]

Mean values (standard deviation). Values with different letters in the same column are significantly different ($p \leq 0.05$).

Amongst the most interesting functional components of fungal cell walls are β-glucans. These compounds, homo- and heteroglucans with β (1–3) and β (1–6) glycosidic linkages, have been proven to have a number of properties related to the benefits of mushrooms, such as antibacterial, antiviral and immunomodulatory properties, amongst others (Han, Baruah, Cox, Vanrompay, & Bossier, 2020; Joo Seo & Choi, 2021, p. 350). In this work, the antiviral activity of the extracts was evaluated against a human norovirus surrogate in order to elucidate if the extract composition and structural complexity of β-glucans affected their functional properties. Table 3 compiles the results of antiviral activity of the different extracts. Overall, unpurified extracts did not show antiviral activity against MNV ($p > 0.05$), whereas RAFp extract significantly reduced MNV titers below the limit of detection. This points out that a greater structural polysaccharide heterogeneity had a positive effect on the antiviral properties with the additional

advantage that it comes from the stipes. As a result, the antiviral activity of the extracts was mainly ascribed to the quantity and type of polysaccharides and not to the polyphenol content and lower molecular weight compounds present in unpurified samples. Similarly, (Vetvicka et al., 2018) showed that highly purified β-glucans extracts from yeast had biocide activity.

4.3. Technological properties of β-glucans-based extracts.

The rheological behavior of the different β-glucan extracts was also evaluated in order to understand the role of extract composition and structure on the viscosity and gelling properties of the corresponding extracts. Complete flow curves presented in Fig. 1 show a shear-thinning behavior (pseudoplastic), which was accentuated in extracts obtained from the stipes and in purified samples. Similar results were obtained for purified β-glucan-based solutions obtained from *Pholiota nameko, Agaricus brasiliensis, Lentinula edodes* and *Dictyophora rubrovolvat* (da Silva Milhorini et al., 2022). The rheological data were accurately fitted to the Ostwald-de-Waale model and, the flow (n) and consistency (k) indexes together with the apparent viscosity (η) values at a shear rate of 250 s^{-1} are shown in Table 4. The lower viscosity found for AF solutions can be ascribed to the higher content of other water-soluble compounds (polyphenol, ashes and proteins) that did not contribute to the viscosity. Higher viscosity values and shear-thinning behavior were obtained in purified samples (RAFp and AFp) which can be ascribed to the removal of the lower molecular weight compounds (See Table 1) and the higher relative content of β-glucans. It is remarkable that AFp provided higher viscosity values probably due to the higher hydrodynamic radius and the presence of a higher amount of glycoproteins (as deduced from the higher protein content). Branched biopolymers are usually more compact than their linear counterparts at a given molecular weight (Shi, Li, Yin, & Nie, 2019). They will therefore exhibit lower intrinsic viscosities. These results confirm that the molecular structure of β-glucans significantly affects the steady-shear flow behavior of the solutions (Zhu,

Du, Bian, & Xu, 2015). Furthermore, (Abreu et al., 2019) pointed out that differences inside branch sizes provides different molecular interactions and consequently solutions with different viscosities.

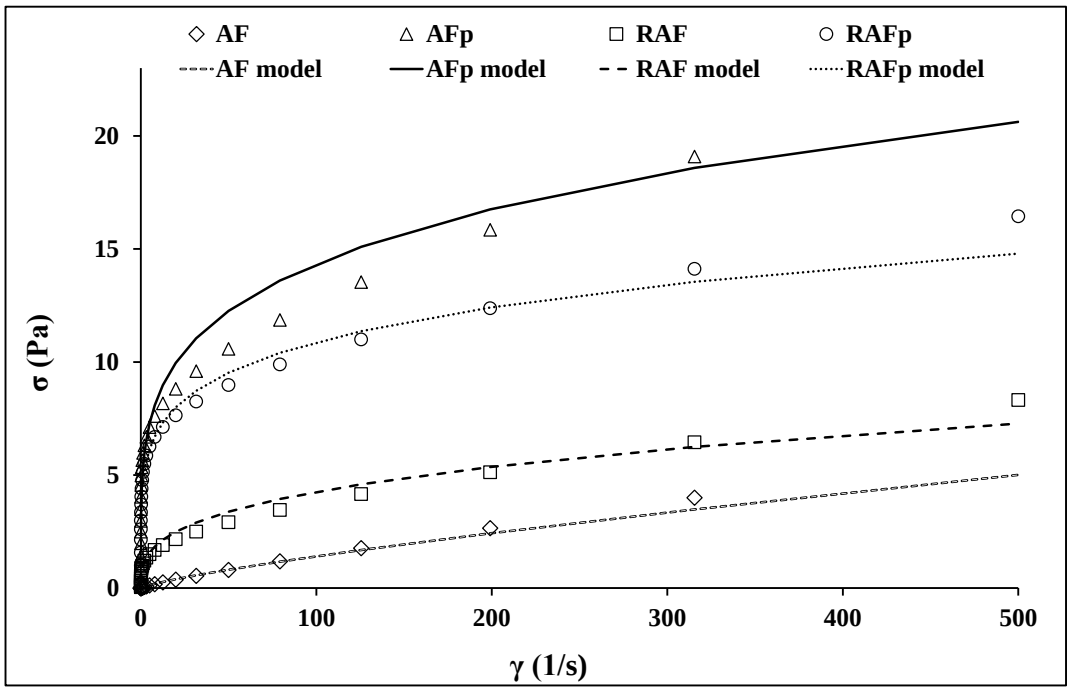


Figure 1. Rheological behavior of AF, AFp, RAF and RAFp solutions at 1% (w/v).

Table 4. Parameters of flow curves obtained by fitting the Ostwald–de Waele model for the different AF, AFp, RAF and RAFp solutions at 1% (w/v).

Sample	n*	k	r^2	η_{ap} (250 Pa.s)
AF	0.82	0.03	0.99	0.013
AFp	0.25	4.74	0.96	0.076
RAF	0.37	0.85	0.98	0.025
RAFp	0.26	3.80	0.97	0.065

*Depending on the value of the flow index (n), the fluid can be classified as being pseudoplastic (n < 1), Newtonian (n = 1), or dilatant (n > 1).

The effect of the composition of the extract in the gel properties was also investigated by performing dynamic oscillatory test. G' and G" were measured as a function of frequency (rad s^{-1}), at a fixed strain depending on previous amplitude sweep test (see Fig. 2). Hydrogels could not be formed with AF extracts probably ascribed to the high content of non-gelling compounds (such as polyphenols, proteins or peptides and proteins) which could interfere on the formation of a packed gelling network. As observed, all the hydrogels formed exhibited a storage modulus (G') higher than the loss modulus (G"), evidencing the solid-like characteristics. Nonetheless, very weak hydrogels were formed with unpurified extracts (RAF). Stronger hydrogels were formed with purified samples and there were not obvious differences between G' values of the hydrogels formed with AFp or RAFp. These results suggest that the gelation mechanism of β-glucan hydrogels depends on its composition and structure. It seems that AFp was able to form a gelling network with a more tightly packed structure, ascribed to the presence of less branched β-glucans, which agrees with SAXS results (as it will be detailed below). Furthermore, the higher protein content could also favour the formation of glycoproteins complexes which contribute to the gel structure (Rodríguez-Seoane, Torres Perez, Fernández de Ana, Sinde-Stompel, & Domínguez, 2022). In contrast, the presence of side chains in RAFp could act as reinforcement agents in hydrogels formed by means of intermolecular associations.

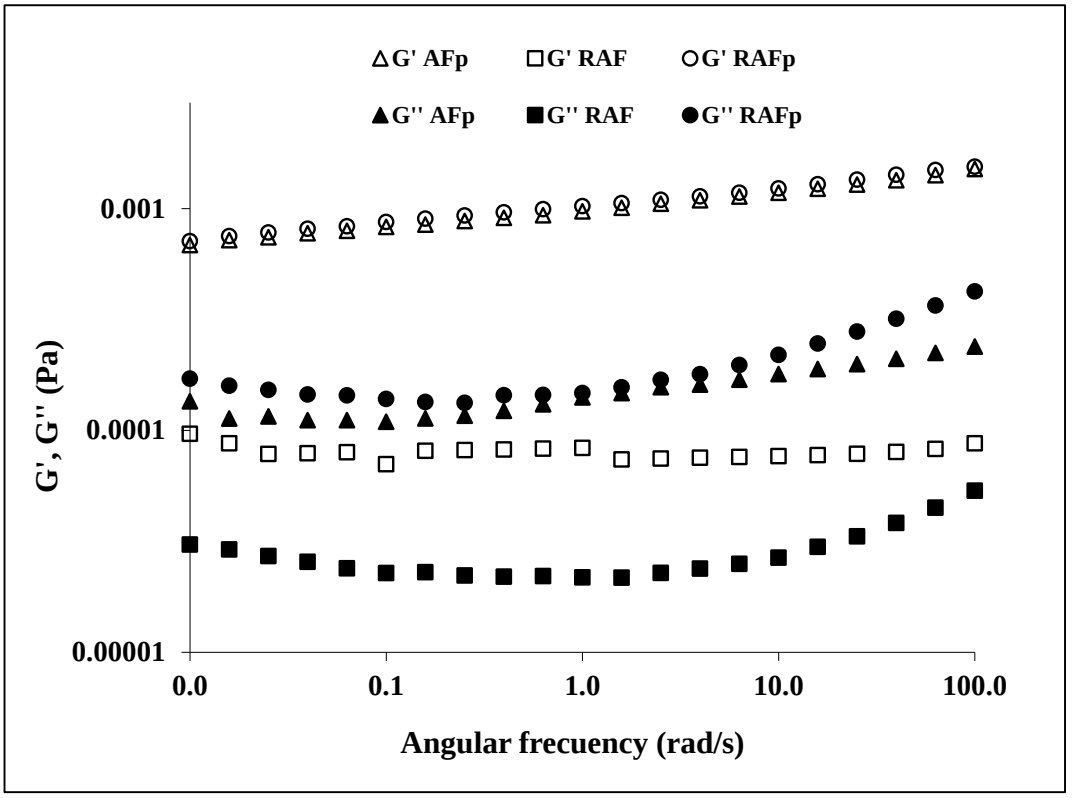


Figure 2. Dynamical oscillatory frequency sweep test curves of hydrogels from AFp, RAF and RAFp fractions. Open and filled symbols correspond to storage modulus (G') and loss modulus (G''), respectively.

4.4. Texture profile analysis.

Table 5 compiles hardness, chewiness, springiness and cohesiveness parameters of the developed hydrogels which directly affect the oral behavior of products (Wee, Goh, Stieger, & Forde, 2018). Specifically, hardness indicates the force required to compress a substance between the teeth. Springiness refers to how the gel structure is broken down by the initial compression. Cohesiveness is the degree of deformation after the first chewing and chewiness is the energy required to chew a solid or semi-solid food product to a state where it can be swallowed. As expected, purified samples displayed the maximum hardness, being higher in those obtained from the whole biomass, probably related to the higher viscosity of the

corresponding solutions. In contrast, there were not significant differences in springiness and the values of all of the hydrogels were close to 1, indicating that they were all highly elastic (Ge et al., 2018). The cohesiveness was lower for hydrogels formed with purified fractions, which is related with the higher hardness values and lower chewiness obtained for these samples.

Table 5. Texture profile analysis (TPA) of β-glucan-based hydrogels of gel-forming, unpurified (RAF) and purified (AFp and RAFp) samples.

Sample	Hardness (N)	Cohesiveness (N)	Springiness (N)	Chewiness (N)
AFp	2.87±0.27[a]	2.70±0.23[a]	1.00±0.00[a]	7.93±0.51[a]
RAF	1.93±0.31[b]	4.74±0.55[b]	1.00±0.00[a]	9.08±0.87[b]
RAFp	2.29±0.26[b]	3.16±0.41[a]	1.00±0.00[a]	7.05±0.44[a]

Mean values ± standard deviation. Values with different letters in the same column are significantly different ($p \leq 0.05$).

4.5. Nanostructural properties of the hydrogels

The nanostructure of the hydrogels (or the viscous suspension in the case of AF) was studied by means of SAXS and the obtained scattering patterns are shown in Fig. 3. As observed, no marked scattering features were detected for the hydrogel samples and the experimental data could be properly fitted to a simple correlation length model; however, a broad peak, centred at ca. 0.011 Å□ 1, corresponding to a real distance of 57 nm, was detected for the AF sample. This peak could be ascribed to the formation of polymeric clusters due to self-aggregation of the β-glucan chains, which would in turn hinder the capacity of the polysaccharide to form a gelling network (Gunness, Flanagan, Mata, Gilbert, & Gidley, 2016). Amongst the other samples, AFp showed the highest scattering intensity. This could not be due to a concentration effect, since neither the β-glucan concentration nor the total solid content of this sample were the highest. It seems that this particular extract was able to form a gelling network with a greater physical density, i.e. a more tightly packed structure, which is in agreement with the

rheological characterization (cf. Fig. 1). The fitting parameters, summarized in Table 6, evidenced the presence of rigid rod-like polymeric chains, as suggested by the Lorentzian exponents close to 1. The correlation length, i.e. the average distance between adjacent polymeric chains, ranged from 2 to 11 nm. The difference in this correlation length between samples must be related to their degree of branching and presence of side chains. Interestingly, while this distance increased in the case of the AFp as compared to AF, the opposite trend was observed in the RAFp and RAF samples. With regards to the power-law exponents, associated to the scattering from polymeric chain clusters, the values ranged between 2.4 and 3.0, thus indicating the presence of mass fractal structures at the higher size range. The greater exponents of the RAFp and RAF samples are indicative of a greater degree of branching, leading to the formation of more "clustered" network, as depicted in Fig. 4.

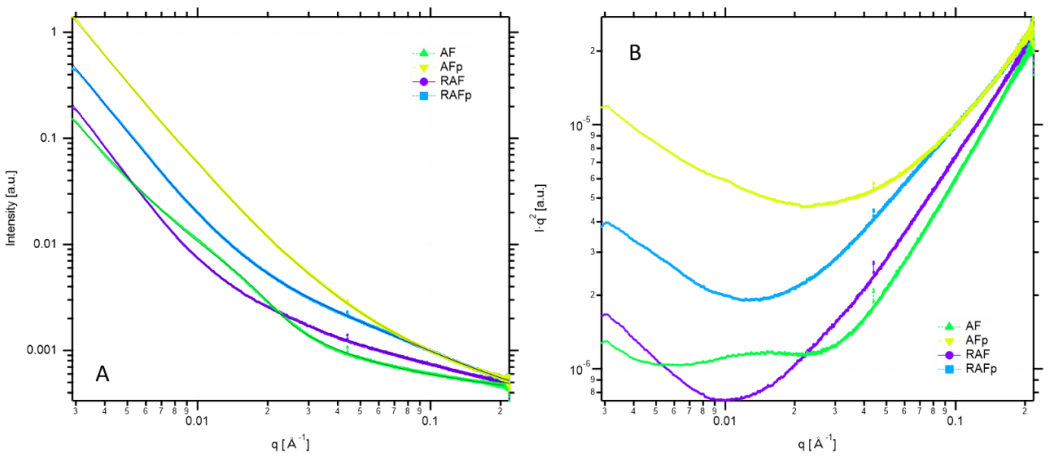


Figure 3. (A) SAXS patterns and (B) corresponding Kratky plots from the β-glucan-based hydrogels or aqueous suspensions. Markers represent the experimental data and solid lines show the fits obtained using the corresponding fitting models.

Table 5. Parameters obtained from the fits of the SAXS data. (*n*: power-law exponent, *m*: Lorentzian exponent, ξ: correlation length, d: real distance corresponding to the Gaussian peak).

Sample	n	m	ξ (nm)	d (nm)
AF	2.4	1.2	2.2	57
AFp	2.6	1.2	11.2	---
RAF	3.0	1.0	8.8	---
RAFp	2.7	1.5	2.5	---

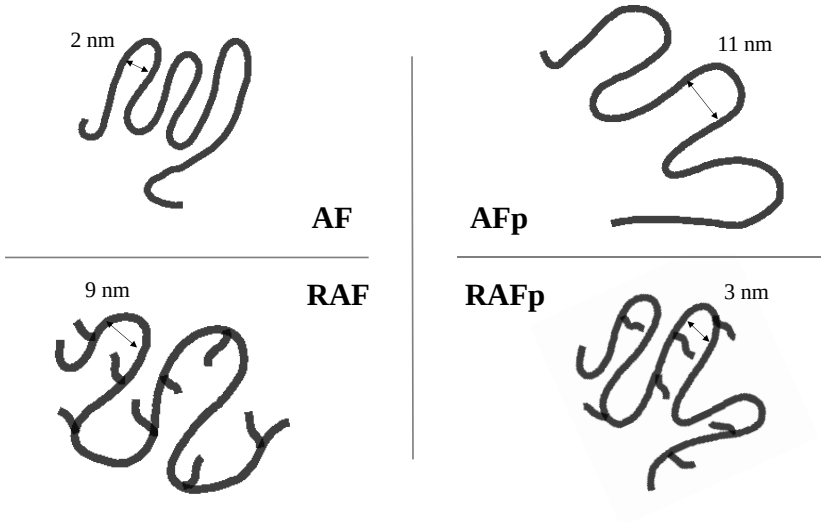


Figure 4. Schematic representation of the nanostructure proposed for the suspension (AF) and the different hydrogels (AFp, RAF and RAFp).

5. Conclusions

In this work, compositional analysis, functional and technological properties of aqueous β-glucans fractions obtained from discarded *P. ostreatus* biomass and its stipes were characterized, before and after a purification step to enrich its content in β-glucans. The purification process increased the β-glucan content (greater in those obtained from the stipes), which resulted in a greater viscosity and elastic

behavior and hardness of their corresponding gels. These improvements were clearly increased in the sample generated from the residue (RAFp). Furthermore, RAFp had a strong antiviral activity against murine norovirus, which was related to the structural complexity of β-glucans. These results evidence that the structure and composition of β-glucan fractions will determine their techno-functionality and, highlight the enormous potential of this stipe as a source of novel food ingredients combining their good technological (texturizing and gelling capacity) and functional (antioxidant and antiviral activities) properties.

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Supplementary Material

Table S1. Compositional analysis of discarded biomass and the stipes.

Composition (Dry wt. %)	*P. ostreatus*	*P. ostreatus* Residue
Lipid	5.35±0.39	7.97±1.07
Ash	7.82±0.01	9.72±0.39
Protein	24.05±1.09	9.36±0.34
Total polyphenol content (mg GA/g DW)	20.11±1.00	10.21±0.78
Carbohydrates (%) *	51.79±3.15	63.57±2.25
of which (g/100g fraction)		
Fucose**	< 0.1	< 0.1
Rhamnose**	0.10±0.02	0.20±0.03
Arabinose**	0.05±0.01	0.22±0.01
Galactose**	1.34±0.06	1.59±0.04
Mannose**	0.80±0.11	0.80±0.03
Xylose**	1.10±0.05	1.34±0.15
GalA**	0.16±0.06	< 0.1
GlcA**	1.50±0.16	2.21±0.18
GlcN (chitin) **	0.31±0.02	0.32±0.03
Glucose **	46.43±2.66	56.89±1.78
of which (g/100 g fraction)		
β-glucans ***	45.97±2.71	56.56±1.82
α-glucans ***	0.46±0.05	0.33±0.04

NMR assays were performed to try to elucidate the main structural differences of the glucans found in the AFp and RAFp fractions using the ^{1}H proton signal. The largest number of peaks of interest were found within the range of 3 to 4.5 ppm and are shown in figure S2. As it is clearly seen, the samples were very heterogeneous even after the purification step, so these results were used as a support of the other analysis carried out in the work. The main signals with the highest relative intensity for RAFp were located at a δ 3.40 which correspond to (→3)-β-D-Glcp-(1→) on H3, or (→6)-β-D-Glcp-(1→ end group at H4). Furthermore, peaks centered at δ 3.81 ascribed to β-D-Glcp-(1→), at δ 4.12 and δ

4.16 related to (→3,6)-α-D-Glcp-(1→) backbone and at δ 4.43, δ 4.46 ascribed to (→3)-β-D-Glcp-(1→) end group (da Silva Milhorini et al., 2022; Sovrani et al., 2017), were also higher in RAFp samples. In the case of AFp sample, the highest relative intensity was centered at δ 3.73 and δ 3.77 related to (→6)-α-D-Glc*p*-(1→) backbone (Castro-Alves et al., 2017; Morales et al., 2020).

Therefore, even though both α- and β-glucans were found in both samples, some differences in the structure of β-glucans were evidenced by NMR. Specifically, AFp showed a higher content in α-glucans. In contrast, an increase in the relative intensity of the peak located 3.40 ppm was observed in the RAFp sample, evidencing a higher content of β (1-3) and β (1-6) glycosidic linkages, suggesting a higher branching degree.

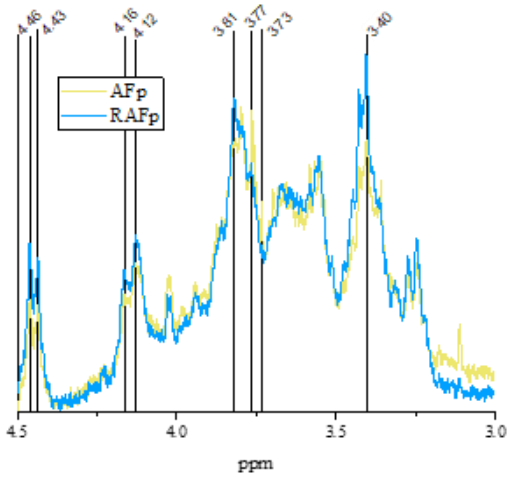
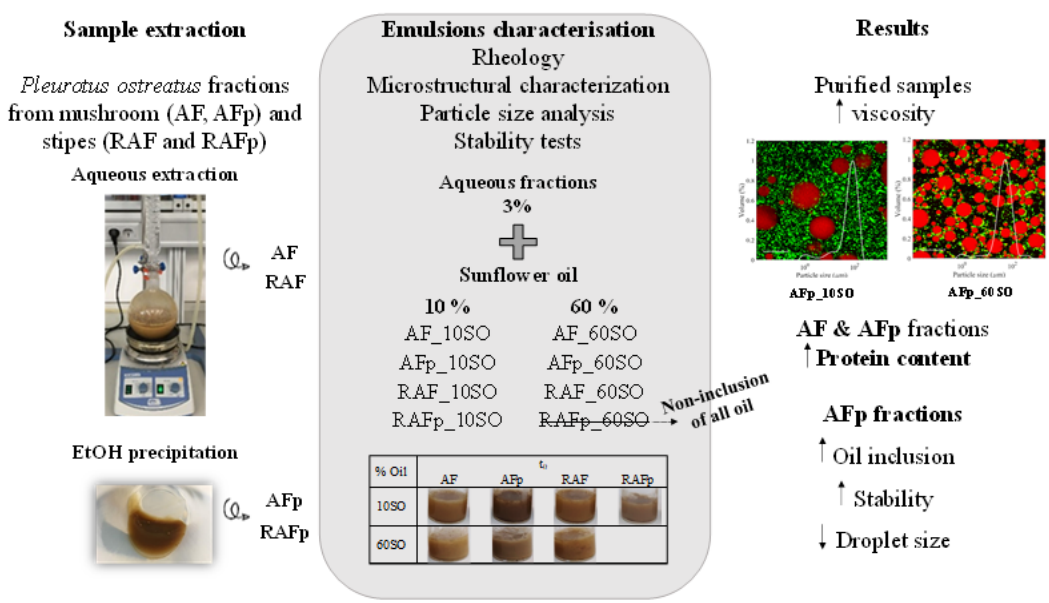


Figure S2. ^{1}H NMR spectra of purified samples from the discarded biomass (AFp) and stipes (RAFp) in the range of 3-4.5 ppm.

2.2. Emulsifying properties of β-glucan-rich extracts obtained from *Pleurotus ostreatus* mushroom and stipes.



This section is an adapted version of the following **submitted** research article:

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"Emulsifying properties of β-glucan-rich extracts obtained from *Pleurotus ostreatus* mushroom and stipes."

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Abstract

In this work, the effect of the composition and structure of different aqueous extracts obtained from *Pleurotus ostreatus* (whole biomass aqueous fraction (-AF) and purified aqueous fractions (-AFp), and from stipes, residue aqueous fraction (-RAF) and purified residue aqueous fractions (-RAFp)), on the physical stability, rheological properties and microstructure of oil-in-water (O/W) emulsions was investigated. For this purpose, two types of emulsions were made, at minimum and maximum oil concentration (10 and 60%, respectively) looking for the extremes in their potential food application. Better emulsifying properties were obtained for AFp-based emulsions (being able to incorporate greater amounts of oil phase), ascribed to the relatively high protein content (acting at the O/W interphase) and high viscosity of the continuous phase which slowed down the oil droplet movement. The lower emulsifying properties of β-glucan extracts obtained from the stipes was ascribed to the lower content of proteins, which were less accessible to act as surface-active compounds due to the greater glucan structural complexity with which they are interacting. Freeze-thaw treatments gave a much more negative effect on emulsion stability than heat treatments. AFp emulsions prepared with the highest oil content were the most stable under sterilization process ascribed to the relatively smaller droplet size and higher viscosity values.

1. Introduction

Food emulsions are colloidal dispersions of liquid droplets in another non-miscible continuous liquid phase with limited stability, and will, over time, separate out into their oil and aqueous phases. Surface-active biomolecules such as low molecular weight emulsifiers, proteins and some hydrocolloids are used regularly to create dispersed emulsions, through their ability to adsorb onto the oil surface droplets and reduce the interfacial tension, thus reducing or preventing coalescence, flocculation and Ostwald ripening. The major challenge for emulsifying and stabilizing food emulsions is the selection of the emulsifying agent. Natural

emulsifiers such as proteins and fibres are preferred from synthetic additives for both health and sustainability. Emulsifiers are sometimes used not only for texture modification towards greater consumer acceptability but are also necessary to ensure the stability and shelf-life of food products containing them (Castel, Rubiolo & Carrara, 2017). Among the different types of emulsifiers used, biopolymers have received great attention in recent years due to the growing interest in the use of natural and sustainable food ingredients and some of the most studied natural macromolecules have been β-glucans, vegetable proteins or pectin (Ashraf Khan, Gani, Masoodi, Mushtaq & Silotry Naik, 2017; Gallotti, Turchiuli, & Lavelli, 2022; Umaña, Turchiuli, Eim, Rosselló & Simal, 2021). Furthermore, with the aim of reducing the dependence on animal proteins together with the circular economy strategies, many researchers are focused on the valorisation of agrifood waste to look for alternative emulsifiers and stabilizers (i.e. extracted from plants, cereals or mushroom-based alternatives, for instance), which also have their own technical and scientific challenges.

The rapid development of edible mushroom industry has also resulted in the generation of great amount of mushroom waste each year (Wan Mahari et al., 2020). Mushroom by-products, including misshapen mushroom and mushroom stem whose size or shape does not meet commercial standards, although it is still edible, contain significant amounts of polysaccharides and proteins that can be extracted for food applications. Specifically, mushroom β-glucans have received increased attention from scientist and product manufacturers, and many researches are focusing on efficient extraction methods of these polysaccharides (Leong, Yang & Chang, 2021). β-glucans are long- or short-chain polymers formed by glucose units via β-1,3 linkages (forming linear structures) with β-1,6 branching. Their structure may vary depending on the mushroom species, their chain length, branching degree and interactions with other cell wall components which will directly affect their molecular weight and water solubility (Du, Meenu, Liu & Xu, 2019). They present very interesting properties not only in terms of bioactivity, but

also for technological applications in the food industry, such as moisture retention, foam formation, gel formation or emulsifying capacity (Zhu, Du & Xu, 2016). Therefore, easily extractable water-soluble β-glucans obtained from one of the most cultivated mushrooms (*Pleurotus ostreatus*) could be an eco-sustainable option for the production of natural emulsifiers. It is hypothesized that the structure and composition of β-glucan-rich extracts will depend on the source (stipes or whole biomass of *P. ostreatus*) and extraction conditions (more or less purified samples) and, thus, these aspects will also affect their emulsifying properties. The composition of β-glucan rich extracts and the presence of proteins or other minor compounds could play an important role on the emulsifying properties of the obtained extracts. Thus, this work was aimed at evaluating the effect of extract composition and β-glucan properties on their emulsifying capacity.

2. Methods and materials

2.1. Materials

The starting raw material used to obtain the aqueous fractions for the different emulsions was *Pleurotus ostreatus* mushroom biomass (*P. ostreatus*) and the residue generated by the mushroom stipes (*P. ostreatus* residue), cultivated by Centro Tecnológico de Investigación del Champiñón –Asochamp- (Rioja, Spain). Sunflower oil (SO) was obtained from a local supermarket and used as the lipid phase. Ethanol (96 % (v/v)) was purchased from Panreac Applichem and used for the purification of β-glucans aqueous fractions. For the characterisation of monosaccharides, trifluoroacetic acid (TFA), the standards (fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid) were purchased from Sigma-Aldrich. Other reagents used, Folin-Ciocalteu's reagent, sodium carbonate, phosphate buffered saline (PBS), potassium persulphate, gallic acid, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

(ABTS) and 6-hydroxy-2. 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were also obtained from Sigma-Aldrich. An enzyme kit for the determination of yeast and fungal β-glucan (K-YBGL 09/2009), and β-glucan from yeast (49%) were both obtained from Megazyme.

2.2. Aqueous extractions and ethanol precipitation

P. ostreatus mushrooms and its residues (stipes) were used as starting material to obtain β-glucans aqueous fractions. This raw material was initially freeze-dried, ground and then, sieved (500 μm), prior to aqueous extractions, as previously reported by (Pérez-Bassart, Fabra, Martínez-Abad & López-Rubio, 2023). Briefly, 25 grams of each biomass (whole biomass or stipes) were dispersed in 300 mL of distilled water and left under reflux extraction at boiling temperature for 7 hours. The samples were then centrifuged for 10 minutes at 8000 rpm in an Avanti J-26 XPI centrifuge with a JLA-16,250 rotor and, the supernatant was concentrated using a Heidolph rotavapor apparatus and subsequently lyophilized to obtain the aqueous fractions of the *P. ostreatus* whole biomass (AF), and of the *P. ostreatus* residue (RAF). The purified fractions were obtained by adding three times the volume of the aqueous sample (after aqueous extraction and before evaporation), in 96% (v/v) ethanol, keeping the mixture under magnetic stirring for 1 hour. Then, the samples were centrifuged and the precipitate was recovered and left to air dry overnight to remove the solvent. Finally, the samples were freeze-dried, thus obtaining the purified fractions for the whole biomass (AFp) and for the stipes (RAFp).

2.3. Proximate analysis

As mushrooms have two sources of nitrogen, protein and chitin, the protein content was determined by the difference between the total elemental nitrogen and that obtained from chitin, using a correction factor of 6.25. Total nitrogen was determined using a Rapid Surplus elemental nitrogen analyser (rapid surplus N

analyser) based on the modified Dumas method. Chitin nitrogen was calculated after acid hydrolysis to glucosamine (GlcN) with HCl, as described in (Pérez-Bassart et al., 2023). Monosaccharide content was determined by acid hydrolysis with trifluoroacetic acid (TFA) as in (Pérez-Bassart et al., 2023), and were separated by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using glucose, fucose, rhamnose, arabinose, galactose, mannose, xylose, GalA and GlcN as standards for calibration. The determination of β-glucans was calculated by the difference between the total glucose content after TFA hydrolysis and the glucose content of the α-glucans determined using an enzyme kit (Cat. No. K-YBGL) with the enzymes amyloglucosidase plus invertase (Megazyme, Ireland). The chemical structure of b-glucans has been illustrated in Figure S1 of the Supplementary Material. Finally, the ash content was determined according to the standard TAPPI T211 method. All experiments were performed in triplicate.

2.4. Preparation of fraction solutions and emulsions

β-glucans aqueous solutions (3% (w/v)) were prepared by dissolving the required amount of powdered freeze-dried samples of the extracted fractions (AF, AFp, RAF or RAFp) into distilled water at 80°C for 30 minutes. The concentration of purified and unpurified fractions were fixed based on literature (Banerjee et al., 2020; Choe et al., 2018; Umaña et al., 2021) and screening studies carried out to obtain the corresponding stable emulsions. Then, oil-in-water emulsions were obtained by incorporating 10 or 60 % (v/v) of sunflower oil (SO) into the aqueous solutions using a high-speed homogenization equipment (Ultra-Turrax, MICCRA D-9 Homogenizer, IKA, Müllheim, Germany) (UT) at 10000 rpm for 2 min and one drop of oil per second, obtaining samples of 10 mL final volume for each fraction and carrying out the mixing immediately with the aqueous fraction still hot. The SO concentration was established taking into account the highest amount of the oil fraction that did not affect the stability of the emulsions. Samples'

nomenclature was x_ySO where ‘x’ refers to the aqueous fraction (AF, AFp, RAF or RAFp) and ‘y’ indicates the concentration of SO used.

2.5. Rheology

The rheological behaviour of the emulsions and the corresponding solutions was analysed, in triplicate, using a rheometer HR20, (TA Instruments, Montreal, QC, Canada) with a 40 mm parallel plate geometry and a gap of 0.5 mm. Samples were left to rest for 3 min before the measurements were taken. The shear stress (σ) was obtained as a function of shear rate (γ) between 0 and 200 s^{-1}. The power law model Eq. (1) was applied to determine consistency index (k) and flow behaviour index (n). Apparent viscosities were determined at 100 s^{-1}.

$$\sigma = K \gamma^{n} \quad \textbf{(1)}$$

2.6. Emulsion microstructural characterization

The microstructure of the o/w emulsions was studied using laser scanning confocal microscopy following the method described by (Mendez et al., 2021). Fluorescence images of the different emulsions were processed using a FV 1000-IX81 laser scanning confocal microscope (CLSM, Olympus, Japan) with excitation wavelengths of 559 nm and 635 nm and with emission wavelength of 572 nm and 647 nm for Nile red and fast green colouring agents, respectively. For staining of the samples, the oil was stained prior to emulsion formation by adding 0.01% (w/v) Nile red and then, once the emulsion was formed, 0.01% (w/v) fast green was added to stain the proteins in the system. Images were analysed and processed using FV10-ASW Version 4.02.03.06 (Olympus Corporation, Tokyo, Japan).

2.7. Emulsion particle size analysis

The size distribution and volume weighted mean diameter ($D_{4,3}$) of the SO droplets in the different emulsions were determined, in triplicate, using a laser scattering instrument (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK).

These measurements were carried out on freshly prepared samples (10 mL) and after 7 days of storage under refrigerated conditions (4°C). The droplet size distribution was determined based on the best fit between the experimental measurement and the theory of Mie (McClements, 2005). All experiments were performed in triplicate.

2.8. Stability tests of emulsions prepared with β-glucans rich extracts

2.8.1. Storage stability

Emulsion creaming stability was determined using the method described by (Gao & Wu, 2023). To this end, 10 mL of the freshly prepared emulsions were put into cylindrical glass tubes with a tightly closed plastic cap to prevent evaporation. Samples were stored under refrigerated conditions (4 ºC ± 0.5 °C) for 7 days. The height of the upper cream layer (H_S) and the total height of the emulsion (H_E) were measured and, the creaming index (CI %) was expressed as the ratio of the height of the serum layer over the total height of emulsion (Koocheki, Kadkhodaee, Mortazavi, Shahidi & Taherian, 2009).

2.8.2. Freeze-thaw stability

The freeze-thaw stability was measured using the method reported by (Gao & Wu, 2023). The different emulsion samples (10 mL) were incubated at -20 ºC in a freezer for 24 h followed by 2 h incubation in water bath at 40 °C. The freeze-thaw cycle was repeated a total of 3 times and the effect on the creaming index (CI) was measured.

2.8.3. Heat stability

The heat stability of the emulsions was also measured as in (Liang et al., 2017). Emulsion samples (10 mL) were made into glass tubes as in the other stability assays, then the sealed tubes were put in the autoclave under 121 °C during 20 min, to mimic a sterilisation treatment. After thermal sterilization, the emulsions

were immediately cooled down to 25 °C and the effect on the CI was measured. All experiments were performed in triplicate.

2.9. Statistical analyses

Statistical analysis of the results was carried out using Statgraphics Centurion 18.1.13 and one-way analysis of variance (ANOVA), then a LSD Fisher test was applied to determine the significant differences between samples (n= 3) at a significance level of $P \leq 0.05$.

3. Results and discussion

The four samples used in this work have been previously characterized and the most significant characteristics related to their emulsifying properties are reproduced in **Table 1** (Pérez-Bassart et al., 2023). Briefly, a higher protein and α-glucan content were observed for aqueous fractions obtained from the discarded biomass (AF and AFp) than their counterparts obtained from stipes (RAF and RAFp). The β-glucan content was increased in purified samples, reaching values of 47.6 and 55.5 % for AFp and RAFp, respectively. Furthermore, low molecular weight compounds such as minerals, polyphenols, some proteins and peptides or even glucans or other carbohydrates with very low molecular weight were removed after the purification step. Interestingly, the protein content in RAFp was similar to its unpurified counterpart (RAF), evidencing that a certain protein fraction precipitated together with the polysaccharides (glycoproteins). The monosaccharide composition revealed that the structural complexity of β-glucans was higher in those from the stipes, which was also confirmed by NMR (Pérez-Bassart et al., 2023). The stipes, showed a greater abundance of β-1,3 and β-1,6 linkages which are directly related to longer chains and a greater degree of branching. In order to comparatively evaluate the emulsification capacity of each fraction, oil-in-water emulsions were prepared using a fixed total solid

concentration of 3% (w/v) and two different sunflower oil (SO) concentrations (10 and 60% (v/v)) and an in-depth analysis was carried out for the O/W emulsions.

Table 1. Compositional characterization of each fraction (adapted from Pérez-Bassart et al., 2023).

Composition (Dry wt. %)	**AF**	**AFp**	**RAF**	**RAFp**
Ash	9.70±0.64^{a}	3.80±0.24^{b}	22.37±0.09^{c}	7.46±0.10^{a}
Protein	22.97±0.53^{a}	16.53±0.91^{b}	7.84±0.77^{c}	9.26±0.16^{c}
Carbohydrates (%) *	48.77±8.48^{a}	67.58±5.17^{bc}	49.33±3.85^{ab}	75.04±5.10^{c}
of which (g/100g fraction)				
Fucose**	< 0.1	0.47±0.01^{a}	0.32±0.03^{b}	0.42±0.03^{ab}
Rhamnose**	0.11±0.03^{a}	0.23±0.02^{b}	0.32±0.01^{b}	0.43±0.02^{c}
Arabinose**	0.15±0.03^{a}	0.42±0.03^{b}	0.25±0.04^{a}	0.87±0.01^{c}
Galactose**	1.82±0.09^{a}	6.71±0.42^{c}	3.87±0.09^{b}	6.03±0.07^{c}
Mannose**	1.13±0.07^{a}	5.56±0.44^{b}	2.84±0.11^{c}	3.83±0.08^{d}
Xylose**	0.56±0.03^{a}	0.66±0.06^{a}	0.80±0.04^{a}	0.75±0.18^{a}
GalA**	< 0.1	0.88±0.06^{a}	0.72±0.01^{a}	0.91±0.20^{a}
GlcA**	0.73±0.01^{a}	2.44±0.01^{b}	2.14±0.14^{b}	3.57±0.18^{c}
GlcN (chitin) **	0.64±0.09^{a}	0.90±0.13^{b}	2.16±0.11^{c}	1.32±0.14^{d}
β-glucans ***	42.70±3.27^{ab}	47.62±4.05^{bc}	35.42±0.56^{a}	55.48±1.42^{c}

n=3, Mean values ± standard deviation. Means in the same row with different superscripts are significantly different (P≤0.05). AF and RAF are aqueous fractions obtained from the whole biomass and from the stipes, respectively, and p refers to purified extracts.

* As the sum of all detected monosaccharide constituents

** Monosaccharide composition determined by HPAEC-PAD.

***Measured with mushroom and yeast β-glucan assay kit

3.1. Oil droplet size and emulsion stability

Instability of O/W emulsions such as creaming, coalescence, flocculation is promoted as the droplet size of emulsions gradually increases. Emulsification to a smaller oil droplet size tends to decrease creaming and improve stability. Therefore, the droplet size of emulsions is an important indicator in order to evaluate the emulsion stability and it was determined on the different β-glucan

based emulsions using integrated light scattering (Table 2). As observed in **Figure 1**, a thicker consistency was observed for the ones prepared with purified β-glucan fractions, being even higher in those obtained from AFp. This is consistent with the higher relative content of β-glucan in purified samples and the different β-glucan structural properties. Furthermore, the higher consistency observed in AFp samples could be ascribed to the more homogenous distribution of the oil droplets in the continuous phase and the smaller particle size found in this case. Furthermore, the structural complexity of β-glucans and the composition of the extracts will also influence texture and viscosity of the resulting emulsions, as it was previously reported by Pérez-Bassart et al., 2023, and thus will affect their stability, as it will be detailed below. In fact, it is worth mentioning that stable RAFp emulsions could not be obtained for the greater oil content (60% (v/v)) since a slight oil phase separation was observed in freshly prepared samples.

The stability of the emulsions was evaluated during storage for 7 days at 4 ºC. The first thing to highlight is that the stability of refrigerated emulsions depended not only on the composition of the extracts but also on the structural complexity of β-glucan. After 7 days of refrigerated storage, no phase separation was observed in AFp samples (see **Figure 1** and **Table 2**), whereas the emulsions prepared with AF and 60% (v/v) SO (AF_60SO) displayed a slight phase separation indicating that this was the least stable emulsion obtained among AF and AFp samples. This fact was further corroborated by a significant increase in the average oil droplet size. In contrast, unpurified RAF extracts led to enhanced emulsion stability compared to their counterparts prepared with RAFp, as no phase separation was observed during refrigerated storage. Since the protein content was similar in RAF and RAFp extracts, the difference in the emulsion stability can be ascribed to the lower particle size of SO droplets in RAF-10SO as compared to its counterpart RAFp-10SO. Interestingly, the mean particle size of the oil droplets of the most stable AFp emulsions was smaller than their counterparts prepared with AF, and did not

increase after the refrigerated storage. However, the average droplet size of emulsions prepared with RAF increased after the 7 days of storage, despite having a negligible creaming index. This suggested different forms of stabilisation of the different β-glucan extracts. As already mentioned, β-glucans possess high potential for being used as stabilizers but the mechanism of stabilization can be ascribed to some structural features of the polysaccharides as well as the composition of the β-glucans-rich extracts. As observed in the work of (Pérez-Bassart et al., 2024), the structures found in the aqueous fractions of the residue suggested containing a more complex type of β-glucan, with more branches than those found in the whole mushroom.

Table 2. Average oil (SO) droplet size of the emulsions $D_{4,3}$ (μm) measured for freshly prepared samples (t_0) and after 7 days (t_f) of storage at 4 °C. Creaming Index (CI%) after refrigerated storage.

Sample	Particle size $D_{4,3}$ (μm)		CI
	t_0	t_f	%
AF_10SO	76.28±0.10^{ab}	157.87±30.00^{a}*	-
AF_60SO	109.03±2.60^{e}	139.29±10.66^{a}	12.23±1.60^{a}
AFp_10SO	71.56±2.23^{ac}	76.15±4.15^{b}	-
AFp_60SO	55.85±9.48^{c}	56.45±5.38^{b}	-
RAF_10SO	59.41±0.70^{c}	145.22±14.27^{a}*	-
RAF_60SO	88.68±8.21^{bd}	135.82±28.65^{a}*	-
RAFp_10SO	96.79±2.34^{de}	328.25±52.14^{c}*	9.85±2.81^{a}

Values are mean ± standard deviation. Means in the same column with different superscripts are significantly different (P≤0.05). (*) indicates significant differences (P≤0.05) in the same row (related to storage time). (-) indicates the absence or such a small value that it could not be quantified.

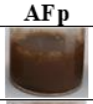
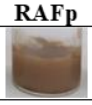
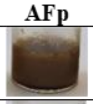
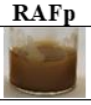
SO (%)	t_0				t_f			
	AF	AFp	RAF	RAFp	AF	AFp	RAF	RAFp
10								
60								

Figure 1. Macroscopic images of the different freshly prepared emulsions (t_0) and after 7 days (t_f) of storage at 4 °C. The RAFp sample did not emulsify 60% oil. AF and RAF are aqueous fractions obtained from the whole biomass and from the stipes, respectively, and p indicates the purified extracts.

3.2. Rheological properties

The rheological behaviour of the emulsions depends on the characteristics of their composition and interactions between the emulsion components, thus providing valuable information about the structure and intermolecular interactions within emulsions. Complete flow curves of aqueous β-glucans solutions and their corresponding O/W emulsions are shown in **Figure 3**. The obtained curves were fitted to the Ostwald de Waele model, since it is the most widely employed model for non-Newtonian fluids, and the flow (n) and consistency (k) indexes, together with the apparent viscosity (η_{ap}) values at a shear rate of 100 s^{-1} are summarized in **Table 3**. All samples (both solutions and emulsions) exhibited a shear thinning (pseudoplastic) behaviour, as previously reported for other β-glucans (Aljewicz, Mulet-Cabero & Wilde, 2021; Wang, Yin, Huang & Nie, 2020). As observed in **Figure 2** and **Table 3**, the addition of a greater SO content (60% (v/v)) made the emulsions more viscous and more shear thinning that their counterpart solutions, whereas no significant differences were observed between β-glucans solutions and those containing the lowest SO concentration, in agreement with the visual appearance (see **Fig. 1**). In general, emulsions prepared with purified samples (AFp and RAFp) were more viscous and showed a much higher flow consistency index (k) than their counterparts prepared with AF and RAF, probably ascribed to

the higher molecular weight of the corresponding β-glucans and the removal of low molecular weight compounds.

Interestingly, both extracts obtained from the stipes provided more viscous emulsions than those from the whole biomass (AF and AFp). Nonetheless, although an increased viscosity of the continuous phase will contribute to emulsion stability, this seems not to be the determining factor for the stabilization of AFp samples, these having lower viscosity and consistency index than RAFp but greater emulsion stability. The higher stability of AFp emulsions was ascribed to the higher content of accessible protein able to adsorb at the O/W interphase as it will be detailed below.

Table 3. Flow behavior index (n), consistency index (k) and apparent viscosity (η_{ap}) of fraction solutions and oil-in-water emulsions.

Emulsion(*)	Ostwald - de Waele parameters			
	n	k	r^2	η_{ap} (100 Pa.s)
AF_10SO	0.65	0.20	1.00	0.04
AF_60SO	0.40	2.71	0.99	0.17
AFp_10SO	0.53	1.39	0.99	0.16
AFp_60SO	0.35	15.92	1.00	0.79
RAF_10SO	0.53	1.41	0.99	0.16
RAF_60SO	0.57	1.74	0.98	0.24
RAFp_10SO	0.47	3.69	1.00	0.32

(*) AF and RAF are aqueous fractions obtained from the whole biomass and from the stipes, respectively, and p indicates the purified extracts

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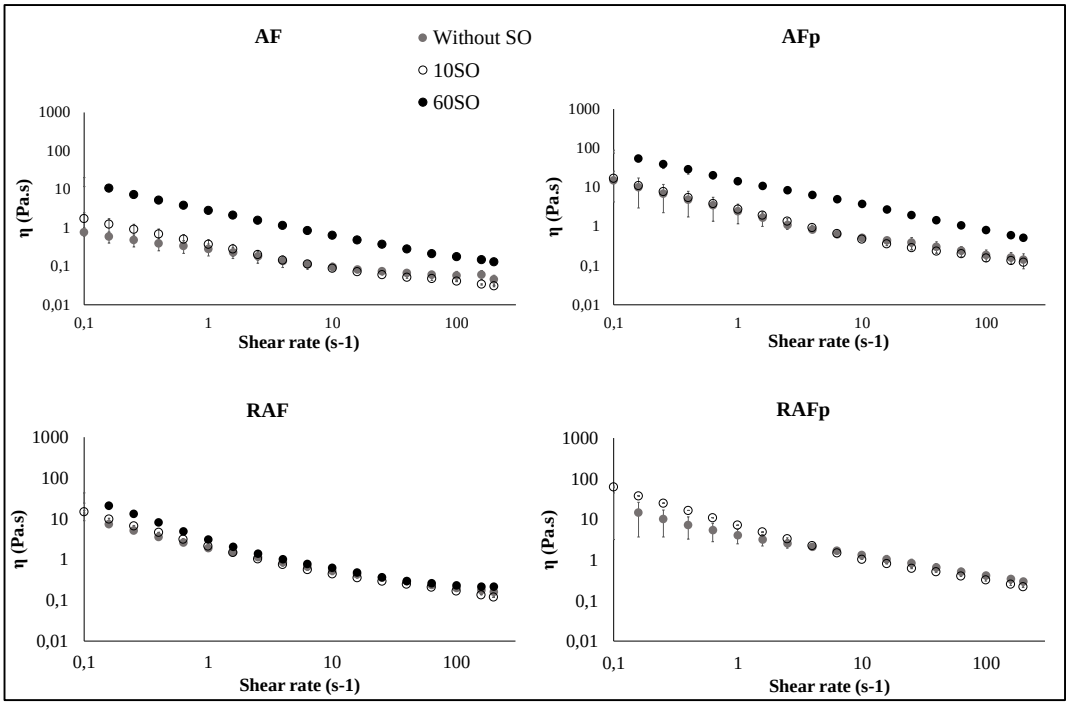

Figure 2. Apparent viscosity as a function of shear rate curves for solutions and emulsions prepared with AF, AFp, RAF, RAFp fractions and sunflower oil (SO) at different concentrations (10 and 60 %, named as 10SO and 60SO respectively). AF and RAF are aqueous fractions obtained from the whole biomass and from the stipes, respectively, and p indicates the purified extracts.

3.3. Microstructural characterization of the emulsions

To gain more structural insight, the microstructure of the freshly-prepared emulsions was further investigated by means of confocal microscopy by staining the SO and proteins with Nile red and fast green dye, respectively.

Representative images are compiled in **Figure 4,** together with the size distribution of the oil droplets. A different pattern was observed depending on the extraction source and thus, the β-glucan structural properties. A much higher protein content was present in the aqueous phase of the AF and AFp-stabilized emulsions, as compared to their counterparts prepared with RAF and RAFp samples. This is

consistent with the measured protein content in β-glucans extracts obtained from the whole biomass (AF). These proteins were located at the O/W interphase, playing a major role in emulsion stabilization. It is interesting to note that increasing the SO content in the emulsion stabilized with AFp, a more homogeneous particle size distribution and smaller average oil droplet particle was attained, leading to a very compacted microstructure formed by a great number of highly packed oil droplets and in agreement with the higher viscosity found in this sample. In fact, this emulsion displayed the lowest average oil droplet size and excellent storage stability. In contrast, a different pattern was observed for extracts obtained from the stipes. As observed, a lower protein content was present in the aqueous phase, consistent with the measured protein content of RAF and RAFp samples (cf. **Table 1**). Interestingly, although some protein could be discerned at the O/W interphase of RAF emulsions prepared with the higher SO concentration, the protein layer was not continuous and less visible. This again suggests a different stabilization mechanism of the different β-glucan extracts. In the case of AF, it is hypothesised that the production of a thicker absorbed protein layer could prevent coalescence of oil droplets, together with the intrinsic viscosity provided by the β-glucans present (Damodaran, 2005). Furthermore, the higher molecular weight of purified samples (AFp) would contribute to increased viscosity of the aqueous phase (as it will be detailed below) of O/W emulsions and to improve the emulsion stability, as the movement of the oil droplets is slowed down, thus limiting coalescence (Kontogiorgos, Biliaderis, Kiosseoglou & Doxastakis, 2004). However, the viscosity of the continuous phase was not the key factor for the stabilization of emulsions prepared with RAF, as having lower viscosity than their purified counterparts (RAFp) it displayed higher emulsion stability. In the case of emulsions prepared with RAF and RAFp, the protein content was similar in both samples, suggesting that a certain protein fraction precipitated together with the polysaccharides and thus, it is hypothesised that most of the proteins are interacting with polysaccharides (glycoproteins) and they could not act as surface

active materials at the w/o interphase. Furthermore, the greater degree of branching found in β-glucans from the stipes could limit the accessibility of bound proteins at the O/W interphase in a greater extent than in the case of less complex β-glucans obtained from the whole biomass. As a result, the emulsifying properties are conditioned upon their potential interactions with other compounds, the complexity of β-glucans at which they could be bound and, probably, the type of proteins present in the different samples. In fact, having lower viscosity and similar protein content, unpurified RAF extracts provided emulsions with greater stability than their purified counterparts. This can be ascribed to the presence of low molecular weight β-glucans or proteins (see Supplementary Material S2) which stabilize the emulsion through the formation of a network in aqueous phase, as it was reported by (Burkus & Temelli, 2000). Furthermore, the higher structural complexity of branched β-glucans found in RAF samples could contribute to the stabilization of these samples by steric repulsions between droplets at lower SO concentration.

Therefore, since β-glucans lack surface activity, their emulsifying properties can be attributed to the presence of protein impurities in the extracts (Xu et al., 2018) and to the viscosity imparted to the aqueous phase of the emulsions. Not only the presence of proteins but also the protein accessibility and the structural complexity of polysaccharides at which they are bound will determine the emulsifying properties of β-glucans. Similar effects have been reported for pectins (Alba, Bingham, Gunning, Wilde & Kontogiorgos, 2018; Mendez et al., 2021; Ngouémazong, Christiaens, Shpigelman, Van Loey & Hendrickx, 2015).

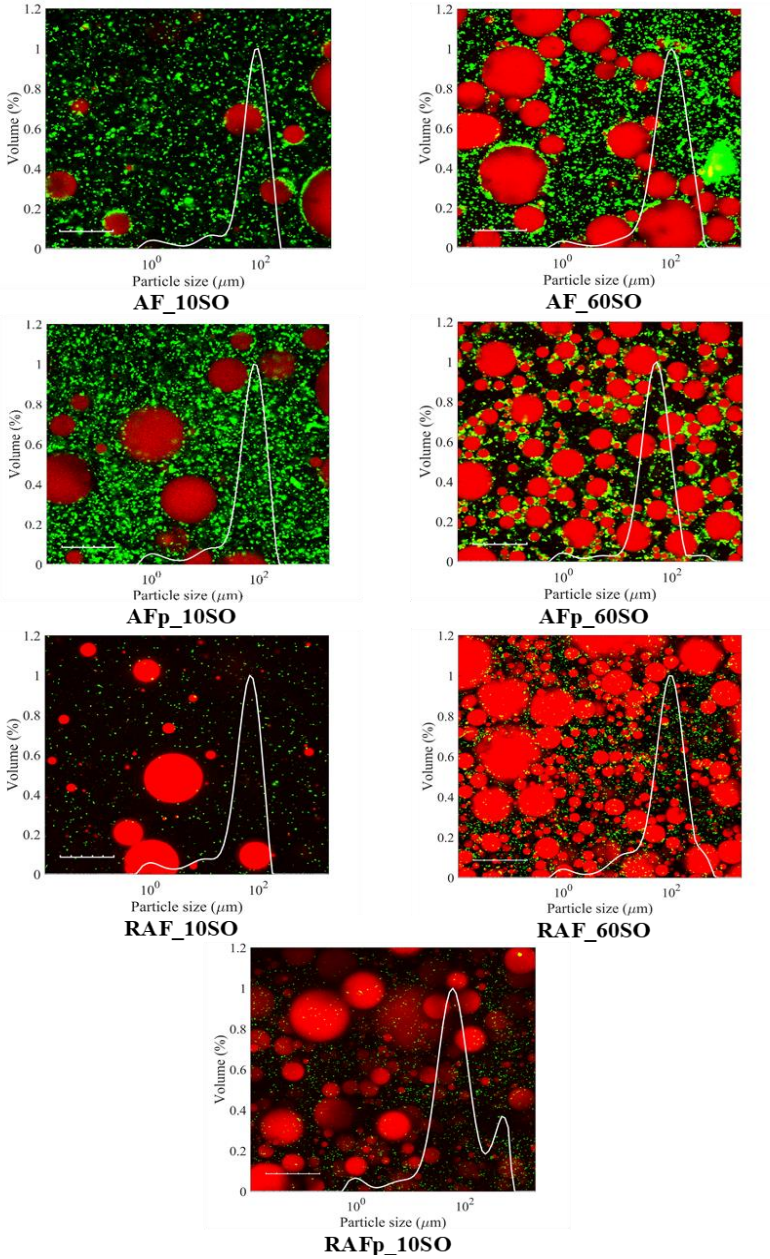

Figure 3. Confocal microscopy images of β-glucan-based emulsions and particle size distribution at day 0. The oil phase was stained with Nile red and the protein phase with fast green. All pictures were taken with the same light intensity and magnification (scale bar corresponds to 60 μm). AF and RAF are aqueous fractions obtained from the whole biomass and from the stipes, respectively, and p indicates the purified extracts.

3.4. Freeze-thaw and thermal stability of β-glucan based emulsions.

The stability under extreme temperatures such as the sterilization and freeze-thaw cycles are important for food applications. In general, freeze-thaw treatments gave a much more negative effect on emulsion stability than heat treatments (see **Table 4;** images shown in supplementary material). After freeze -thawing treatment, the emulsion samples prepared with the unpurified AF fraction showed higher creaming index values than their counterparts AFp, being accentuated as SO content increased. This can be related to the viscosity values of the resulting emulsions. As described by (Degner et al., 2013), if the oil droplets do not crystallize before the aqueous phase freezes, they are progressively concentrated into the areas of nonfrozen aqueous phase as ice freezing progresses. The close contact between oil droplets induces flocculation/coalescence and then, a complete oiling off during the thawing procedure. It should be highlighted that the higher viscosity of the RAF -based emulsions prevented phase separation after freezing when low SO concentration was used, as compared to their AF counterparts. However, the higher viscosity found in those obtained from the stipes (RAF) did not prevent droplet flocculation for higher SO concentration, although the creaming index was significantly lower than their counterparts prepared with β-glucan fractions from the whole biomass (AF), evidencing that the viscosity of the emulsion played a key role on the freeze-thaw stability.

After heat treatment, the emulsion stability of all emulsions was also determined. As summarized in **Table 4**, the creaming index value of all the samples increased, except in AFp-based emulsions prepared with the highest SO content, and in RAF containing 10% (v/v) SO. Considering that, according to the Stokes' law, the velocity of creaming is proportional to the droplet size and inversely proportional to the viscosity (Xu et al., 2020), it seems that the relatively smaller droplet size of RAF_10SO and AFp_60SO together with higher viscosity values of AFp-60SO sample inhibited the creaming of the emulsions.

Table 4. Creaming index (CI) after freeze-thaw treatment (three freeze-thaw cycles at -20 ºC), and after sterilization treatment (121 ºC, 20 min).

Creaming index (%) with respect to fresh samples		
Sample	**Freeze-thaw cycle (-20 °C) (%)**	**Heat treatment (121 ºC) 20' (%)**
3AF_10O	12.51±0.74^{c}	2.86±0.68^{d}
3AF_60O	59.32±0.40^{a}	51.74±0.46^{a}
3AFp_10O	0.00±0.00^{d}	8.04±0.50^{c}
3AFp_60O	34.85±2.62^{b}	-
3RAF_10O	0.00±0.00^{d}	-
3RAF_60O	37.86±0.37^{b}	13.85±1.27^{b}
3RAFp_10O	0.00±0.00^{d}	8.08±1.25^{c}

Values are mean ± standard deviation. Means in the same column with different superscripts are significantly different (P≤0.05). (-) indicates the absence or such a small value that it could not be quantified.

4. Conclusions

The results evidenced the capacity of AFp extracts to stabilize O/W emulsions, being able to incorporate greater amounts of oil phase (60 % (v/v)) than its counterpart RAFp and producing a much more homogeneous distribution of smaller oil droplets. The emulsifying capacity of AF and AFp extracts was explained by the presence of greater and more accessible proteins (acting as surface-active materials) and the higher viscosity of the continuous phase of purified samples (AFp). The lower emulsifying properties of β-glucan extracts obtained from the stipes (RAF and RAFp) was ascribed to the lower content of proteins. Unpurified RAF extracts provided emulsions with greatest stability than their purified counterparts (RAFp). In this case, the presence of low molecular weight compounds and the more structural complexity of branched β-glucans found in RAF samples could contribute to the stabilization of RAF emulsions by steric repulsions between droplets at lower oil concentration. When the emulsions were subjected to extreme temperatures, freeze-thaw treatments gave a much more

negative effect on emulsion stability than heat treatments. These results evidence the high relevance of microstructure and composition of β-glucan aqueous extracts in their behaviour upon emulsifying properties, and offer a simple and economical clean label ingredient (used as emulsifiers), rich in protein and fibre, which can be of great interest in the food industry.

Funding

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Supplementary Material

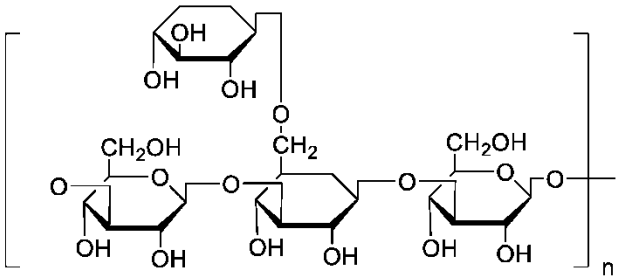


Figure S1. Ideal structure of a pleuran or characteristic β-glucan of the genus *Pleurotus*. Modified from (Cerletti et al., 2021).

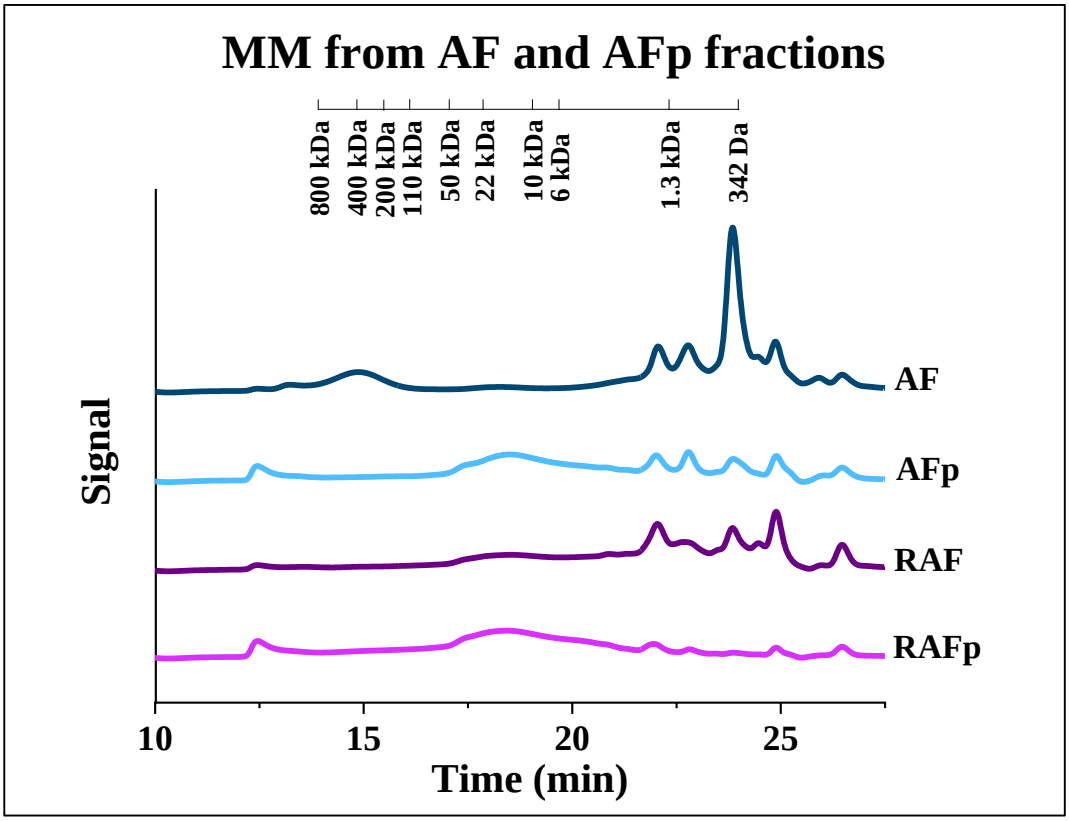


Figure S2. Molar mass corresponding to the fractions used in the emulsions (AF, AFp, RAF and RAFp). In the upper part, as a reference, the known molecular weights of pullulan standard are shown. Image modified from (Pérez et al, 2024).

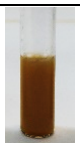
Freeze-thaw treatment									
Fraction	Oil	t_0				t_f			
		AF	AFp	RAF	RAFp	AF	AFp	RAF	RAFp
3%	10%								
	60%								

Figure S3. Macroscopic images of the different freshly prepared emulsions and after 3 freeze-thaw cycles.

Heat treatment (121 ºC)									
Fraction	Oil	t_0				t_f			
		AF	AFp	RAF	RAFp	AF	AFp	RAF	RAFp
3%	10%								
	60%								

Figure S4. Macroscopic images of the different freshly prepared emulsions (t_0) and after a heat treatment of 121 ºC for 20 min (t_f).

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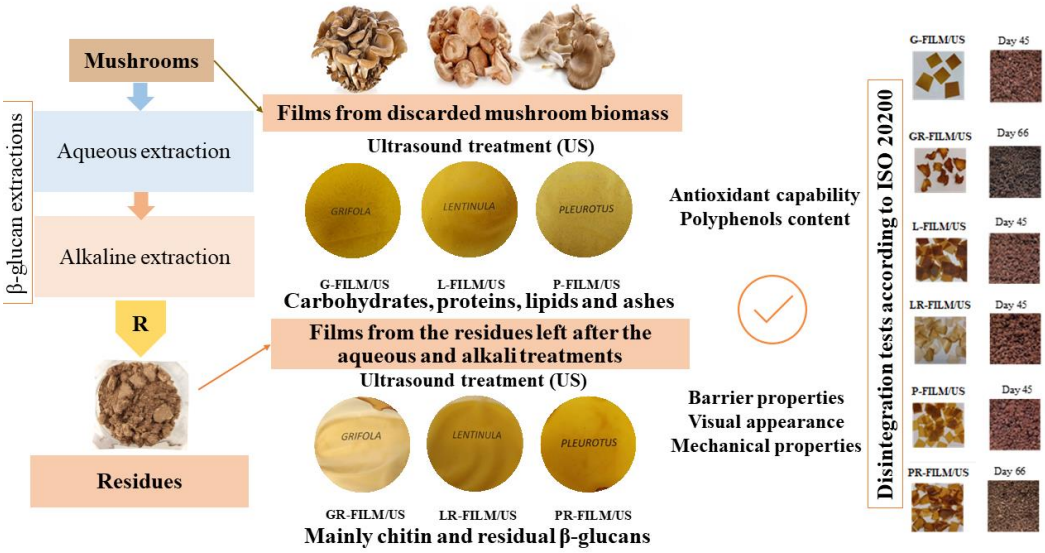
CHAPTER 3

Use of mushrooms waste biomass for the formation of biodegradable film-like materials for food packaging applications

3.1. Ultrasound-treatment as a promising strategy to develop biodegradable films obtained from mushroom waste biomass.

3.2. Feasibility of *Agaricus bisporus* waste biomass to develop biodegradable food packaging materials.

3.1. Ultrasound-treatment as a promising strategy to develop biodegradable films obtained from mushroom waste biomass.



This section is an adapted version of the following published research article:

Zaida Pérez-Bassart, Antonio Martínez-Abad, Alcira Reyes, Amparo López-Rubio, María José Fabra, 2023.

"Ultrasound-treatment as a promising strategy to develop biodegradable films obtained from mushroom waste biomass"

Food Hydrocolloids, volume 135, 108174.

Abstract

This work reports on the valorisation of discarded mushroom biomass generated in the industry and the waste produced after a β-glucan extraction process from three different species (*Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*) for the production of biobased and biodegradable films. Initially, the composition of the starting material was characterized before film production. The results evidenced no significant compositional differences between the discarded mushrooms, proteins, and β-glucans being the main components in all varieties. In contrast, the residues were mainly composed of carbohydrates (glucans and chitin).

The films obtained from the residues presented a very rigid behaviour, with higher elastic moduli (ca. 2-4.5 GPa) and lower elongation values (ca. 1%) compared to their counterparts prepared with the mushroom's discards. The developed films outperformed benchmark biopolymers in terms of barrier properties with the additional advantages that they can be directly produced from fungal biomass (without plasticizers or any other additives) and they proved to be easily disintegrated according to the standard ISO 20200.

1. Introduction

The global mushroom cultivation market is considerably growing and is expected to reach $52 billion by 2026, posing an environmental challenge for the main industries that market these products worldwide (Fortune Business Insights, 2019). Mushroom waste is mainly composed of mushrooms with misshapen caps and/or stalks that do not meet the specifications set by retailers but have high nutritional value (Aguiló-Aguayo, Walton, Viñas & Tiwari, 2017). Therefore, researchers are looking for ways to turn the significant amount of waste produced from mushroom cultivation into valuable products since they offer significant economic potential as fungal cell walls contain chitin, hemicelluloses, and, among the most interesting functional components, β-glucans (Muzzarelli et al., 2012; Synytsya & Novák,

2013). Their valorisation has been gaining attention not only because of the health-promoting effects of β-glucans but also due to the different potential industrial applications, their impact on the economy, and the support for sustainability. Several valorisation schemes have been proposed from mushroom' waste and by-products to obtain valuable compounds (Grimm & Wösten, 2018; Zhu, Du, & Xu, 2016). Of especial interest is the integrated value chains as one of the most promising pathways to achieve the zero-waste goal and to accelerate the transition of the mushroom industry to a circular bioeconomy. For instance, the use of the waste streams, generated after β-glucans extraction from mushrooms´ residues and by-products, to develop biopolymers is an underexplored area but with a great potential since some of the components present in the native mushrooms (i.e. chitin and recalcitrant β-glucans), remain in the residue (Ifuku, Nomura, Morimoto, & Saimoto, 2011).

Over the last decades, plastics have been widely used with an alarmingly growing rate of global production (Payne, McKeown & Jones, 2019), raising concerns related to their waste management and environmental contamination, thus urging to look for alternatives. In this context, it is worth mentioning the current open debate and activity around plastic packaging and its environmental impacts that have mainly focused on the grocery and food retail sectors. Packaging has become an essential element in the commercialization of food products, being fundamental to ensuring food quality and safety and facilitating purchasing and transportation activities throughout "the farm to fork chain", contributing to reducing food waste (Bhargava, Sharanagat, Mor, & Kumar, 2020). Food packaging is a challenging part of the global plastics waste management challenge, representing the most significant demand for plastic packaging (Europe, 2021). Therefore, replacingsynthetic plastics by biodegradable polymers from renewable natural resources (i.e. biopolymers) is envisaged as the most sustainable and long-term solution (George, Sanjay, Srisuk, Parameswaranpillai, & Siengchin, 2020;

Savadekar & Mhaske, 2012). There is an increasing research interest in developing environmentally and economically sustainable packaging materials using more environmentally friendly alternatives that follow the Circular Economy principles (European Bioplastics, www.european-bioplastics.org). However, biopolymers´ physicochemical properties (mechanical and barrier performance) are not comparable to benchmark synthetic polymers, and their production costs are too high to compete in the market. Most of these biopolymers need the addition of plasticizers, nucleating agents, or further additives to reach desirable mechanical properties, which pose disadvantages in their barrier properties, compostability, and even ecoxitological effects. Furthermore, the raw materials normally used for the production of biopolymers come from land crops, whose primary use is the food and feed sectors. As an alternative, agrifood waste and by-products are also being explored as a source for biopolymer production.

This work is focused on the valorisation of discarded mushroom biomass generated in the industry and the waste produced after β-glucan extraction from three different species (*Grifola frondosa, Lentinula edodes* and *Pleurotus ostreatus*) for the production of biopolymeric films. The suitability of both the discarded mushrooms (rejected biomass) and the residues to produce biopolymeric films was evaluated, investigating the effect of their composition on the performance of the biopolymeric films for their use as food packaging materials.

2. Methods and materials

2.1. Raw Materials

The three fungal species (*Grifola frondosa, Lentinula edodes* and *Pleurotus ostreatus*) were kindly donated by Centro Tecnológico de Investigación del Champiñón –Asochamp- (Rioja, Spain). The extraction of β-glucans was carried out by means of using hot water treatments followed by an alkali treatment with NaOH, as described in previous work (Pérez-Bassart et al., 2022) with some

modifications. Briefly, 25 g of freeze-dried raw material were subjected to an aqueous treatment at 100°C with reflux with 300 mL of distilled water, stirring for 7h. The resulting soluble part was concentrated and lyophilised. The precipitate obtained from centrifugation was subjected to an alkaline treatment at room temperature overnight with 300 mL of 1M NaOH and 0.05% $NaBH_4$ to prevent peeling at the reducing end of the polysaccharides. The suspension obtained was then centrifuged. The supernatant was reserved for precipitation with 1:3 (v/v) ethanol to remove low molecular weight components and then dialysed and lyophilised to remove traces of NaOH. The remaining precipitate was washed with distilled water until neutral pH to ensure that the remaining NaOH was removed. This insoluble residue was referred to as fraction R, with GR, LR or PR, depending on the species, and was used for film development.

2.2. Chemical analysis of the raw material and waste fungal biomass

The protein content in whole mushrooms was calculated based on the nitrogen content estimated with the Kjeldahl method, multiplied by a factor 4.38 (Kalač, 2009). The protein content was calculated in both the whole mushroom and the residue by difference from the total elemental nitrogen and subtracting the nitrogen from D-glucosamine from the chitin, as previously described in Pérez-Bassart et al., 2022. The lipid content was estimated after performing the Soxhlet extraction according to AOAC method 933.06 with slight modifications. Approximately 5 g of raw material was extracted using Soxhlet apparatus with 250 mL of hexane over 8 hours. The ash content was determined using the standard method TAPPI T211.

The monosaccharide composition, including the chitin content, from the discarded biomass and the residues, was determined as previously described in Pérez-Bassart et al., 2022. All experiments were carried out in triplicate.

2.3. Production of fungal-based films

Fungal- based films were produced by adding 1.5 g of the discarded biomass, or the residue generated after the β-glucan extraction protocol, to 100 mL of distilled water. The solution was dispersed using an ultra-turrax homogenizer (D9, MICCRA GmbH, Müllheim, Deutschland) (UT) at 20000 rpm for 1 min or sonication (US) using an UP-400S ultrasound equipment (Hielcher GmbH, Germany) at 400 W for 1 min. These were then evenly spread over a Teflon casting plate resting on a leveled surface. Films were formed by drying for 48h at 56% RH at 25 ºC. These conditions were selected after previous experiments to guarantee that homogeneous dry films could be peeled intact from the casting plate. The formed films were peeled off the casting plate and preconditioned at 53% RH for one week in a cabinet using magnesium nitrate oversaturated solution before analysis.

2.4. Scanning electron microscopy (SEM)

SEM was conducted on a Hitachi microscope (Hitachi S-4800) at an accelerating voltage of 10 kV and a working distance of 8-12 mm. Two different samples of each film were cryo-fractured after immersion in liquid nitrogen and randomly broken to investigate the cross-section of the samples. Samples were fixed on M4 Aluminium Specimen Mount and sputtered with a gold-palladium mixture under vacuum before their morphology was examined.

2.5. Optical properties

The transparency of the films was determined through the surface reflectance spectra in a spectrophotometer CM-26D (Minolta Co., Tokyo, Japan) with a 10 mm illuminated sample area. Measurements were taken from three samples in each film using both a white and a black background. The transparency was determined by applying the Kubelka–Munk theory for multiple scattering to the reflection spectra. Transparency (Ti) (0-100 theoretical range) was calculated from

the reflectance of the sample layer on a white background of known reflectance and on an ideal black background, as described in (Fabra, Talens, & Chiralt, 2009).

2.6. Fourier transform infrared spectroscopy (FT-IR)

Film samples were analysed by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm^{-1} resolution in a wavelength range between 400−4000 $^{-1}$ and averaged a minimum of 32 scans. The results were processed using Origin Pro 2019 software.

2.7. X-ray diffraction (XRD)

XRD measurements of the films were carried out at room temperature on a D5005 Bruker diffractometer. The instrument was equipped with a Cu tube and a secondary monochromator. The configuration of the equipment was θ–2θ, and the samples were examined over the angular range between 3°–60° with a step size of 0.02° and a count time of 200 s per step.

2.8. Thermal properties

Thermogravimetric curves (TG) of the discarded biomass and the residues were recorded with a TA 550 (Waters- TA Instruments, New Castle, EEUU). The samples (*ca*. 5mg) were heated from 30 to 700 ºC with a heating rate of 10 ºC/min under a nitrogen atmosphere. Derivative TG curves (DTG) expressed the weight loss rate as a function of temperature and were plotted using Origin Pro 2019 software.

Thermal properties of the films were also evaluated by Differential Scanning Calorimetry (DSC) using a TA Instrument (New Castle, DE, USA)thermal analysis system under nitrogen atmosphere. The analysis was carried out on ~ 3 mg of each sample at a heating rate of 10ºC/min, from 20 ºC to 450 ºC. The DSC equipment was calibrated with indium as a standard and the slope of the

thermograms was corrected by subtracting similar scans of an empty pan. Tests were done in triplicate.

2.9. Water vapor permeability (WVP)

Direct permeability to water vapor was determined from the slope of the weight gain versus time curves at 23 °C, using the ASTM 2010 gravimetric method. Tests were done in triplicate for each sample type, and water vapor permeability was carried out at 0–75% relative humidity gradient. The films were sandwiched between the aluminium top (open O-ring) and bottom (deposit for silica gel) parts of Payne permeability cups (3.5 cm diameter, Elcometer SPRL, Hermelle /s Argenteau, Belgium). A Viton rubber O-ring was placed between the film and bottom part of the cell to enhance sealability. Permeability cups containing silica were placed in an equilibrated cabinet at 75 % RH using an oversaturated sodium chloride salt solution.

2.10. Oxygen permeability (OP)

Oxygen permeability (OP) was calculated from oxygen transmission rate (OTR) measurements recorded in triplicate using an Oxygen Permeation Analyzer 8001 (Systech Illinois, UK). The samples were previously purged with nitrogen in the humidity equilibrated test cell, before exposure to an oxygen flow of 10 mL min^{-1}. The exposure area during the test was 5 cm^2 for each sample. In order to obtain the oxygen permeability, film thickness and gas partial pressure were considered in each case. Experiments were carried out at 23 (±1) °C and 53 (±2) % RH.

2.11. Mechanical properties

A universal test Machine (Instron, USA) was used to determine the tensile strength (TS), elastic modulus (E), and elongation at break (ε_b) of the films, according to ASTM standard method D882.10 (ASTM, 2001). Tensile parameters were determined from the stress-strain curves, estimated from force-distance data obtained for the different films (1 cm wide and 8 cm long). At least eight replicates

were obtained per formulation. Pre-conditioned specimens were mounted in the film-extension grips of the testing machine and stretched at 50 mm min^{-1} until breaking. The relative humidity of the environment was held constant at 54 (±2) % during the tests, which were performed at 23 (±1) °C.

2.12. Water contact angle measurements

The surface hydrophobicity as the wettability of fungal-based films were measured in a DSA25 equipment (Kruss) equipped with image analysis AD4021 software at ambient conditions. A precision syringe deposited a water droplet (~3µL) on the film surface. Contact angle values were obtained by analyzing the shape of the distilled water drop after it had been placed over the film for 10s. Five replicates were analyzed per film formulation.

2.13. Water uptake

The water absorption behavior of the films was determined. The samples were dried prior to testing at 60°C to constant weight. They were then placed in a 75 % RH equilibrated cabinet with a supersaturated sodium chloride salt solution and gravimetric measurements were performed until constant weight. For each type of sample, the tests were carried out in triplicate. The water absorption content was calculated as a percentage of weight gained concerning the initial weight of the sample.

2.14. Disintegration tests

The disintegration test of the mushroom-derived films was carried out following several guidelines of the standard ISO 20200:2016. In this test, a synthetic solid consisting of a mixture of sawdust, rabbit feed, mature compost, corn starch, sucrose, corn oil, urea and water is used to simulate laboratory scale composting conditions and the samples to be tested are composted with this prepared solid matrix.

One test reactor (consisting of a polypropylene container) per sample was prepared and each reactor contained 5g of film and 1 kg of wet synthetic solid. The mass ratio of the films vs. the wet synthetic residue was between 0.5 % and 2.0 %, according to the standard method.

The reactors were incubated at a temperature of 58 ± 2° C for a minimum of 45 days. The contents of the reactors were regularly mixed and moistened (final water content of 55% in total) to guarantee an excellent composting process. During the composting period, the gross mass of the composting container was totally or partially restored according to the schedule established in the calendar indicated by the standard method.

The degree of disintegration was determined after the composting period by sieving the final matrix through standard 10 mm, 5 mm, and 2 mm sieves (according to ISO 3310-1) to recover the non-disintegrated material. The mass reduction of the tested films was considered as disintegrated material and was used to calculate the degree of disintegration.

2.15. Statistical analysis

Statistical analysis of results was carried out using Statgraphics using one-way analysis of variance (ANOVA) to determine the significant differences between samples, at a significance level of $P < 0.05$.

3. Results and Discussion

3.1. Compositional characterization of the mushrooms' discards and the residue

In the first stage of this work, the composition of the three different mushrooms (*Grifola frondosa, Lentinula edodes, and Pleurotus ostreatus*) and the residue generated after β-glucan extraction (applying hot-water treatments followed by alkali treatments) was characterized before film production. The results, shown in Table 1, evidenced no significant compositional differences between the discarded mushrooms, except for the higher lipid content of *Grifola frondosa* and *Lentinula edodes* compared to *Pleurotus ostreatus*. This agrees with a previous work in which very similar composition was reported for the three different mushroom varieties (Pérez-Bassart et al., 2022). As expected, proteins and β-glucans represented all varieties' main components, with *ca*. 22, 23 and 24 % protein contents for *L. edodes*, *G. frondosa* and *P. ostreatus*, respectively. As observed in Figure 1, *P. ostreatus* had the most meaningful β-glucan content (*ca.* 43%), higher than that of the other two mushroom species (*ca.* 36 and 31% for *G. frondosa* and *L. edodes*, respectively). Chitin content was lower in *L. edodes* than in the other two species, within the range reported for wild mushroom species (2-8.5% of the dry matter) (Vetter, 2007). Additionally, the polyphenol content (expressed as mg of gallic acid –GA- per gram of sample) of the discarded biomass was higher for *G. frondosa.* It should be noted that although a lower polyphenol content was found in discarded *P. ostreatus* biomass, no significant differences were observed in the antioxidant activity when *G. frondosa* and *P. ostreatus* were compared. This effect indicates that the antioxidant activity of *P. ostreatus* was not only given by the phenolic compounds but other molecules, such as *β*-glucans with reported antioxidant properties could have also contributed to the observed TEAC values.

Table 1. Proximate composition and antioxidant capacity of the discarded biomass and the residue.

Sample	Protein (%)	Lipids (%)	Carbohydrates (%)	Ashes (%)	Polyphenols (mgGA/gDW)*	TEAC (µg TE/g DW)**
G	23.55 (0.25) ^{ab}	8.23 (0.23) ^{a}	50.87 (1.67) ^{a}	5.95 (0.01) ^{a}	92.03 (6.83) ^{a}	150.59 (5.30) ^{a}
GR	6.59 (0.10) ^{c}	< 0.1	92.05 (5.19) ^{d}	1.13 (0.37) ^{c}	7.33 (0.13) ^{c}	16.28 (1.29) ^{c}
L	22.21 (0.18) ^{a}	7.22 (0.30) ^{b}	41.91 (4.08) ^{a}	6.54 (0.30) ^{a}	58.93 (2.92) ^{b}	124.67 (5.60) ^{b}
LR	6.63 (0.03) ^{c}	< 0.1	69.65 (1.09) ^{bc}	1.03 (0.21) ^{c}	7.42 (0.21) ^{c}	10.89 (0.89) ^{d}
P	24.05 (1.09) ^{b}	5.35 (0.39) ^{c}	51.81 (3.82) ^{ab}	7.82 (0.01) ^{b}	61.51 (1.25) ^{b}	149.20 (4.36) ^{a}
PR	7.52 (0.00) ^{c}	< 0.1	78.71 (5.03) ^{c}	1.30 (0.35) ^{c}	7.14 (0.04) ^{c}	15.08 (2.34) ^{cd}

Mean values (standard deviation). Values with different letters in the same column are significantly different ($p \leq 0.05$).

G: *G. frondosa* whole mushroom; L: *L edodes* whole mushroom; P: *P. ostreatus* whole mushroom; GR: residue from *G. frondosa* left after the aqueous and alkaline treatments, LR: residue from *L. edodes* left after the aqueous and alkaline treatments, PR: residue from *P. ostreatus* left after the aqueous and alkaline treatments. *mg of gallic acid equivalents per 100 g of dry weight (DW). **µg Trolox Equivalent per gram of DW. DW: dry weight.

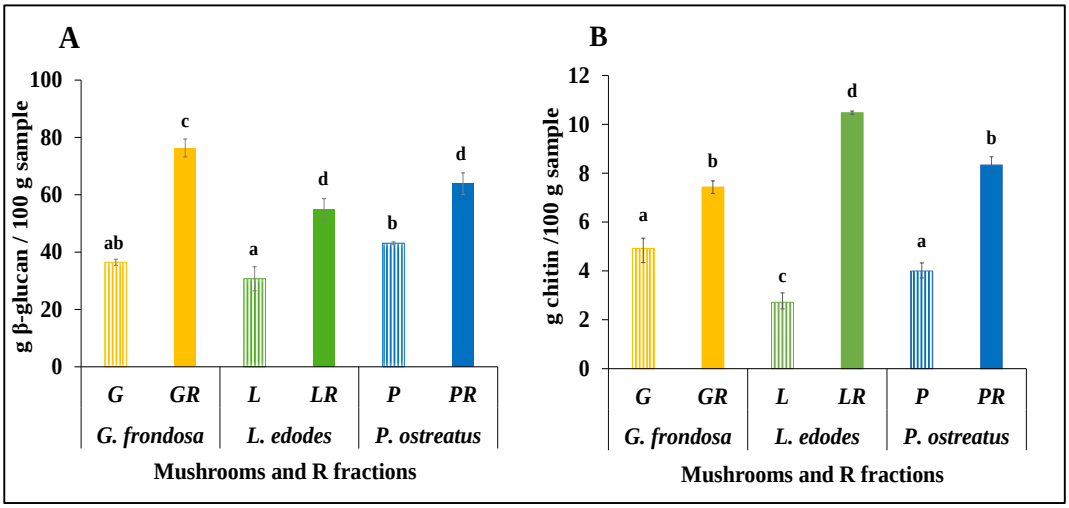


Figure 1. (**A)** β-glucan and **(B)** chitin content of the mushrooms' discards and residues. Values followed by different letters are significantly different ($p \leq 0.05$).

The composition of the residues obtained after subjecting the fungal biomass to the β-glucan extraction protocols previously described is also shown in Table 1. The residual yields were 19.28 ±0.97, 23.36±0.47, and 22.54±0.59 % for *G. frondosa*, *L. edodes*, and *P. ostreatus*, respectively. Not surprisingly, the residues contained a significant amount of chitin and minor amounts of protein. Chitin was more abundant in the *L. edodes* residue than in the other two residues. A significant amount of β-glucan remained in the residues even after aqueous and alkaline extraction. Between the three species, it was significantly higher for *G. frondosa*, thus confirming its excellent recalcitrance nature, probably ascribed to stronger β-glucan-chitin bonds (as it did not have higher chitin content). Compared to the discarded biomass, the higher amount of glucans in the residue indicated that they were concentrated, even after soluble β-glucan extraction since other more soluble components (such as proteins, ashes and polyphenols) were more efficiently removed during the aqueous and alkaline treatments. Although the internal structure and molecular weight of the β-glucans in the residues may substantially differ between species, significant differences in total β-glucan were only observed between *G. frondosa* and the other two species. Although in minor amounts (< 8%), heteropolysaccharides such as xylomannans, mannogalactans, fucomannogalactans or xyloglucans (see Figure S1 for detailed composition) might have also influenced the film´s thermo-mechanical properties. Furthermore, the antioxidant activity and the phenolic compounds of the residues were significantly reduced compared to the discarded mushroom biomass.

Additionally, TGA characterization was carried out to determine the thermal stability of the discarded biomass and the residues, and the results are shown in Figure 2. As deduced from the derivative thermogravimetric profiles, all species from the discarded mushroom biomass degraded at lower temperatures than the residue. Discarded mushrooms showed very similar behavior, typical of multicomponent materials, with an initial degradation shoulder between 135 and

175ºC ascribed to the tightly bound water and the degradation of low molecular weight compounds present in the mushrooms' cell walls. A broad degradation profile, around 230 to 300ºC, was ascribed to the contribution of both β-glucans and chitin (Aburas, İspirli, Taylan, Yilmaz, & Dertli, 2020; Eyigor, Bahadori, Yenigun, & Eroglu, 2018, Puanglek et al., 2016), in agreement to that found for commercial glucans and chitin samples (see Figure S2 from Supplementary Material). The last degradation sharp peak with a maximum at 413 ºC for *P. ostreatus* and 484 ºC for *G. frondosa* and *L. edodes* is related to the degradation of chitin-glucan complexes and pure chitin (Girometta et al., 2020). Analogous to these findings, the DTG curve of the chitin standard reports a peak at around these temperatures (see Figure S2 from Supplementary Material). The different behavior found in the last degradation peak supports the presence of β-glucan-chitin complexes in *G. frondosa* and the less recalcitrant nature of β-glucans from *P. ostreatus*, which clearly showed the degradation peak of chitin centered at 413 ºC.

When compared with the residues, the first thing to highlight is that the maxima of the degradation peaks appeared at temperatures higher than the discarded biomass, indicating the higher thermal stability of the residues. It should be noted that *P. ostreatus* and *L. edodes* residues showed a thermal degradation profile characterized by an initial sharp peak in the temperature range of 305 and 351 ºC, which can be ascribed to the degradation of β-glucans and chitin. The same degradation peak, although with broader distribution, was observed for *G. frondosa*, showing a similar degradation profile to its counterpart discarded biomass and confirming the presence of more recalcitrant β-glucans from the *G. frondosa* residue, which are still linked to the chitin in the residues. The last degradation peak observed in all the residues, mainly ascribed to the contribution of the chitin and glucan-chitin complexes, showed a broader distribution in the *L. edodes* and *G. frondosa* residues than in their counterparts' discarded biomass, being more accentuated in *G. frondosa*.

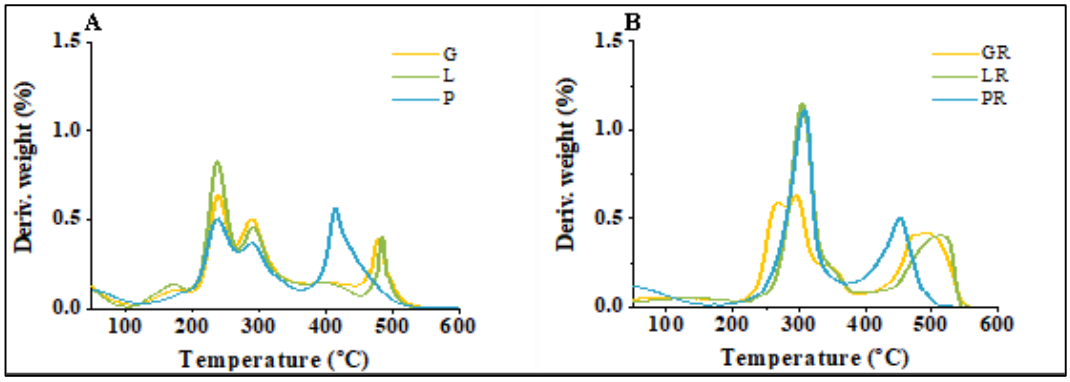


Figure 2. Derivative thermogravimetric (DTG) curves of the **(A)** mushrooms' discards and **(B)** the residue.

3.2. Characterization of mushroom films from the mushrooms' discards and the residue.

The discarded mushroom biomass was used to generate films from aqueous suspensions using UT and US treatments. It is interesting to note that sonication improved the film forming capacity of those obtained from the mushrooms' discards, not being able to produce films when only UT treatment was used, except in the case of *G. frondosa*. Intense sonication conditions can cleave glycosidic bonds, disrupting polysaccharide interactions within the cell wall or reducing their molecular weight (Chen et al., 2015). This, in turn, allows the formation of a continuous film. In contrast, the dispersion of the samples by UT treatment, only enabled film formation in the samples where these components were already present, which in *G. frondosa* might be related to its higher recalcitrance and lower presence of water-soluble β-glucans (Pérez-Bassart et al., 2022).

Furthermore, the residues generated after the extraction of β-glucans from the three different species by applying hot-water treatments followed by an alkali treatment were also valorised in this work to evaluate the possibility of forming films from aqueous suspensions using US. In this case, residues were already subjected to aqueous and alkaline treatments, removing specific components (salts, small peptides and oligosaccharides, polyphenols, pigments, and other secondary

metabolites, proteins), which might hinder film formation. Therefore, films were formed from these residues by UT or US treatments for all tested species.

3.2.1. Optical properties and morphology.

Figure 3A shows the visual appearance of the produced films. All the films were translucent and presented a yellow-brownish tonality. It is well-known that the transparency of the films is directly linked with the internal structure of the developed films (Fabra et al., 2009). Thus, the effect of discarded mushroom composition and the residues was quantitatively assessed through the internal transmittance (Ti) measurements. As observed in Figure 3B, Ti´s spectral distribution curves depended on the matrices´ internal microstructure and the composition and distribution of the films' components. The discarded mushroom biomass films presented much lower transmittance values than their counterparts prepared with the residues, making these films opaquer and heterogeneous. This effect could be attributed to the differences in refractive indices among films' components such as proteins, lipids and polyphenols (an increase in light scattering). Also, the selective light absorption of some compounds (i.e polyphenols) at low wavelengths was more accentuated in the case of *G. frondosa* films which had a higher polyphenol content (see Table 1).

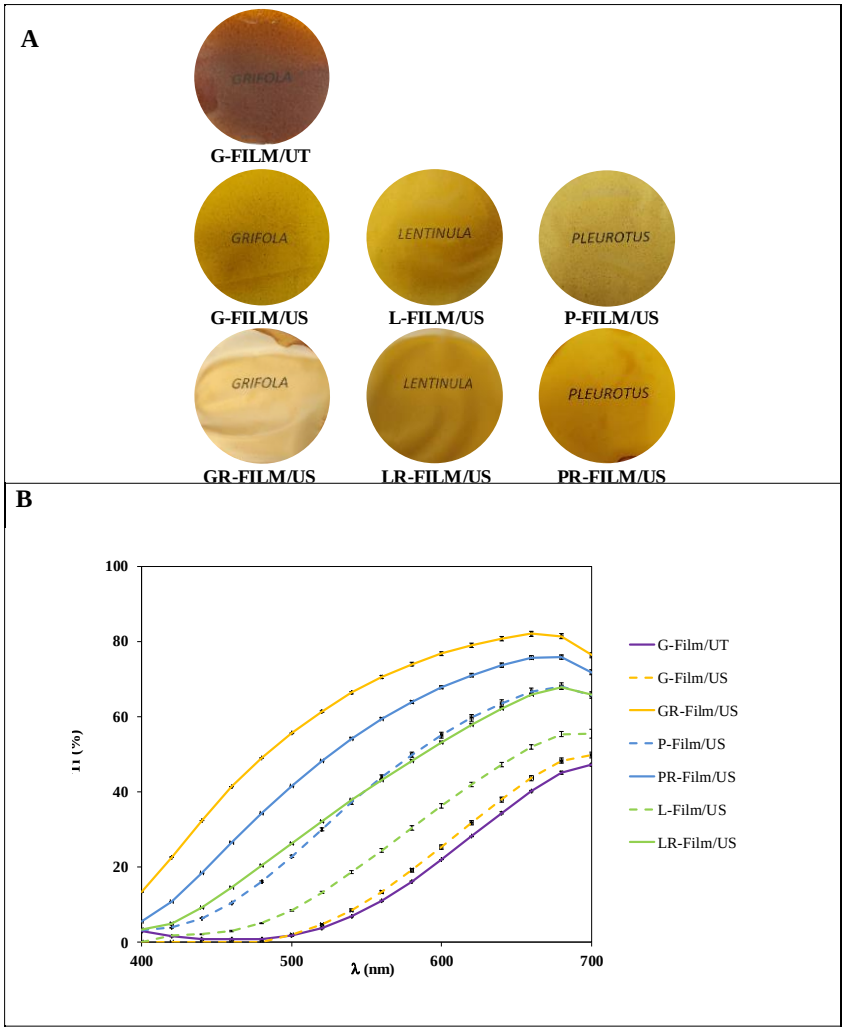


Figure 3. **(A)** Visual appearance and **(B)** Spectral distribution of internal transmittance (Ti) of the developed films using the whole mushrooms (G: *G. frondosa*, L: *L edodes* and P: *P. ostreatus*) or the residues from GR (*G. frondosa*), LR (*L edodes*) and PR (*P. ostreatus*) left after the aqueous and alkaline treatments. US and UT refer to ultrasound or UltraTurrax treatments, respectively, used for the production of fungal-based films.

SEM evaluated the produced films' morphology and representative images are shown in Figure 4. Generally, a different internal arrangement was observed as a function of the raw material used and the homogenization conditions. *G. frondosa*

discards films prepared with UT (Fig. 4A) showed a more heterogeneous cross-section and structural discontinuities suggesting a lack of miscibility of the components in this film. In contrast, films obtained from discarded biomass using the US treatment, showed a more continuous and softer microstructure than their counterparts prepared using UT, less compact than those obtained from the residue.

When the three species were compared, films obtained from *L. edodes* discards (Fig. 4D) exhibited the most homogeneous structure with no brittle areas or bubbles, consistent with forming a compact arrangement of biopolymer components (β-glucans and proteins). This is substantial evidence of differences in the cell wall architecture among the three species, which deserve further study.

A more compact and laminar-like structure was observed in the films obtained from the residues, which could be ascribed to the fibrillar structure of chitin embedded in the biopolymer matrix. The residual β-glucans and proteins remaining in the residue acted as a continuous amorphous matrix in which chitin fibrils were embedded. Fibrils from chitin were more clearly discerned in the *L. edodes* films (Fig. 4E) in agreement with its higher chitin content in the residue and the lower amount of β-glucans (see Figure 1). Chitin fibres seem more embedded in the continuous β-glucan matrix in *G. frondosa* and *P. ostreatus* films produced from the residues (Fig. 4C and 4G).

As detailed below, these differences in the internal morphology of the films' cross-section could also explain the differences found in the mechanical and barrier properties.

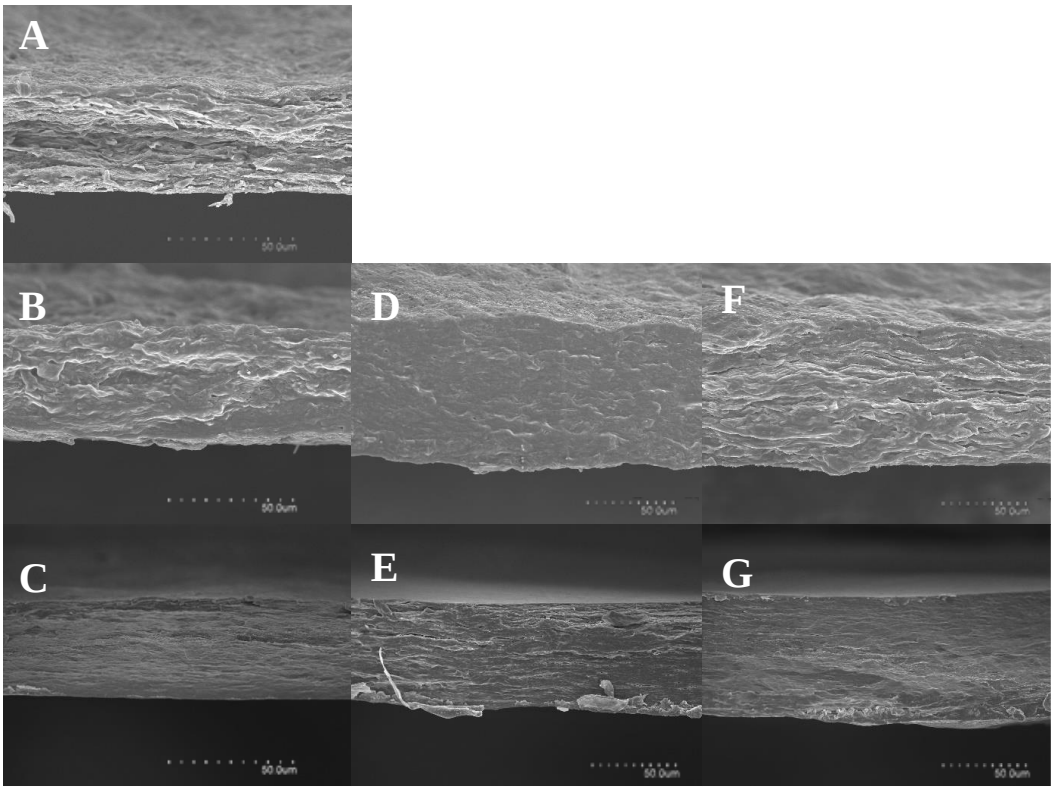


Figure 4. SEM images of the cross-section from the developed films: **A** G-FILM/UT; **B** G-FILM/US; **C** GR-FILM/US; **D** L-FILM/US; **E** LR-FILM/US; **F** P-FILM/US; **G** PR-FILM/US.

3.2.2. ATR-FTIR and XRD analysis.

To better understand the differences between the films obtained from the discarded biomass and the residues, they were characterized by FT-IR, and the obtained spectra are shown in Figure 5. All samples generally showed broad bands at 3600-3200 cm^{-1} and 3000-2900 cm^{-1} attributed to O-H and C-H stretching, respectively, which presented very similar shapes. The broad band centred around 3300 cm^{-1} also contains contributions from the amide N-H stretching (3540-3125 cm^{-1}), which causes a sharpening of this band and further indicates the presence of higher amounts of proteins in the discarded biomass, in agreement with the proximate composition (see Table 1). Moreover, a sharper peak at 2924 cm^{-1} ascribed to

vibrations of lipids was observed in the discarded mushrooms indicating the presence of small amounts of lipids in these samples, more accentuated in the case of *G. frondosa*. As expected, these peaks became less intense in the residues. This result evidences, in line with the proximate composition (see Table 1), that most proteins and lipids were removed during the aqueous and alkaline extraction processes and were in minor amounts in the residues. Furthermore, several intense, highly overlapped IR bands in the regions 950-1200 cm^{-1}, mainly ascribed to C-C and C-O stretching vibrations in pyranoid rings, indicated the presence of polysaccharides as the main components. The presence of glucans in the discarded mushroom biomass and residues left after the aqueous and alkaline extractions was confirmed by the appearance of several characteristic bands located at 1100 and 1374 cm^{-1}. The weaker band located at 894 cm^{-1}, indicated the presence of β-glycosidic bonds in all the samples, mainly ascribed to β-glucans (Synytsya et al., 2009). This suggests that, as already anticipated, β-glucans remained in the residues obtained and were concentrated in the residue since other components have been proportionately removed after applying the aqueous and alkaline treatments. The peaks centered at 1650 cm^{-1} and 1540 cm^{-1} were assigned to amide I and amide II vibrations, respectively, of proteins and chitin.

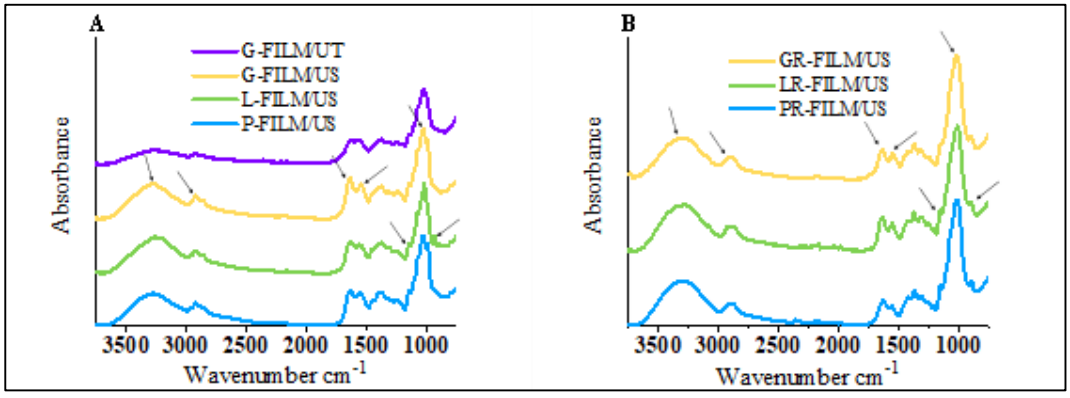


Figure 5. FT-IR spectra of the developed films. Spectra have been offset for clarity. Arrows point out to the spectral bands displaying the most significant changes amongst the different samples.

To evaluate the impact of the mushroom´s composition on the crystallinity of the films, the films were assessed by XRD analysis and the patterns are plotted in Figure 6. All the patterns from the discarded biomass films showed several sharp and intense peaks arising from crystalline components such as minerals and salts, being more accentuated in the case of *L. edodes*. However, none of these peaks were visible in the spectra from the residue, suggesting that these components were removed upon the applied extraction processes.

In the case of the films obtained from the discarded biomass, all the samples were characterized by the appearance of a peak located at 19.3 º, indicating the presence of crystalline GlcN (N-acetyl glucosamine) sequences from chitin, corresponding to the (110) crystal plane (Cárdenas, Cabrera, Taboada, & Miranda, 2004). As expected, the crystal structure changed notably in the films obtained from the residue. The relative intensity of this peak was significantly higher, except in the case of those obtained from *G. frondosa* residue, which almost disappeared after the aqueous and alkaline treatments. This effect may be related to the stronger chitin-glucan interactions and deserve further study. Interestingly, all the films obtained from the residue presented two prominent diffraction peaks located at 6.2 º and 9.1 º, which suggest a preferential orientation of (020) and (101) chitin crystal planes in the residues (Farinha et al., 2015). It is worth noting that the chitin peak at 9.1º has been associated with the most ordered regions involving the acetamide groups (Andrade et al., 2002) and also, with the randomly interplane layered structure (Wu, Mushi & Berglund, 2020), shown in Figure 3. A weak new diffraction peak was observed at 40º in films prepared from the *P. ostreatus* residue which may correspond to a second order peak of the previous intense diffraction peak centered at 20º The diffraction peak at 40 º, although more tenue, was also observed in films prepared from *L. edodes*.

The crystallinity of the films was estimated by fitting the areas under the diffraction patterns, resulting in values of *ca.* 25-27% for films prepared from discarded biomass and *ca.* 41, 30, and 29% for GR, LR and PR films, respectively.

The resulting crystallinity degree can be ascribed to the presence of chitin in a crystalline form and other components from mushrooms that can be arranged during the film-forming process and they could contribute to the crystallinity degree. In fact, as deduced from Figure 6, the peaks are wide suggesting that crystals were not perfectly defined and thus, not only chitin crystals but also other ordered components could contribute to the crystallinity.

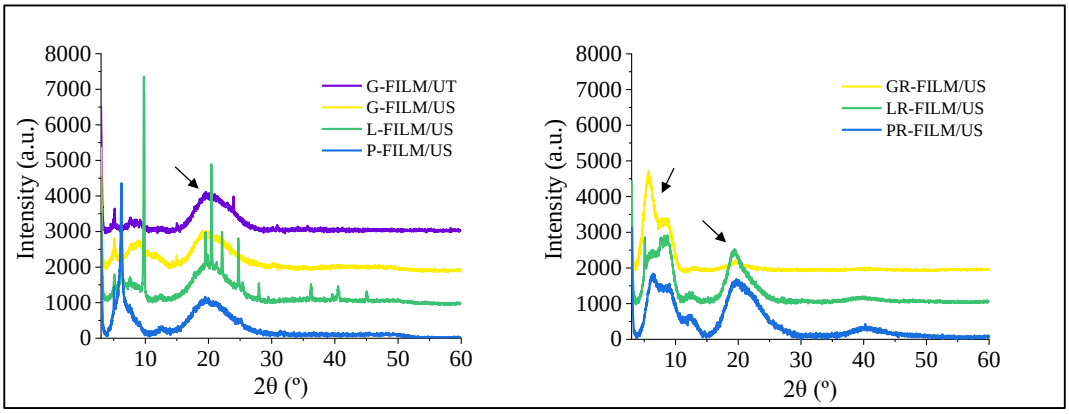


Figure 6. XRD patterns of the films from (A) mushrooms' discards and (B) the residue. The spectra from *Grifola* and *Lentinula* have been offset for clarity.

3.2.3. Thermal properties of the films.

DSC analysis was also carried out to study the thermal behaviour of the films. As shown in Figure 7, a first endothermic peak was detected between 80 and 150 ºC, ascribed to the bound water´s evaporation (Kittur, Harish Prashanth, Udaya Sankar, & Tharanathan, 2002). On the other hand, a triple helix-single coil transition of (1,6) branched (1,3)-β-glucans was previously reported in the same range (Kitamura et al., 1990; Liu, Wang, Cao, Xu, & Zhang, 2014). It is worth pointing out the main differences in this endothermic peak. While films from the residues left after the aqueous and alkaline treatment showed a sharper transition, the thermogram from films of discarded biomass showed a broader endothermic peak, which occurred at higher temperatures, indicating much more heterogeneous structures. According to previous reports, the triple helix of glucans can be

dissociated to random coils when heated above 135 ºC in aqueous solutions (Kitamura et al., 1990), evidenced by the higher temperatures at which the heat transition occurs in the films from discarded biomass. The sharp endothermic peak observed in the residues left after the aqueous and alkaline extractions can be mainly ascribed to the evaporation of water bound to the polysaccharides since the native structure of β-glucans were presumable lost during the harsh conditions used during the extraction protocol.

The exothermic event, including two exothermic decomposition peaks, evidences the presence of a mixture of chitin-glucan complexes usually associated with fungal cell walls (and detected during the compositional analysis), being more pronounced in the residues. Based on literature (Nováka et al., 2012; Pawlak & Mucha, 2003); the exothermic effect might result from cross-linking reaction between polysaccharides (glucan and chitin), involving the destruction of free amino groups in chitosan generated during the alkali digestion. Differences in the residue composition and the recalcitrant nature of the β-glucans were also patent in the exothermic peaks. As clearly observed in Figure 5b, the most recalcitrant *G. frondosa* residue showed two evident populations. Meanwhile, *L. edodes* showed a more intense peak at a higher temperature, probably related to chitin degradation (see Figure 5) and ascribed to its higher chitin purity.

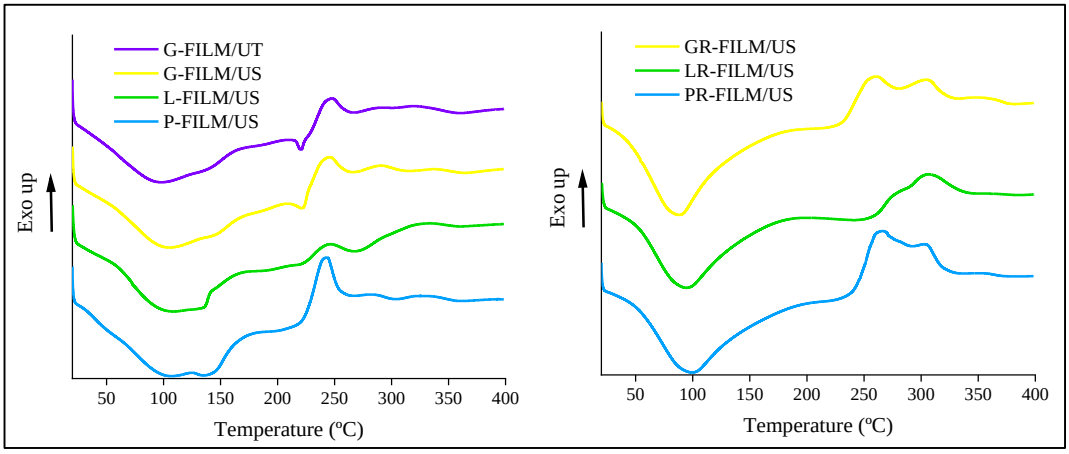


Figure 7. DSC results of the obtained films.

3.2.4. Mechanical, barrier, water sorption and wettability properties of the films.

The tensile properties of the films are useful parameters to describe their mechanical behavior and are closely related to their internal structure. The most representative parameters from the stress-strain curves are summarized in Table 2. The mechanical properties of the films obtained from the discarded mushroom biomass were affected mainly by the species used and the homogenization conditions.

The first clear observation is that *L. edodes* films were more flexible, elastic, and deformable (presenting lower elastic modulus and tensile strength, and higher elongation at break than the films from the other two mushrooms, in agreement with a more homogeneous distribution of mushroom´ components in the film (see Figure 3). This flexibility might be ascribed to higher hydrophilicity and plasticizing effect of the polysaccharide components. This could be related to a different structural organization of the β-glucans or/and to the higher abundance of mannogalactans, more hydrophilic than other heteropolysaccharides present in the other two species (Fig S1). Furthermore, it has been demonstrated that a certain fraction of β-glucans present in the cell walls of *L. edodes* is bound to soluble proteins (Pérez-Bassart et al., 2022). Thus, it seems reasonable to hypothesize that the US treatment disrupts this interaction. A certain fraction of β-glucans and proteins are released into the aqueous medium, favouring the formation of a continuous matrix in which most of the components are well-integrated, interacting with the biopolymer matrix.

Regardless of the films obtained from the residues, all of them presented a very rigid behaviour, with higher elastic moduli and lower elongation values, compared to their counterparts prepared with the mushrooms discards and ascribed to the higher crystallinity degree. The *L. edodes* residue provided less ductile films with higher TS values in agreement with the more fibrillar structure and its higher chitin

content (see Figures 1 and 3). In contrast, the residues from *P. ostreatus* gave rise to films that were more flexible and more ductile (lower E and TS values) than *G. frondosa*, an effect that can be explained by its lower crystalline degree and the type of crystals.

Table 2. Tensile properties (E: Elastic Modulus, TS: Tensile Strength and ε_b: Elongation at Break).

Film	E (MPa)	TS (MPa)	ε_b(%)
G-Film/UT	398 (34) ^{ab}	6.40 (0.19) ^{ab}	4.79 (0.59) ^{a}
G-Film/US	226 (50) ^{b}	4.64 (0.78) ^{ab}	3.00 (0.55) ^{ab}
GR-Film/US	3149 (46) ^{d}	33.59 (4.12) ^{c}	1.74 (0.43) ^{b}
L-Film/US	89 (15) ^{c}	3.63 (0.48) ^{a}	20.53 (2.71) ^{c}
LR-Film/US	4494 (180) ^{e}	28.68 (8.77) ^{c}	1.00 (0.18) ^{b}
P-Film/US	202 (25) ^{ac}	4.73 (0.82) ^{ab}	8.34 (2.67) ^{d}
PR-Film/US	2435 (196) ^{f}	11.08 (1.93) ^{b}	0.90 (0.14) ^{b}

E: Young's modulus; TS: tensile strength; ε_b: elongation at break; Values with different letters are significantly different ($p \leq 0.05$).

The films´ oxygen and water vapor barrier properties were also measured, and the results are summarized in Table 3. Although there were no significant differences in the water vapor permeability of the films prepared with the three different species, *L. edodes* films were substantially less permeable. This is most likely due to the more compacted film structure, as evidenced by SEM. Similarly, this more homogenous and compact structure also resulted in better oxygen barrier properties than the films obtained with the other two mushroom species. After the aqueous and alkaline extractions, films obtained from the residues displayed lower barrier performance. Removing some lipids, proteins and polyphenol compounds from the residues could have negatively affected the barrier properties.

Table 3. Water vapor permeability (measured at 0-75% RH), water uptake (measured at 75% RH) and oxygen permeability values (measured at 50% RH) of the obtained films.

Film	**WVP *** 10^{-14} **(Kg·m·Pa^{-1}· s^{-1}·m^{-2})**	**OP** $*10^{-19}$ **(m^3·m·Pa^{-1}·s^{-1}·m^{-2})**	**Water uptake (%)**
G-Film/UT	18.58 (0.74) [a]	0.34 (0.17) [a]	23.9 (1.2) [a]
G-Film/US	9.44 (0.09) [b]	2.61 (0.47) [bc]	28.8 (2.3) [b]
GR-Film/US	18.73 (0.80) [a]	2.98 (0.18) [c]	17.2 (0.8) [cd]
L-Film/US	7.68 (0.29) [c]	0.91 (0.08) [d]	25.1 (2.8) [a]
LR-Film/US	19.88 (1.8) [a]	2.39 (0.12) [b]	15.8 (0.3) [d]
P-Film/US	8.36 (0.64) [bc]	1.25 (0.16) [d]	20.2 (0.7) [c]
PR-Film/US	22.37 (0.30) [d]	2.15 (0.12) [b]	13.6 (0.3) [d]
PLA[*]	13.1 (0.1)	17.8 (1.3)	0.95 (0.15)
Starch[**]	155.2 (1.0)	410 (23)	-

WVP: Water vapor permeability, OP: oxygen permeability. Values with different letters are significantly different ($p \leq 0.05$). [*] Martínez-Sanz et al., 2012. [**]Fabra et al., 2018.

Compared to similar studies, in terms of barrier properties and production, the developed films in this study, outperformed benchmark biopolymers such as polylactic acid (PLA) (Martínez-Sanz, Lopez-Rubio, & Lagaron, 2012) and starch (Fabra, Martínez-Sanz, Gómez-Mascaraque, Gavara & López-Rubio, 2018) with the additional advantages that they can be directly produced from fungal biomass.

As expected, the water sorption capacity of the films, measured through gravimetrical assays (see Table 3), was higher in films formulated with the whole mushroom biomass than their counterparts from the residues. This can be directly related to the more amorphous character of the matrices, thus being more accessible to moisture than the more crystalline fractions left after the extraction processes. In fact, the discarded biomass had a higher content on water soluble β-

glucans, proteins, ashes and polyphenols which had a prominent hydrophilic nature which directly contributed to an increase in the water sorption. These results suggest that the lower permeability of the films prepared with the discarded mushroom biomass was partially related to the lower water diffusion because of the increased water sorption.

The water affinity of the films' surface was also evaluated through contact angle measurements which are normally used to estimate the degree of hydrophobicity of the material. The results are displayed in Figure 8. The estimated contact angle of films prepared with the discarded mushroom biomass ranged between 23 and 65º. The mushroom residues yielded materials with much more hydrophobic surfaces after the extraction processes; may be due to the removal of hydrophilic compounds and amorphous compounds after the water and alkaline treatment, respectively, and also to a lesser amount of free hydroxyl groups in the surface of the films due to β-glucan-chitin interactions. Other biopolymeric films made of starch (Jiménez, Fabra, Talens, & Chiralt, 2012), PLA (Bonilla, Fortunati, Vargas, Chiralt & Kenny, 2013), gelatin (George & Siddaramaiah, 2012) and PHB (polyhydroxybutyrate) (Pérez-Arauz et al., 2019; Urbina, Eceiza, Gabilondo, Corcuera & Retegi, 2019) showed lower values than the ones obtained in the present work from the residues, demonstrating the high hydrophobicity of these materials and their potential applicability for food packaging (Urbina et al., 2019; Wang et al., 2020). Therefore, the residues promoted a more hydrophobic behavior at the surface of the films due to the absence of water-soluble molecules (including β-glucans). However, they had higher water vapor permeability values since the water molecules could quickly diffuse through the biopolymer network. In contrast, in the films obtained from the discarded biomass, thickness could increase during the WVP assays with the hydration shell of the biopolymer network and the subsequent decrease in mobility, impeding water diffusion, thus being a determining factor in the WVP. Therefore, the more amorphous character

of the films obtained from discarded mushroom biomass may have promoted water sorption (higher values), producing greater swelling of the film matrix (lower contact angle) and, hence, yielding a structure that impedes water diffusion to a greater extent (lower WVP values).

A relevant aspect being highlighted is that, in contrast to the results observed for the discarded mushroom biomass, films from the residues could be generated by means of both UT and US. However, they did not show significant differences on the mechanical and barrier performance (see Supplementary Material S3).

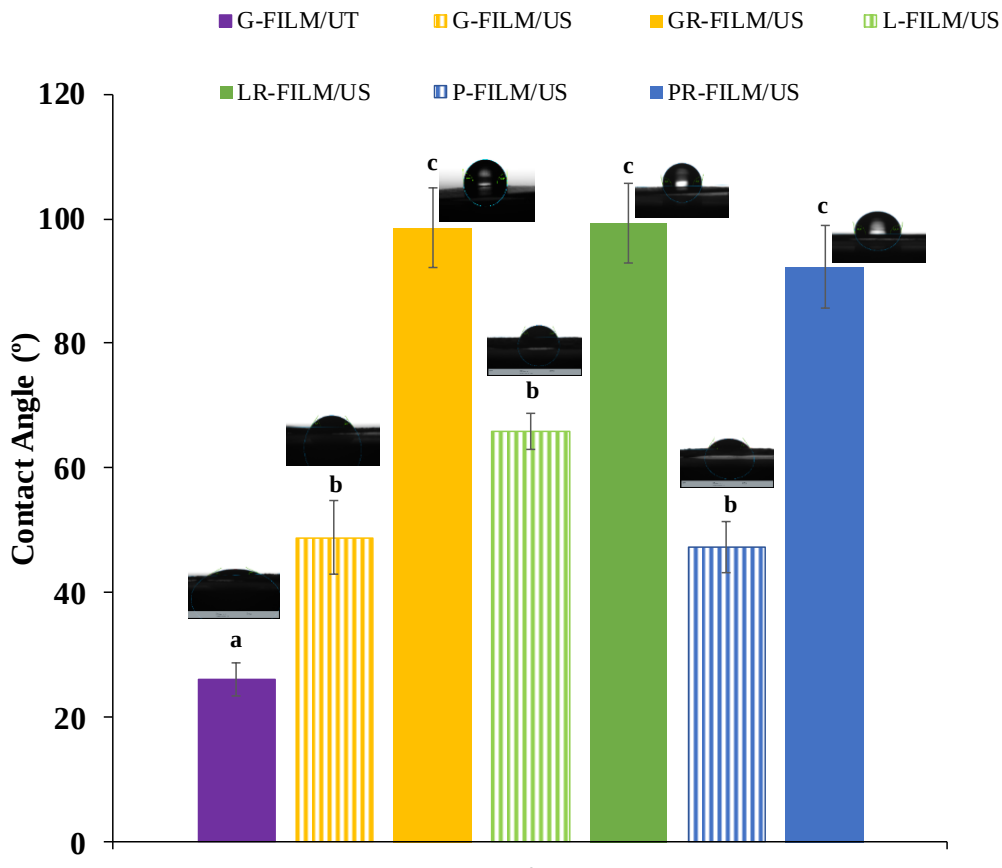


Figure 8. Contact angle values and representative images of water droplets formed on the air-side of the developed films. Values followed by different letters are significantly different ($p \leq 0.05$).

3.3. Disintegration tests according to ISO 20200.

One of the main advantages of biopolymers is that microorganisms can compost them within a certain time after use. The degree of disintegration is crucial to determine the bioplastics´ compostability. In this work, the disintegration behavior of the developed films was compared and the results are shown in Figure 9. In this process, the mass loss is considered as disintegrated material. Therefore, it calculates the degree of disintegration reached after the corresponding time.

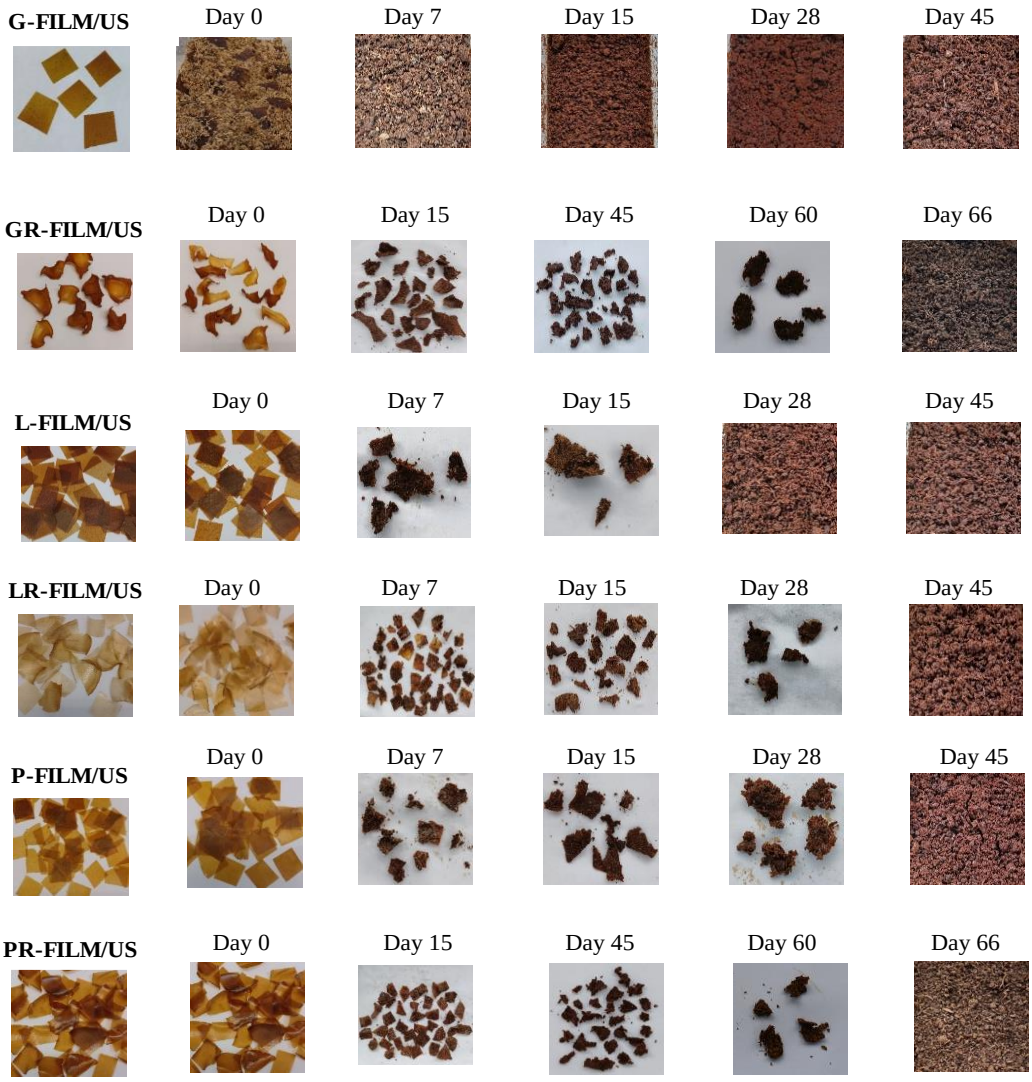


Figure 9. Visual appearance of the samples before, during and after of the disintegration process.

Interestingly, significant differences were observed between films prepared from the discarded biomass and those obtained from the residue generated after the aqueous and alkaline extraction processes. As clearly observed in Figure 9, although all of them were disintegrated before the end of the assay (90 days), thus indicating that the films were biodisintegrable, those prepared from the residue took more time than those prepared from the discarded biomass. This effect could be explained by the different compositions of the starting material and the physicochemical properties of the corresponding films. For instance, higher water uptake values (see table 3) were obtained for films prepared from discarded biomass, being these ones more easily disintegrated than their counterparts obtained from the residues.

Specifically, films prepared from *G. frondosa* discarded biomass were disintegrated entirely during the first week of the test. However, according to the standard, the test was maintained for a minimum of 45 days. In contrast, films generated from the residue obtained after the aqueous and alkaline treatments were more resistant than the film generated from the discarded biomass. In the first two weeks, the material absorbed water, doubling its thickness and giving it a gelly appearance. On day 45 of the test, the material´s structure was soft and gradually disintegrated into smaller pieces. Total disintegration of the sample occurred on day 66.

In the case of films prepared from *L. edodes*, L-Film/US samples had an amber color, and they became darker and softer, and a quick fragmentation started during the first week of the disintegration assay. The mass loss was evident and the physical aspect corresponded to a wet paper. On day 15, very few sample pieces remained and it was wholly disintegrated after four weeks (28 days). Regarding the LR-Film/US samples, it was observed that they initially presented a light shade and became more opaque once the incubation period started. Similar to *G. frondosa* films, the disintegration time was the main difference concerning to films prepared from *L. edodes* discarded biomass; while the samples from the discarded

mushroom disintegrated faster (28 days), the film from the residue took longer to reach a complete disintegration (45 days).

Similarly, films prepared from *P. ostreatus* discarded biomass (P-Film/US) disintegrated after 30 days of the assay. In contrast, those prepared from the residue (PR-Film/US) did not entirely degrade until day 66 of testing.

In general, the mass loss during the disintegration process until the films completely disappeared in the synthetic medium was an indicator that these materials could meet the compostability standard (EN13432) but, before affirming this, other tests corresponding to EN-13432:2001 should be carried out.

4. Conclusions

Fungal-based films were prepared from the discarded biomass of three different species (*Grifola frondosa, Lentinula edodes,* and *Pleurotus ostreatus*) and the corresponding residues were obtained after β-glucan extraction. The fungal biomass was mainly composed of carbohydrates, but significant amounts of proteins, lipids, and ashes were also detected. After the β-glucans extraction, polysaccharides were concentrated in the residue, mainly composed of chitin and residual β-glucans with some residual protein. Furthermore, the antioxidant activity and the phenolic compounds of the residues were significantly reduced compared to the mushrooms' discards. Chitin was more abundant in the *Lentinula* residue than in the other two residues and, although a significant amount of β-glucan remained in the residues, it was significantly higher for *G. frondosa* due to its greatest recalcitrance nature probably ascribed to stronger β-glucan-chitin bonds (as it did not have greater chitin content).

The discarded fungal biomass and the residues generated after β-glucan extraction were used to produce films (without plasticizers or other additives), which were characterized to evaluate their potential as food packaging materials. Overall, the films from the residue presented more desirable characteristics in terms of barrier, and mechanical properties, and visual appearance. Furthermore, according to the

ISO 20200, all the films were disintegrable since they completely disappeared in a synthetic medium before 90 days of the assay.

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Supplementary Material

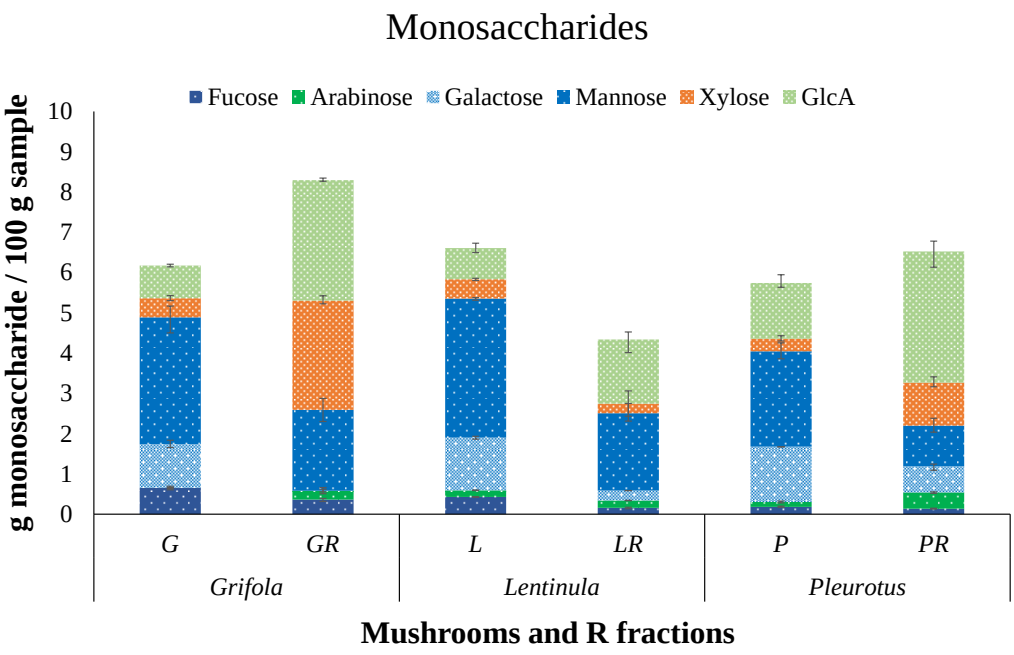


Figure S1 Relative carbohydrate composition of the discarded mushroom biomass and the residues generated after the aqueous and alkaline extraction. The results from the sugar constituents are expressed as g polysaccharide per 100 g total sample. Data correspond to the mean calculated values, n = 3.

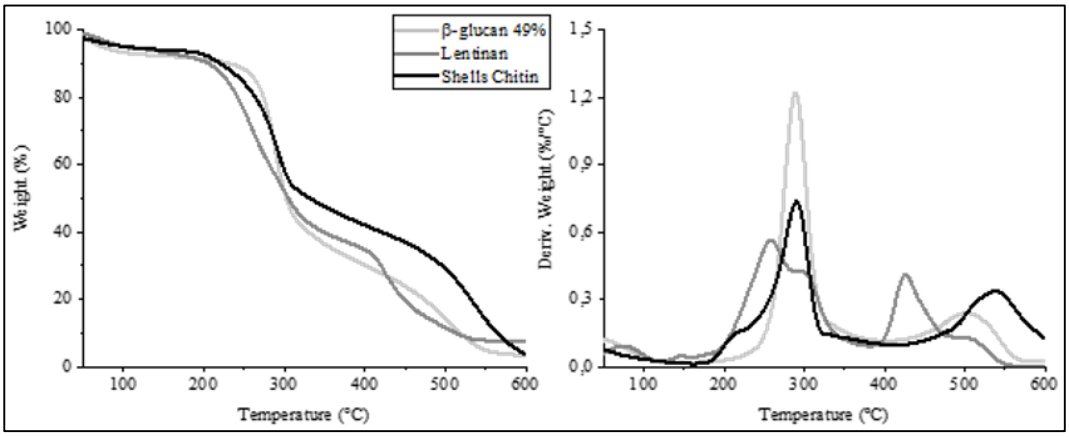


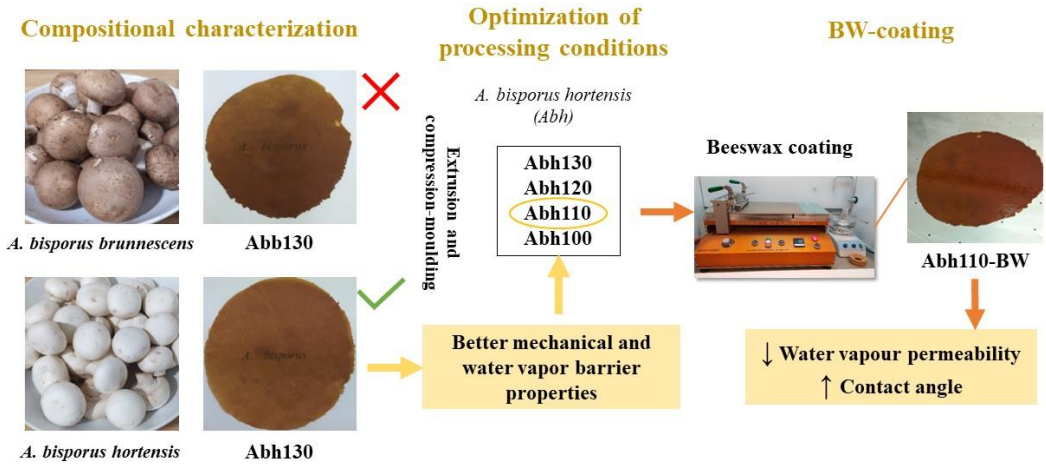
Figure S2 Derivative thermogravimetric (DTG) curves of commercial b-glucan 49%, lentinan and shells chitin.

Table S1. Tensile properties (E: Elastic Modulus, TS: Tensile Strength and εb: Elongation at Break), water vapour permeability (measured at 0-75% RH) and oxygen permeability values (measured at 50% RH) of the obtained films.

Film	E (MPa)	TS (MPa)	EAB (%)	**WVP** 10^{-14} **(Kg·m·Pa^{-1}· s^{-1}·m^{-2})**	**OP** $*10^{-19}$ **(m^3·m·Pa^{-1}·s^{-1}·m^{-2})**
GR-Film/UT	4595.22 (81.06)	28.60 (9.88)	0.87 (0.21)	19.01 (0.30)	2.74 (0.32)
LR-Film/UT	4205.86 (382.76)	27.72 (2.69)	0.92 (0.16)	21.34 (1.39)	0.93 (0.30)
PR-Film/UT	2573.34 (209.35)	19.08 (0.11)	1.14 (0.07)	20.41 (0.85)	1.56 (0.26)

Mean value (standard deviation).

3.2. Feasibility of *Agaricus bisporus* waste biomass to develop biodegradable food packaging materials.



This section is an adapted version of the following published research article:

Zaida Pérez-Bassart, Alcira Reyes, Antonio Martínez-Abad, Amparo López-Rubio, María José Fabra, 2023.

"Feasibility of *Agaricus bisporus* waste biomass to develop biodegradable food packaging materials"

Food Hydrocolloids, volume 142, 108861.

Abstract

This work aimed to valorise *Agaricus bisporus* biomass residues to produce bio-based and biodegradable films. To this end, two of the most widely consumed varieties, *Agaricus bisporus brunnescens* (Abb) and *Agaricus bisporus hortensis* (Abh), were compositionally characterised and then, films were formed by extrusion and compression-moulding. Proteins and β-glucans were the major components in both varieties. Abb showed a higher protein content, whereas the amount of polysaccharides and lipids was higher in Abh. Thermally-processed Abh-films showed better mechanical and barrier performance than their counterparts prepared with Abb, mainly ascribed to the higher carbohydrate and lipid content. Thus, Abh was selected to optimize the processing conditions in terms of energy consumption (from 100 to 130 °C) and barrier properties. As a result, 110 °C was selected as the optimum processing temperature for Abh-films since the mechanical properties in terms of strength were not significantly modified and barrier properties were significantly improved. The resulting film was coated with a beeswax layer to reduce its hydrophilic nature. The coated films showed similar mechanical properties than their counterparts prepared without the beeswax coating but displayed significantly better water vapour barrier performance. Furthermore, both films were easily disintegrated under composting conditions according to ISO 20200.

1. Introduction

Nowadays, the consumption of mushrooms has remarkably increased due to their excellent nutritional and health benefits ascribed to the presence of fungal polysaccharides (especially β-glucans), proteins, vitamins, antioxidants, and minerals. More than thirty-five species of edible mushrooms are commercially cultivated throughout the world, being around twenty of them produced at industrial scale (Ramos et al., 2019). Amongst them, *Agaricus bisporus* (*A. bisporus*) is one of the most consumed mushrooms in the world (Kała et al., 2021).

Specifically, the global white mushroom market (*A. bisporus hortensis*) accounted for USD 16.73 billion in 2020 and is expected to reach USD 27.39 billion by 2028 (Zion Market Research, 2021). Unfortunately, the sharp increase in industrial edible mushroom production has also resulted in the generation of large amounts of mushroom waste each year, including mushroom stems and misshapen mushrooms whose size or shape do not meet commercial standards. Therefore, recent academic and industrial interest in mushroom's waste is increasingly being focused towards its valorisation to fulfil the pursuit of circular economy and sustainability principles.

Discarded mushrooms and mushroom stems contain structural carbohydrates, such as β-glucans and chitin, very interesting for the development of biodegradable materials. This is a highly underexplored area with potential interest to shift the food packaging market towards more sustainable packaging solutions to develop bio-based and biodegradable polymers. In a recent work, biodegradable films were directly produced (without plasticizers or any other additives) by means of the solvent-casting method, either from discarded mushroom biomass generated in the industry or from the waste produced after a β-glucan extraction process from three different species (*Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*) (Feng et al., 2022; Pérez-Bassart, Martínez-Abad, Reyes, López-Rubio, & Fabra, 2023). However, the solvent-casting methodology lacks industrial applicability and it remains a challenge to process fungal biomass through industrial processing methodologies for plastics or bioplastics (such as extrusion or injection) which, to the best of our knowledge, remains unexplored.

Moreover, there are additional characteristics from bio-based polymers which need to be improved if these materials are intended for food packaging applications. Specifically, their oxygen and water vapour barrier properties, which are not comparable to those of benchmark synthetic polymers (Cheng et al., 2023), greatly limit their application in the field of food packaging. Thus, in order to overcome this aspect, different strategies such as multilayer coextrusion, layer-by-layer

assembly nanotechnology and surface coating (bilayer structures) have been considered for decades (Fabra, López-Rubio, & Lagaron, 2016; Fabra, López-Rubio, Ambrosio-Martín, & Lagaron, 2016; Koppolu et al., 2019; Rocca-Smith et al., 2019).

It is worth mentioning that organic biopolymeric coatings from lipid-based compounds have shown their advantages in improving water vapour barrier of packaging materials, as compared to the simple biodegradable blends or bionanocomposites, because of their good film-forming ability from the viscoelasticity behaviour (Bayer & Bayer, 2020; Péroval, Debeaufort, Despré, & Voilley, 2002; Reis, de Santana, Bilck, Grossmann, & Yamashita, 2018). Therefore, a bilayer film using hydrophobic food-grade substances is being proposed as a simple and feasible route to improve barrier performance and applicability (Youssef & El-Sayed, 2018; Zhang et al., 2019) of fungal-based films. Among natural waxes, beeswax (BW) is a food-grade additive (E-901) produced by honeybee. It contains a high proportion of fatty acid esters and long-chain alcohols, which give them interesting properties for edible coatings or films due to their effectiveness in reducing moisture and permeability (Cheng et al., 2023; Mohamed, El-Sakhawy, & El-Sakhawy, 2020).

Therefore, this work is aimed at evaluating the suitability of one of the most cultivated mushrooms (*Agaricus bisporus*) to produce biodegradable films by means of extrusion processing. Then, a bilayer film was produced and characterized in terms of mechanical and barrier performance and the disintegration of the developed films was compared.

2. Methods and materials

2.1. Reagents and materials

Two fungal varieties (*Agaricus bisporus brunnescens* -Abb- and *Agaricus bisporus hortensis* -Abh-) were kindly supplied by Centro Tecnológico de Investigación del

Champiñón, “Asochamp” (La Rioja, Spain). For this work, mushrooms were harvested at maturity, once the gills were exposed and the cap margin completely extended.

HCl (37%), sodium carbonate, gallic acid, Folin-Ciocalteu reagent, potassium persulfate, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2.5.7.8-tetramethylchromane-2-carboxylic acid (Trolox), phosphate buffered saline (PBS), trifluoroacetic acid (TFA), monosaccharide standards (including fucose, rhamnose, galactose, glucose, arabinose, xylose, mannose, galacturonic acid, glucuronic acid) and shrimp shell chitin were purchased from Sigma-Aldrich. Lentinan (min. 30% purity) was purchased from Carbosynth Biosynth (UK). Yeast β–glucan (49% purity) was obtained from Megazyme, and beeswax (BW) was provided by VWR (Madrid, Spain).

2.2. Chemical analysis of the raw material

A proximate analysis was made to each mushroom variety. The protein content was determined taking into account the two sources of nitrogen in mushrooms (proteins and chitin), so it was calculated on each mushroom by subtracting the nitrogen from D-glucosamine of chitin from the total elemental nitrogen. The chitin content and monosaccharide composition was determined by acid hydrolysis with HCl and TFA respectively, as previously described in (Pérez-Bassart, Fabra, Martínez-Abad, & López-Rubio, 2023). Carbohydrates were calculated as the sum of all sugar constituents. The lipid content was estimated after performing Soxhlet extractions, using 5 g of raw material with 250 mL of hexane for 8 h. The total phenolic content and the antioxidant capacity were determined using the assay based on the Folin-Ciocalteu method as modified by Singleton (Singleton, Orthofer, & Lamuela-Raventós, 1999) and by the ABTS method, respectively, as described in (Pérez-Bassart, Martínez-Abad, et al., 2023). The ash content was determined according to the standard method TAPPI T211. Finally, the β-glucan content was calculated by subtracting the α-glucan glucose from the total glucose

content and α–glucan content was determined colorimetrically upon enzymatic hydrolysis (K-YBGL from Megazyme, Ireland). All experiments were carried out in triplicate.

2.3. Production of fungal-based films

Initially, films were formed from mushrooms of the genus *Agaricus*, specifically with the varieties *Agaricus bisporus brunnescens* (Abb) and *Agaricus bisporus hortensis* (Abh), two of the most widely consumed mushroom varieties worldwide. Freeze-dried and ground mushrooms (<500 μm) were extruded at 130 °C and 100 rpm for 4 min, to ensure a homogeneous mixing of each sample. These conditions were selected based on literature (Alonso-González, Felix, Guerrero, & Romero, 2021; Andrade et al., 2022; Cebrián-Lloret, Martínez-Abad, López-Rubio, & Martínez-Sanz, 2022) and based on preliminary trials, guaranteeing a low torque for the good operation of the equipment. Subsequently, the films were formed by compression-moulding (Carver 4122, USA) at a pressure of 12 tonnes and 130 °C for 4 min and, then, two more minutes to improve homogeneity.

In the second part of this work, in an effort to reduce energy consumption, production cost and thus, the environmental impact, the processing conditions of Abh-films were optimized to determine the lowest temperature needed without negatively affecting the mechanical and barrier performance of the films. Thus, Abh-films were obtained at different temperatures (100, 110 and 120 °C).

Finally, Abh-films were coated with a BW layer using an automatic film applicator (Elcometer 4340 motorised) with a standard carriage speed of 30 mm/s and a separation distance of 200 μm.

Sample's nomenclature was “xT-y” where ‘x’ refers to the species (Abb or Abh), “T” specifies the processing temperature and “y” refers to BW (in the case of coated samples).

All films were stored in equilibrated relative humidity cabinets at 53% and 23 (±1) °C for 5 days prior to their characterization.

2.4. Scanning electron microscopy (SEM)

The cross-sectional morphology of the films before and after coating was examined by Field Emission Scanning Electron Microscope (FESEM) with a SCIOS 2 FIB-SEM at an accelerating voltage of 10 kV. Samples of each film were cryo-fractured after immersion in liquid nitrogen to investigate the cross-section of the samples. The samples were then fixed in the M4 aluminium sample holder and sputtered with a gold - palladium mixture under vacuum during 120 s.

2.5. Optical properties

The transparency of the films was estimated from the internal transmittance values measured using a spectrocolorimeter CM-26D (Minolta Co., Tokyo, Japan), using the methodology described by (Fabra, Talens, & Chiralt, 2009).

2.6. Water vapour permeability (WVP)

To determine the direct water vapour permeability of the films, the slope of the weight increase versus time curves was used, as in the ASTM 2010gravimetric method. The films were placed between the top (open O-ring) and bottom aluminium parts of a Payne permeability cup containing silica gel (3.5 cm diameter, Elcometer SPRL, Hermelle/s Argenteau, Belgium). A Viton rubber O-ring was placed between the film and the bottom of the cup to ensure a better seal. Permeability cups containing silica were then placed in a 75% RH equilibrated cabinet using a supersaturated sodium chloride salt solution, thus measuring water vapour permeability with a relative humidity gradient from 0 to 75% RH and at 23 (±1) °C. The tests were performed in triplicate for each sample.

2.7. Oxygen permeability (OP)

Oxygen permeability (OP) was calculated from recorded oxygen transmission rate (OTR) measurements using an 8001-oxygen permeability analyser (Systech Illinois, UK). Prior to exposure to an oxygen flow rate of 10 mL/min, with an exposure area of 5 cm^2, the samples were purged with nitrogen in the test cell,

which was also previously equilibrated in humidity. To obtain the oxygen permeability, the film thickness and the partial pressure of the gas were determined in each case. The experiments were carried out at 23 (±1) °C and 53 (±2) % RH and all tests were performed in triplicate.

2.8. Mechanical properties

Tensile tests were performed using a universal testing machine (Instron, USA). The tensile parameters (Tensile Strength -TS-, Elastic Modulus -E- and Elongation at Break -ϵb-) of the films were determined from the stress-strain curves, according to ASTM standard method D882.10 (ASTM, 2001). Pre-conditioned specimens (1 cm wide by 8 cm long) were mounted in film-extension grips of the testing machine and stretched at 50 mm min^{-1} until breaking. The relative humidity of the environment was held constant at 53 (±2) % during the tests, which were performed at 23 (±1) ◦C. At least eight replicates were obtained per formulation.

2.9. Water contact angle measurements

Contact angle values were estimated from measurements performed on a DSA25 equipment (Kruss) equipped with AD4021 image analysis software at ambient conditions. For this purpose, a drop volume of 3 μL of the probe liquid (distilled water) was placed on the film surface using a micro-syringe. Contact angle values were obtained by analysing the shape of the droplet after it was placed on the film for 10s and only on the beeswax side for the coated films. Ten replicates per sample were tested.

2.10. Water uptake

To estimate the water sorption capacity of the films, samples were previously dried at 60 °C to constant weight and then, they were placed in a 75% RH equilibrated cabinet with a supersaturated sodium chloride salt solution. Gravimetric measurements were performed until constant weight, and water sorption was

calculated as a percentage of weight gained with respect to the initial weight of the sample. The tests were carried out in triplicate for each different sample.

2.11. Disintegration tests

The disintegration test of the mushroom-derived films was carried out following several guidelines of the standard ISO 20200:2016. In this test, a synthetic solid consisting of a mixture of sawdust, rabbit feed, mature compost, corn starch, sucrose, corn oil, urea and water is used to simulate laboratory scale composting conditions and the samples to be tested are composted with this prepared solid matrix.

Three test reactors (consisting on a polypropylene container) per sample were prepared and each reactor contained 5g of film and 1 kg of wet synthetic solid. The mass ratio of the films vs. the wet synthetic residue was between 0.5% and 2.0% according to the standard method.

The reactors were incubated at a constant temperature of 58 ± 2 °C for a minimum period of 45 days. To guarantee a good composting process, the contents of the reactors was regularly mixed and moistened (final water content of 55% in total) and during the composting period the gross mass of the composting container was totally or partially restored according to the schedule established in the calendar indicated by the standard method. The degree of disintegration was determined after the composting period, by sieving the final matrix through standard 10 mm, 5 mm and 2 mm sieves (according to ISO 3310-1) in order to recover the non-disintegrated material. The mass reduction of the tested films was considered as disintegrated material and was used to calculate the degree of disintegration.

At the beginning of the test, the Total Organic Carbon (TOC) and Nitrogen (N) were analysed using an ICP-MS (Agilent Technologies. ICPMS7900, Spain) in order to ensure that the C/N ratio was between 20:1 and 40:1. TOC and N were also measured at the end of the assay.

2.12. Statistical analysis

The statistical analysis to determine the significant differences between samples was carried out with Statgraphics software, using one-way analysis of variance (ANOVA) at a significance level of $P < 0.05$.

3. Results and discussion

3.1. Compositional characterization of *Agaricus bisporus brunnescens* and *Agaricus bisporus hortensis*

Initially, the proximate composition and antioxidant activity of Abb and Abh species were determined before film production and the results are compiled in **Table 1**. As expected, proteins and β-glucans were the major components. The protein content ranged between 27 and 32 %, in accordance to previously reported values (Pérez-Bassart et al., 2023; Ramos et al., 2019). While Abb showed a higher protein content, the amount of polysaccharides was higher in Abh, being in both cases within the content previously reported for mushrooms (Ramos et al., 2019; Wang et al., 2014). The ash content was around 8%, in agreement with the existing literature (Banerjee et al., 2020; Krishnamoorthi, Srinivash, Mahalingam & Malaikozhundan, 2022) and the lipid content was higher in Abh than Abb, but in accordance to previously reported values, which may range from 0.1 to 16.3% (Kalač, 2012; Sande et al., 2019). The carbohydrate composition in both species consisted mainly of glucose, ascribed to the presence of glucans. Interestingly, the β-glucan content did not significantly differ between both species (ca. 22-23%), but Abh showed a higher α-glucan content than Abb, as previously reported in the literature (Sari, Prange, Lelley & Hambitzer, 2017). Chitin content was around 8-9% with no significant differences between samples and within chitin content values previously reported for mushrooms (Jones, Kujundzic, John & Bismarck, 2020). Galactose, mannose and xylose were also present in both species, being enriched in Abh, and probably arising from xyloglucans, xylomannans, and

mannogalactans. Negligible quantities of rhamnose, arabinose and galacturonic acid were detected in Abb. In contrast, Abh contained typical pectin components such as galacturonic acid, rhamnose, arabinose and galactose in higher amounts than Abb. Fucose, although in minor quantities, was also present in Abb, probably arising from fucomannogalactans. As observed in **Table 1**, both species contained polyphenol compounds, being greatest for Abb and closely related to its antioxidant activity (Pérez-Bassart et al., 2023).

Table 1. Raw material characterization and antioxidant capacity of *Agaricus bisporus* mushrooms (Abb and Abh).

Mushroom composition (dry wt. %)	**Abb**	**Abh**
Ash	8.21 ± 0.04 [a]	8.35 ± 1.15 [a]
Lipids	5.00 ± 0.52 [a]	9.89 ± 0.15 [b]
Protein	31.60 ± 0.76 [a]	27.58 ± 0.61 [b]
Total polyphenol content (mgGA/g DW)	8.92 ± 0.33 [a]	7.69 ± 0.33 [b]
TEAC (µg TE/g DW)	58.67 ± 1.06 [a]	52.28 ± 2.31 [b]
Carbohydrates (%) [*]	35.23 ± 2.01 [a]	43.63 ± 3.83 [b]
of which (g/100g dry basis)		
Fucose [**]	0.22 ± 0.05	< 0.1
Rhamnose [**]	< 0.1	0.10 ± 0.02
Arabinose [**]	< 0.1	0.40 ± 0.00
Galactose [**]	1.29 ± 0.07 [a]	1.75 ± 0.13 [b]
Mannose [**]	0.76 ± 0.04 [a]	1.30 ± 0.20 [b]
Xylose [**]	0.79 ± 0.24 [a]	0.89 ± 0.01 [a]
GalA [**]	< 0.1	0.47 ± 0.11
GlcA [**]	< 0.1	1.75 ± 0.13
GlcN (chitin) [**]	8.12 ± 1.20 [a]	9.05 ± 0.35 [a]
Glucose	24.05 ± 0.41 [a]	25.56 ± 2.31 [a]
of which (g/100g dry basis)		
β-glucans [***]	23.05 ± 0.44 [a]	22.31 ± 2.51 [a]
α-glucans [***]	1.00 ± 0.03 [a]	3.26 ± 0.20 [b]

Values with different letters in the same line are significantly different ($p \leq 0.05$).

The antioxidant capacity of the samples was expressed as μmol of Trolox equivalents (TE) per g of sample, and polyphenols content as mg of gallic acid equivalents (GA) / g of sample. GalA (galacturonic acid), GlcA (glucuronic acid) and GlcN (N-acetyl-glucosamine).

* As the sum of all detected monosaccharide constituents

** Monosaccharide composition determined by HPAEC-PAD.

3.2. Characterization of *Agaricus bisporus*-based films

Once the mushroom biomasses were characterized, Abb130 and Abh130 films were prepared and compared. The processing conditions for the production of the films were set based on previous literature data (Cebrián-Lloret et al., 2022) where other biomasses were thermally processed to prepare films. **Figure 1** shows the visual appearance, the spectral distribution of Ti values (as an indicator of transparency) and representative SEM micrographs of the films pre-conditioned at 53% RH. As observed, both films were translucent with a brownish hue. In fact, the spectral distribution of Ti of both films was typical of a multicomponent material and, associated with low transparent films, having heterogeneous refractive indices through their structures. Abb130-film presented an opaquer appearance which may be due to the presence of higher polyphenol content. This agrees with the lower Ti values in the range of 400 and 550 nm (see Figure 1), caused by a selective absorption of the coloured polyphenol compounds, being higher in Abb-based films.

Regarding the morphology of the films, Abb130 exhibited a smooth and homogeneous appearance, suggesting the formation of a compact arrangement of biopolymer chains. In contrast, a course aspect was appreciated in Abh130 films. These differences could be ascribed, among others, to the higher lipid content and, probably, to more heterogeneous polysaccharides in Abh samples (see **Table 1**), somehow hindering polymer chain packing.

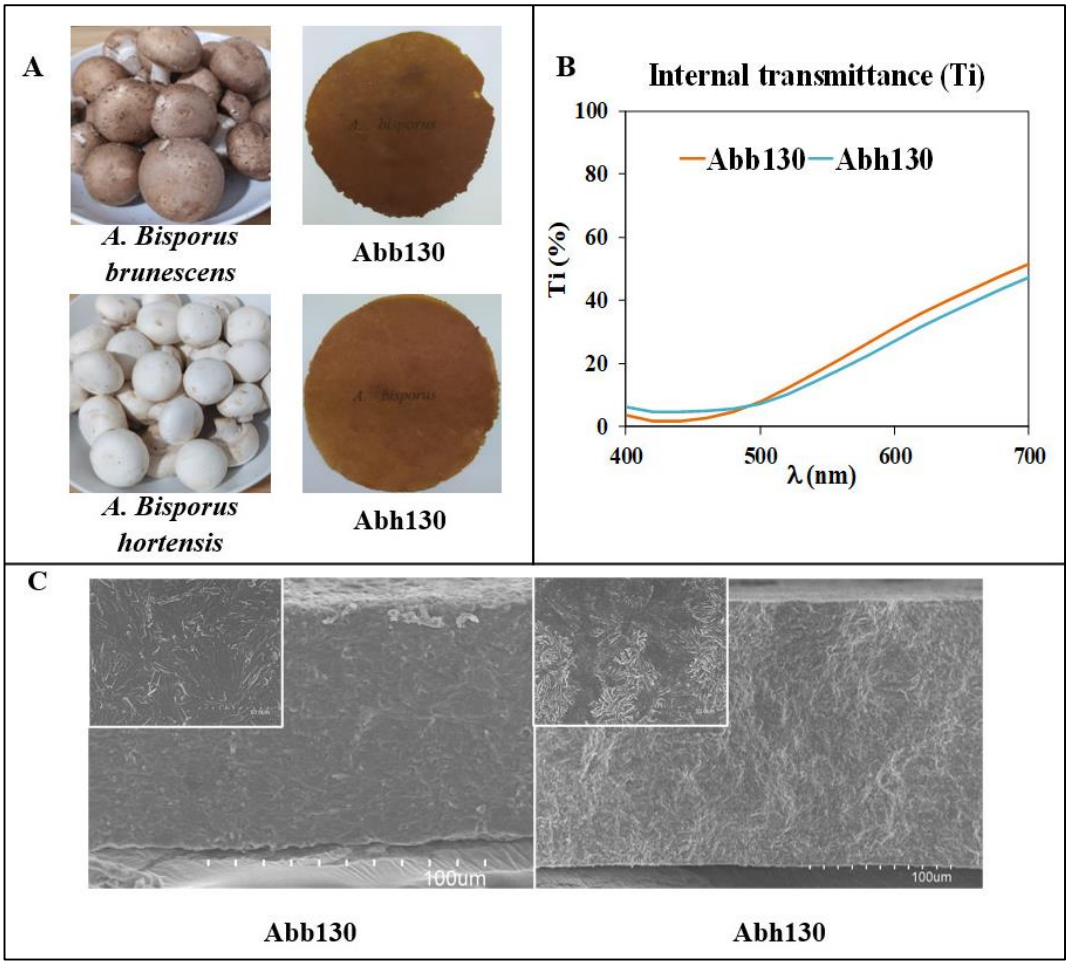


Figure 1. A) Visual appearance of mushrooms (left) and their respective films (right), B) spectral distribution of internal transmittance (Ti) and C) cross-section micrographs of Abb130 and Abh130 films.

Mechanical and barrier properties of the films were also determined to evaluate their potential as a food packaging material. The tensile parameters (E: elastic modulus, TS: tensile strength, εb: Elongation at break) obtained from the stress-strain curves of the fungal-based films are summarized in **Table 2**. In general, the mechanical response of the films was largely affected by the mushroom species. Abb130-films presented poorer tensile properties than other biodegradable packaging materials, such as thermoplastic starch and PLA films (Fabra, Martínez-

Sanz, Gómez-Mascaraque, Gavara & López-Rubio, 2018; Martínez-Sanz, López-Rubio & Lagaron, 2012). Specifically, Abb130-films were less rigid and mechanically resistant (lower E and TS values). The higher flexibility obtained for Abb130-films might be ascribed to its higher hydrophilic nature (lower lipid content, see Table 1) and the presence of higher relative content of mannogalactans, more hydrophilic than other heteropolysaccharides present in the other species (Abh). The mechanical properties of Abb-films were comparable to those reported for *Lentinula edodes* films processed by casting (E~89 MPa, TS~3.6 MPa) (Pérez-Bassart et al., 2023).

In contrast, the films obtained from Abh showed higher elastic modulus and tensile strength values, suggesting that the presence of a higher amount polysaccharides had a positive impact on the mechanical performance of the films. When compared with other fungal-based films, the mechanical response of Abh130-films agreed with the results previously obtained for discarded *Grifola frondosa* and *Pleurotus ostreatus* biomass produced by means of the solvent-casting method. Nonetheless, these films showed lower mechanical performance, in terms of strength, than other fungal-based films obtained from the waste produced after a β-glucan extraction process (E~ 2.5-4.5 GPa, TS~11-33 MPa, Pérez-Bassart et al., 2023) and biomass-based films obtained from marine seaweeds (green and red seaweed) (Cebrián-Lloret et al., 2022; Saedi, Kim, Shokri, Kim & Shin, 2023).

Table 2 also gathers the WVP results, which suggest that the mushroom species with higher polysaccharide and lipid contents produced films with better barrier capacity. This may be due, amongst other factors, to the hydrophobic nature of lipid compounds and the less hydrophilic character of heteropolysaccharides present in Abh samples. Accordingly, Abh130-films presented lower hydrophilic behaviour (higher contact angle value) and lower water uptake capacity (w_e) than their counterparts prepared with Abb (see **Table 2**).

Therefore, films prepared with Abh showed better mechanical and barrier performance, with the additional advantage that greater volumes of these mushroom species are industrially produced and thus, a large number of by-products and misshapen mushrooms are generated at industrial scale.

Table 2. Tensile properties (E: Elastic Modulus, TS: Tensile Strength and ε_b: Elongation at Break), water vapour permeability (WVP, measured at 0-75% RH), water uptake (measured at 75% RH) and contact angle values of films obtained from two different mushrooms (Abb and Abh).

	Abb130	**Abh130**
E (MPa)	56 ± 7 [a]	440 ± 38 [b]
TS (MPa)	1.2 ± 0.2 [a]	5.2 ± 0.7 [b]
εb (%)	6.4 ± 2.1 [a]	4.0 ± 0.8 [a]
WVP * 10^{-14} (Kg·m·Pa^{-1}· s^{-1}·m^{-2})	64.2 ± 0.7 [a]	30.1 ± 0.4 [b]
Water uptake (%)	23.3 ± 0.5 [a]	16.8 ± 0.8 [b]
Contact angle (º)	22.8 ± 1.0 [a]	32.8 ± 2.9 [b]

Values with different letters are significantly different ($p \leq 0.05$).

3.3. Optimization of processing conditions of Abh-based films

In an effort to determine the most suitable processing parameters, different processing conditions were evaluated with the aim of producing the fungal-derived films in a more energy efficient way without negatively affecting the physicochemical properties of the materials. Specifically, Abh-biomass was thermally treated in the mini-extruder at lower processing temperatures (100, 110 and 120ºC) and the films obtained were compared to those previously obtained at 130ºC. It should be noted that temperatures lower than 100ºC were not used due to the excessive torque of the equipment, which hindered proper processing.

Table 3 summarizes the tensile parameters, barrier properties, water contact angle and wettability of the developed films. When comparing the films obtained at different temperatures, no major differences on the mechanical properties were observed amongst them, except in those prepared at the lowest temperature (Abh100) which were slightly less deformable and ductile. These values are in accordance to those previously reported by (Pérez-Bassart et al., 2023) for other fungal biomass and, even higher than those obtained for other types of biomass, such as rice bran (Alonso-González et al., 2021). It should be noted that TS values were higher than 3.5 MPa, which is the setting parameter established by (Kim, Lee & Park, 1995) to determine if a material is suitable for packaging. The lower TS and εb values found for films treated at 100ºC suggested that this temperature was not effective in causing cell wall disruption, thus resulting in a less homogeneous mixture in which the different components could not be perfectly dispersed.

Interestingly, water vapour permeability was improved at lower temperatures although the hydrophilic nature of these films (measured by means of contact angle) was not significantly increased. This can be ascribed to a partial degradation of low molecular weight compounds present in the mushrooms' cell walls and protein (Pérez-Bassart et al., 2023).

As a result, 110 ºC was selected as the optimum processing temperature for Abh-films since the mechanical properties in terms of strength were not significantly modified and barrier properties were significantly improved, with the additional advantage that they were produced in a more energy efficient way.

Table 3. Tensile properties (E: Elastic Modulus, TS: Tensile Strength and ε_b: Elongation at Break), water vapor permeability (WVP) and contact angle values of Abh-based films processed at different temperatures.

	Abh130	**Abh120**	**Abh110**	**Abh100**	**Abh110-BW**
Processing Temp. (ºC)	**130**	**120**	**110**	**100**	**110**
E (MPa)	440 ± 38 ^{a}	481 ± 39 ^{a}	450 ± 60 ^{a}	460 ± 58 ^{a}	491 ± 42^{a}
TS (MPa)	5.2 ± 0.7 ^{ab}	5.7 ± 0.6 ^{a}	5.7 ± 0.8 ^{a}	4.3 ± 0.7 ^{b}	5.4 ± 0.3^{a}
εb (%)	4.0 ± 0.8 ^{a}	3.9 ± 1.1 ^{a}	4.6 ± 0.3 ^{a}	2.3 ± 0.1 ^{b}	3.7 ± 0.6^{a}
WVP * 10^{-14} (Kg·m·Pa^{-1}·s^{-1}·m^{-2})	30.1 ± 0.4 ^{a}	21.9 ± 1.0 ^{b}	24.6 ± 0.3 ^{bc}	26 ± 1.1 ^{c}	3.3 ± 0.5^{d}
Contact angle (º)	32.8 ± 2.9 ^{a}	33.9 ± 3.3 ^{a}	35.4 ± 3.4 ^{a}	29.7 ± 2.2 ^{a}	103.4 ± 3.8^{b}

Values with different letters are significantly different ($p \leq 0.05$).

3.4. Development of bilayer structures

A strategy to limit the moisture uptake of these materials is to develop bilayer structures (Zhang et al., 2020) in which fungal-based films are coated with a more hydrophobic food-grade material with good water vapour barrier properties. Therefore, Abh-films processed at the optimal conditions (110 ºC) were coated with beeswax to develop bilayer films. **Figure 2** shows the contact transparency images of the obtained Abh110 films, with and without the BW- coating, which were very similar. These results agreed with those found for the internal transmittance values (**Figure 2B**) in which a very similar pattern was observed for both samples. Both films displayed low transmittance values (i.e. low transparency) since they had a translucid appearance with a strong brownish hue. **Figure 2C** displays representative SEM micrographs of the cross-section in which a laminar-like structure was clearly observed in the BW-coated sample. The outer BW-layer was thinner than the Abh-based layer, which will have an impact on the hydrophobic nature and barrier properties of the developed films.

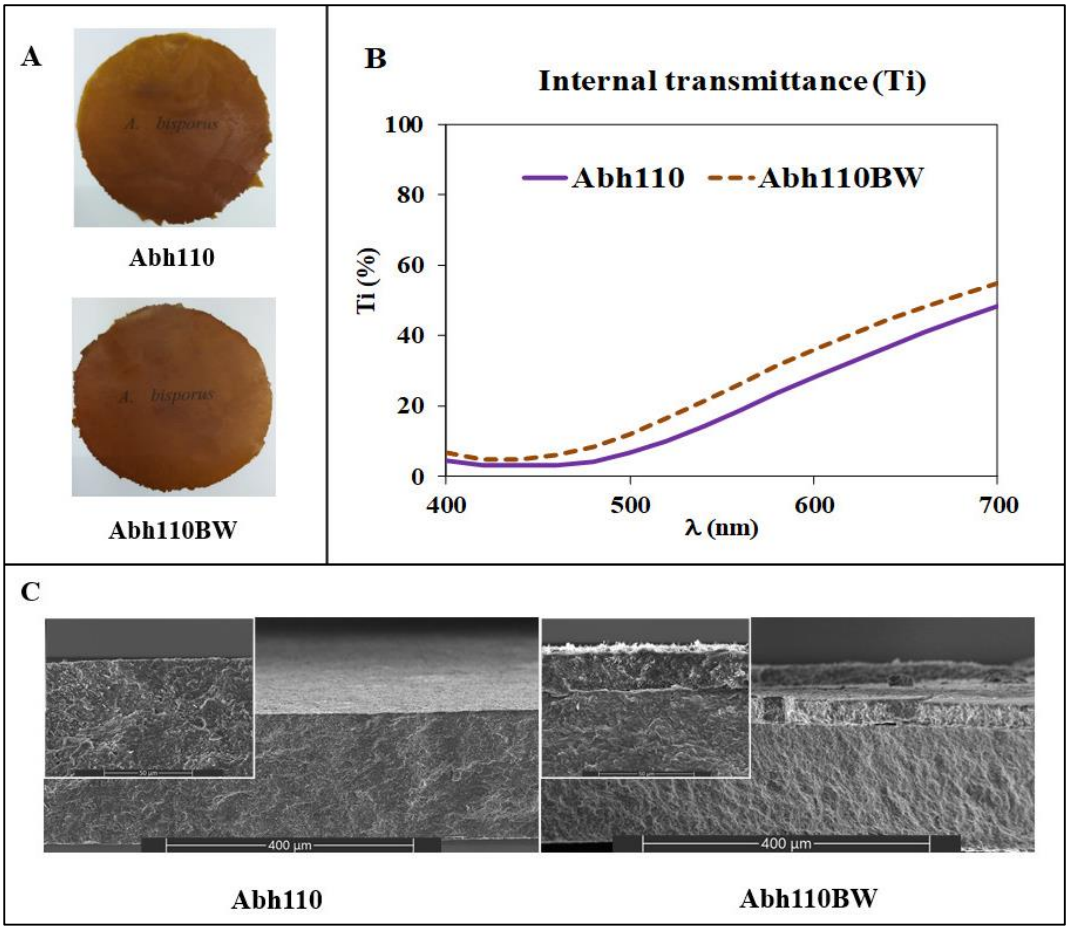


Figure 2. Visual appearance (A), spectral distribution of internal transmittance (Ti) (B) and cross-section micrographs (C) of Abh110-films with and without BW.

The mechanical and barrier properties of the Abh110 films with and without BW were evaluated, and the Abh110-BW results are summarized in **Table 3**. When comparing the films, the first clear observation was that the presence of the BW coating did not have a great impact in the mechanical performance of the films, as the tensile parameters did not significantly differ. No significant differences between the two samples were observed for oxygen barrier properties, having 1.88 ± 0.13 and 1.95 ± 0.40 $m^3 \cdot m \cdot Pa^{-1} \cdot s^{-1} \cdot m^{-2}$ for Abh110 and Abh110-BW, respectively. In contrast, the water vapour permeability was significantly improved up to~87% by adding the hydrophobic layer (see **Table 3**). As expected, the most

hydrophobic nature of Abh100-BW films was also confirmed by its higher contact angle values reported for this film. The improvement in barrier properties of biobased films upon lipid addition or the development of nanocomposites and multilayer strategies have been reported in previous works (El Mouzahim et al., 2023; Fabra et al., 2016; Gharibzahedi, Ahmadigol, Khubber & Altintas, 2023; Tagrida, Nilsuwan, Gulzar, Prodpran & Benjakul, 2023). However, not only the presence of lipids affect the barrier properties but also the morphology of the coating and how it is incorporated in the film (Mujtaba, Lipponen, Ojanen, Puttonen & Vaittinen, 2022; Susmita Devi, Kalita, Mukherjee & Kumar, 2022). In this regard, the development of a continuous, homogeneous and smooth BW coating at the film´s surface, contributed to a more efficient water vapour permeability reduction of the resulting bilayer films with the additional advantage that it can be easily scaled-up.

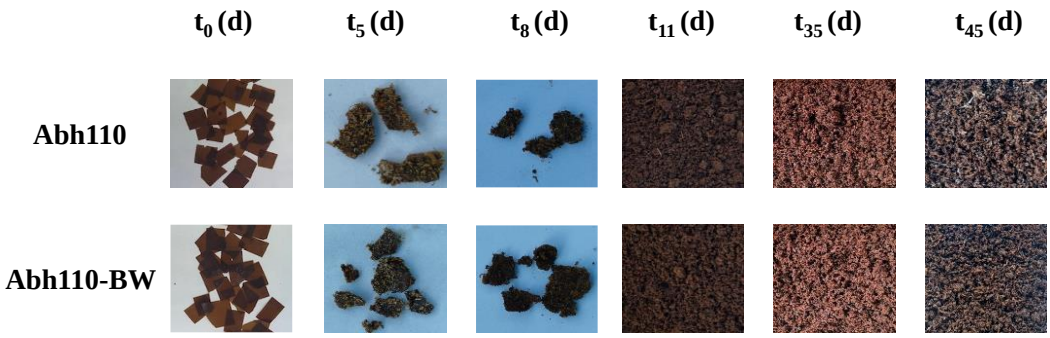
3.5. Disintegration tests according to ISO 20200

Disintegration tests of the optimized films (Abh110 and Abh110-BW) are shown in **Figure 3**. As expected, films were disintegrated before the end of the assay (90 days), thus evidencing that they were biodisintegrable. However, although both samples showed a high rate of disintegration, reaching 100 % after 11 days of storage, small differences were observed between the samples. On day 8, the mass loss was evident in both samples but fewer and smaller Abh110 pieces were observed as compared to the coated film. This can be ascribed to the lower hydrophobic nature of the uncoated samples. Specifically, the higher water absorption in Abh100-film could accelerate the hydrolysis activity, which contributed to the disruption of biopolymer bonds and, thus, to a faster disintegration process (Wongphan, Panrong & Harnkarnsujarit, 2022).

Even though films were completely disintegrated after only 11 days of assay, the test was maintained for a minimum of 45 days, according to the standard (ISO 20200). The control parameters (TOC, N and volatile solids) were measured at the

beginning and the end of the assay (see **Supplementary Material S1**) to corroborate they ranged according to the standard.

Figure 3. Visual appearance of the samples before, during and after of the disintegration process.



Supplementary Material

Table S1. Comparison of the values of the different measured parameters.

Parameter	Sample	At the beginning of the assay	At the end of the assay
Volatile Solids (% d.m.)	Abh110	**97.29**	**87.97**
	Abh110-BW		**87.61**
Total Organic Carbon (% d.m)	Abh110	**43.16**	**42.36**
	Abh110-BW		**40.74**
N (% d.m)	Abh110	**1.70**	**2.26**
	Abh110-BW		**1.97**

IV. GENERAL DISCUSSION

General discussion of the results

4.1. Structure and bioactivity study of different fractions obtained from mushrooms biomass

In this chapter, the relationship between composition, structure and bioactive potential of different fractions extracted from mushrooms was studied.

In the first work, biomass from three widely consumed mushrooms (*Grifola frondosa*, *Lentinula edodes* and *Pleurotus ostreatus*) was used, on which a series of aqueous and alkaline extractions were consecutively applied to study the release of compounds of interest on each fraction. Mushroom β-glucans are differently structured in the mushroom cell wall and can be more or less bound to proteins or chitin. The highest yields of the water-soluble fraction (F1) were found for *Lentinula edodes* and *Pleurotus ostreatus*, with about 80% of all glucans present in this fraction. This evidenced a great potential for the use of these green aqueous extracts for different applications, as explored throughout this thesis. Although the first alkaline fraction displayed significantly higher purity in β-glucans, of interest for bioactive applications, this was at the expense of lower yields. Furthermore, and despite the consecutive extractions of different aggressiveness, a significant amount of biomass remained in the final solid fraction. Glucan recalcitrance was especially patent in *Grifola frondosa*, with 15.9 ± 0.6 % of the biomass in the final residue and a higher presence of protein in the aqueous fractions. On the other hand, glucan populations from *Lentinula edodes* were extracted either in the aqueous or alkaline fractions, leaving a solid fraction with higher chitin purity. Both cold and hot water extracts showed a high antioxidant capacity (between 250-294 μmol TE/g), closely related to higher polyphenol content (34-41 mg GA/g). The results obtained were enlightening for understanding the complex structure of the mushroom cell wall and the structural heterogeneity among different species. They also highlighted the need for understanding the structural hierarchy in the

fungal cell wall of a specific species before designing strategies to separately valorise protein, glucans or chitin rich ingredients.

The second work of this chapter focused on the use of aqueous fractions of different *Pleurotus* (S1-S6) and a residue from *Pleurotus ostreatus* (stipes, S3R), as potential bioactive ingredients. Aqueous fractions with and without an additional purification step by ethanolic precipitation were studied. Purification removed lower molecular weight compounds reducing protein and chitin contents and enriching β-glucans of medium to high molecular weight, with peaks >800 kDa in the purified S3, S3R and S5 samples which could correspond to some heteropolysaccharides, high Mw glycoproteins or glucan-chitin complexes. Despite evident compositional differences, it was not possible to accurately associate the bioactivity with a single compound in the fractions. Without a proinflammatory stimulus (LPS), extracts from S3 and S3R exerted a strong and significant activation on TLR4 mediated NF-κB response. These results could not be related to a very high increase in β-glucan content after purification. However, these samples did have an increase in other rhamnose-rich heteropolysaccharides and showed a specific degradation pattern of certain thermostable structures. A more specific immunomodulating in-vitro cellular assay showed a substantial increase in the levels of interleukin (IL)-8 in THP-1-differentiated macrophages for S3 and S3R samples, indicating a high immunostimulatory potential. These results evidenced a high potential for aqueous extracts of *Pleurotus* species, while pointing out the need for further research to relate specific compositional or structural patterns with their immunomodulating properties, all of which promoted the recently funded project PANACEA (AGROALNEXT/2022/015) within the NextGeneration EU call AGROALNEXT.

4.2. Relationship between structure and functional properties of mushroom biomass as texturisers for food applications

In this chapter, the technological properties of mushroom extracts were addressed, to further explore alternative uses of discarded stipes or whole mushrooms through the formation of texture-modifying ingredients for food applications. The additional bioactivity, both antioxidant and antiviral, was also analyzed as a means to produce green, affordable ingredients with both technological and bioactive properties.

In this way, a first work was carried out exploring the behavior of gel-like structures obtained from the tested fractions of *Pleurotus ostreatus* (mushroom and stipes), both purified and unpurified. The results of the nanostructural properties analysis (SAXS) revealed very different structures from mushroom and residue (stipe) hydrogels, showing a more branched and clustered structure in the case of the stipes. Gels could actually not be formed with unpurified mushroom samples. Purified samples (RAFp and AFp) showed higher viscosity, which was attributed to the enrichment in β-glucans. Analysis of the texture profile of the hydrogels also revealed a higher hardness in the materials obtained from the purified samples, a consequence of their higher content of β-glucans and glycoproteins. No significant differences in springiness were found, while cohesiveness and chewiness, closely related to strength, was lower for hydrogels from purified fractions. This knowledge advances are relevant for the selection of food matrices where to apply them. In parallel, the antioxidant and antiviral activity of the fractions was measured as β-glucans are also recognized as biologically active carbohydrates. Higher antioxidant activity was quantified for the fractions obtained from the mushroom, both unpurified and purified (138.55±1.20 and 118.31±1.20 μmol TE/g respectively), compared to the stipe. On the other hand, only for the purified extracts from the stipes, murine norovirus (MNV) titers were significantly reduced below the detection limit. It follows that the higher structural complexity of the

stipe fractions, and both their amount and type of polysaccharides had a positive effect on the antiviral activity.

Another property highly valued in the food industry, which was studied in a second work included in this chapter, was the emulsifying capacity. This work was completed with the study of the different emulsions formed with the previously generated fractions (3% solids), with low (10%) and high concentration (60%) of incorporated oil. The results were related to the composition and structure of the fractions. The fractions from the whole mushroom (AF and AFp) showed greater emulsifying capacity than those from stipes, which was related to their higher content of glycoproteins accessible to act as surfactants. The best results were obtained from the purified fraction of *P. ostreatus* mushroom, since it showed greater stability in the incorporation of up to 60% of oil, directly related to a better distribution and smaller size of the oil droplets in the creaming index and particle size measurements. Emulsions were resistant to heat treatments but suffered from creaming after freeze-thaw cycles except for low oil content samples (10 % v/v). The results pointed out the potential versatility of cheaply and sustainably produced aqueous extracts from *P. ostreatus* for food applications, which technological properties (gelling, emulsifying properties) depend on the part of the mushroom and the predominance of either glucan or glycoprotein contents.

4.3. Use of mushrooms waste biomass for the formation of biodegradable film-like materials for food packaging applications

In the last chapter of this thesis, the potential use of fungal biomass as food-contact or packaging materials was explored. In the first work of this chapter, the spent fungal biomass generated after the sequential extraction of β-glucan-rich fractions from the three mushrooms studied (*G. frondosa*, *L. edodes* and *P. ostreatus*) was used for the formation of films by casting of the aqueous dispersion, and compared with films generated from the whole biomass. A very relevant finding of this work

was that a physical pre-treatment, either homogenization or ultrasound significantly promoted the formation of continuous films with relatively good mechanical and barrier properties without the addition of any further additives. The spent biomass, purer in β-glucans and chitin, formed films with much more rigid behaviour, lower elongation at break and higher hydrophobicity than those formed from whole mushrooms. The lower water vapour permeability for films produced from whole mushrooms was related to a greater swelling in the water uptake tests. Oxygen permeability values for both types of films were promising, even improving some values of benchmark bioplastics, such as PLA or starch.

In the last work, we explored the production of films through minimal processing (extrusion and compression-moulding) of the abundant and cheap biomass from discards of two of the most widely consumed mushrooms in the world, *Agaricus bisporus hortensis* (Abh) and *Agaricus bisporus brunnescens* (Abb). Both varieties were characterized in terms of composition and successfully processed by this method, showing significant differences in the properties of the materials. The compositional characteristics of the initial biomass were decisive to understand the mechanical and barrier properties of the obtained films. The presence of significant quantities of lipids and a greater quantity and complexity of polysaccharides had a positive impact on the mechanical and barrier performance of Abh films. This was reflected in a higher hydrophobicity (contact angle values of 32.8 ± 2.9 ° for Abh-films as compared to 22.8 ± 1.0 ° obtained for Abb-films), lower water vapour permeability, lower water uptake, higher elastic modulus and higher tensile strength of Abh films. A further optimization of the production of these films resulted in the decrease of the processing temperature to 110°C and the application of a beeswax coating to enhance water resistance and hydrophobicity (contact angle 103.4 ± 3.8 °), without altering mechanical performance.

All the films tested in both scientific contributions were completely disintegrated within 45 days under composting conditions (ISO 20200), evidencing fungal biomass as a potential cheap source for new all natural (no additives) food-contact

or packaging materials. This new application range adds up to the previous works building on ingredients with technological (gelling, emulsifying) or bioactive (immunomodulating, antioxidant, antiviral) properties. Thus, the results of this thesis open up the possibility of producing a wide portfolio of ingredients, either within a cascade circular approach or through a more directed processing. Further research is being conducted to more specifically relate structural characteristics with their functionalities and generate proof of concept demonstrators at larger scale for some of these applications.

V. CONCLUSIONS

Conclusions

- A sequential extraction process provided valuable information on the hierarchical complex structure of the cell wall from three globally relevant mushroom species and evidenced the potential for the extraction of multiple fractions with varying properties for diverse applications.

- The great differences in the cell wall architecture pointed out the need for designing specific valorisation strategies, customizing or simplifying extraction processes to the most effective treatments. As an example, *Grifola frondosa* contained a β-glucan more bound to chitin and of higher recalcitrance, while that of *Pleurotus ostreatus* was much more bioaccessible producing interesting water-based extracts.

- Aqueous fractions obtained from whole mushrooms of the genus *Pleurotus* or from the stipe, showed both immunostimulatory and anti-inflammatory activity through in-vitro cell assays. The most relevant results were obtained from *P. ostreatus* from both fruiting bodies and stipes. Despite partial purification of these aqueous fractions by ethanol precipitation, a direct relationship between a specific compound and the biological activity could not be obtained.

- Stable gels could be formed from aqueous fractions of the stipes of *Pleurotus ostreatus* or fruiting bodies only after purification. These gels showed higher hardness in the purified samples while the gels obtained from the unpurified stipe were found to be chewier and more cohesive. It therefore seems that a higher contribution of compounds with a higher molecular weight favoured the hardness of the hydrogels.

Conclusions

- These fractions, rich in soluble β-glucans, showed high bioactivity. Extracts from whole mushrooms showed higher antioxidant capacity, while the fractions with highest antiviral capacity were the purified fractions from stipes, probably due to their higher glucan structural complexity.

- On the other hand, unpurified fractions with a higher presence of glycoproteins showed the best emulsifying capacity at 10% or 60% oil concentrations, with better results for fruiting bodies compared to the stipe alone. Emulsions were stable to heat but heavily affected by freezing.

- Solid spent biomass after the cascade aqueous and alkaline extractions could be pretreated with ultrasounds or homogenization processes to obtain continuous films by the casting method, without any further additives. These films were more rigid and hydrophobic when compared with films generated from whole mushrooms, but fully disintegrated in composting conditions.

- Films were also generated by extrusion and compression-moulding of two varieties of *Agaricus bisporus*. The common white button mushroom had the best mechanical and barrier properties, mainly attributed to the presence of lipids and more complex polysaccharides. Optimization of this extrusion process and application of a beeswax coating improved their barrier properties, without affecting biodisintegration (ISO 20200) under composting conditions.

VI. ANNEXES

Annex A: list of publications included in this thesis

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Compositional differences of β-glucan-rich extracts from three relevant mushrooms obtained through a sequential extraction protocol

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ARTICLE INFO

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Waste valorisation
Chitin
Fungal β-glucan
Polysaccharide fractionation

ABSTRACT

In this work, a sequential fractionation protocol (cold and hot aqueous and alkaline extractions) and detailed compositional analysis (gross composition, monosaccharide analysis, FTIR, TGA) was applied to three relevant mushrooms in terms of global production (*Grifola frondosa*, *Lentinula edodes* and *Pleurotus ostreatus*) aiming to understand what is preferentially extracted during fractionation and how β-glucan extraction is affected by mushroom source. Room temperature aqueous extracts showed highest overall yields (56.3–82%) consisting of proteins, sugars and polyphenols. β-glucan content was highest in *P. ostreatus* and was concentrated in the more soluble fractions. On the contrary, a recalcitrant β-glucan in *G. frondosa* was mainly present in the residue (7.38%). *L. edodes* showed β-glucan populations distributed along aqueous and alkaline extracts, higher abundance of non-glucan polysaccharides and higher chitin purity (47.78%) in the residue. This work sets the basis for the rational design of extraction processes aiming to valorise mushroom biomass.

1. Introduction

Edible mushrooms are a good source of interesting carbohydrates, proteins, vitamins, antioxidants and minerals with low fat levels and low caloric content, thus making them a nutritious and healthy food source (Kalač, 2012). Probably linked to the wide recognition of their nutritional and health benefits, the economic importance of mushrooms is also growing, with a considerable global production increase from 7.5 million tonnes in 2009 to 11.8 million tonnes in 2019 (Schill et al., 2021). Market requirements of uniform size, shape and colour for these type of foods result in the removal of excess stipe length and rejection of mushroom biomass with slight discoloration and gross size variations (Ramos et al., 2019). This discarded biomass could be upcycled to obtain added-value compounds, in line with current circular economy practices.

The fungal cell wall is mainly formed by two types of structural polysaccharides, a rigid chitin fibrillary structure, and a matrix-like structure consisting on β-glucans, α-glucans and glycoproteins (Ruthes et al., 2016). Fungal glucans comprise structurally different polymers of D-glucopyranose (D-Glcp), which, despite of their simple monosaccharide composition, show a large diversity regarding molecular mass, anomeric configuration, position and sequence of glycosidic bonds along the chain, branching degree, branch composition and chain conformation (de Jesus et al., 2018). According to the anomeric structure, two main groups of fungal glucans are found, i.e. α-D-glucans and β-D-glucans, the last ones being the most abundant polysaccharides in fungal cell walls (Synytsya et al., 2009). β-glucans consist of β-(1 → 3) and (1 → 6) linkages with a huge structural diversity, being lineal or branched, and amorphous or microfibrillar structures (Bai et al., 2019), which can be embedded in the crystalline chitin (Ifuku et al., 2011). The interest in fungal polysaccharides, and especially in β-glucans, derives from their proven biological activity, e.g. antioxidant, antiinflammatory, anticancer, anti-diabetic, antimicrobial, antilipidemic, hypoglycemic, and immunomodulatory activities (Bai et al., 2019), thus making them attractive as active ingredients with potential applications in food, medicine, pharmacy, cosmetics, chemical and feed industries (Mirończuk-Chodakowska et al., 2021).

Specific fungal species, such as *Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*, are even considered medicinal, being recommended for therapeutic applications (Badalyan et al., 2019). *G. frondosa* (Maitake) has been widely used as a traditional food supplement in China, Japan and Korea (Ji et al., 2019) because of the recognized health

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Composition, structural properties and immunomodulatory activity of several aqueous *Pleurotus* β-glucan-rich extracts

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ARTICLE INFO

Keywords:
Beta-glucan
TGA
FT-IR
Immunostimulatory properties
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ABSTRACT

In this work, aqueous extracts from six different *Pleurotus* species were obtained and their yield, gross composition, β-glucan content, monosaccharide profile, thermal stability, molecular weight distribution, and FT-IR were analyzed before and after purification through ethanol precipitation of the carbohydrate-rich fractions. The bioactivity (anti-inflammatory and immunomodulatory activity) of the various fractions obtained was also analyzed in three different cell cultures and compared with a lentinan control. The trend observed after purification of the aqueous fractions was an increase in the concentration of polysaccharides (especially β-glucans), a decrease in ash, glucosamine and protein content and the elimination of low molecular weight (Mw) compounds, thus leaving in the purified samples high Mw populations with increased thermal stability. Interestingly, all these purified fractions displayed immunomodulatory capacity when tested in THP-1 macrophages and most of them also showed significant activity in HEK-hTLR4 cells, highlighting the bioactivity observed for *Pleurotus ostreatus* (both the extracts obtained from the whole mushroom and from the stipes). This specific species was richer in heteropolysaccharides, having moderate β-glucan content and being enriched upon purification in a high Mw fraction with good thermal stability.

1. Introduction

Edible mushrooms and their extracts have been used both in food and traditional medicine since ancient times, not only due to their nutrient composition, but also because of their beneficial biological activities, including immunomodulatory properties, thus being included in nutraceuticals and pharmaceuticals [1,2]. Mushrooms with known immunomodulatory capacity contain diverse organic compounds (including terpenes and terpenoids, proteins like lectins and various polysaccharides) responsible for this activity with very diverse molecular weight and structure [3]. Immunostimulatory polysaccharides like glucans, mannans, arabinogalactans, fucoidans, galactans and xylans can be found in the cell walls of mushrooms and, specifically, β-glucans have attracted a great deal of research interest, due to their proved bioactive properties [4]. Structurally, fungal β-glucans consist of polysaccharide chains of β-(1,3) linked glucose subunits, with β-(1,6)-linked branches [5]. In fact, β-glucans are considered a key pathogen-associated molecular pattern (PAMP), recognized upon fungal infection and responsible of triggering the immune response [6]. The modulation of the immune system by β-glucans is rather complex, depending on many factors not yet fully understood. Immunostimulation has been described to be mediated through the binding of polysaccharides like β-glucan to several receptors such as Dectin-1, complement receptor 3 (CR3), selected scavenger receptors, and lactosylceramide (LacCer), triggering signal transduction in several immune cells [7]. The activity of these β-glucan receptors seems to be highly dependent on the cell types and, for instance, recognition of β-glucan by Dectin-1 on macrophages activates the downstream signaling pathway, activating phagocytosis, ROS generation, microbial killing, and cytokine production [8]. However, it is important to note that the immunomodulatory effects of glucans depend on their structural characteristics, including branching degree, solubility, molecular weight, polymer charge, and conformation in solution [9]. It is still unclear how structural differences of glucans might affect their biological functions. Moreover, whether these

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Antiviral and technological properties of β-glucan-rich aqueous fractions from *Pleurotus ostreatus* waste biomass

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ARTICLE INFO

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Hydrogels
Antiviral activity
RMN
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ABSTRACT

In the present work, aqueous fractions were extracted from the discarded *Pleurotus ostreatus* biomass (AF) and from a waste product (RAF) generated from it in the industry (stipes) with the aim of valorizing these wastes into new food ingredients. Compositional, structural and functional analysis, before and after a purification step (p) to enrich its content in β-glucans, was carried out. Moreover, the rheological properties and the nanostructure of hydrogels obtained from the β-glucan extracts were analyzed. The unpurified fractions stood out for their significantly higher antioxidant capacity (AF, 138.55 ± 1.20 and RAF, 118.31 ± 1.20). As a result of the ethanolic purification process, purified samples had lower protein, ash, polyphenols and α-glucans content than the unpurified ones. Furthermore, extracts from stipes had a greater structural complexity, with more branched β-glucans than their counterparts obtained from the whole biomass, which had a positive impact on the antiviral activity against murine norovirus. Interestingly, purified samples formed stronger hydrogels but the gelation mechanism depended on its composition and structure. AFp was able to form a gelling network with a more tightly packed structure, ascribed to the presence of less branched β-glucans, showing the highest hardness value (2.87 ± 0.27 N). Unpurified RAF fraction showed hydrogels with greater cohesiveness and chewiness values (4.74 ± 0.55 N and 9.08 ± 0.87 N, respectively). In contrast, the presence of small side chains in RAFp could also act as reinforcement agents in hydrogels formed by means of intermolecular associations, with the additional advantages of having greater antiviral activity and coming from agroindustrial residues (stipes).

1. Introduction

The consumption of edible mushrooms is increasing every year due to their nutritional advantages (rich in valuable proteins, minerals, and dietary fibres) (Lu, Lou, Hu, Liu, & Chen, 2020; Maity et al., 2021). However, its increased production and consumption is causing a large number of by-products (caps, stipes and mushroom that do not comply with commercial standards) and posing an environmental challenge and management cost for the industries that market these products (Antunes et al., 2020; Grimm & Wösten, 2018; Kumar et al., 2021).

Pleurotus ostreatus is one of the most consumed and affordable species of cultivated mushrooms globally (Elhusseiny et al., 2021; Mishra, Tomar, Yadav, Vishwakarma, & Singh, 2022). It is an excellent supplier of health-promoting molecules such as vitamins, amino acids, glucans, and essential fatty acids. Specifically, mushroom cell walls are rich in β-glucans, long and short-chain polymers of glucose units with β-1,3 and β-1,6 linkages, that are responsible of the linear and branching structures, respectively (Ehren et al., 2020). Structural, physical, and technological properties of β-glucans vary from different species, cultivars, growing environments, drying conditions and isolation/extractions methods (Bai et al., 2019; Shaheen et al., 2022), with also important changes on their bioactive properties. For instance, β-glucans are used in the food industry as gelling agents and thickeners in the production of low-fat foods with improved texture properties (milk, bread, or yogurt) (Du, Meenu, Liu, & Xu, 2019; Kaur, Sharma, Ji, Xu, & Agyei, 2020). Specifically, hydrogels are three-dimensional, hydrophilic, polymeric networks capable of holding large amounts of water. Moreover, β-glucans also constitute a soluble fibre with a powerful prebiotic effect and

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Ultrasound-treatment as a promising strategy to develop biodegradable films obtained from mushroom waste biomass

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ARTICLE INFO

Keywords:
Valorisation
Biomass
Biopolymers
Packaging
Fungi

ABSTRACT

This work reports on the valorisation of discarded mushroom biomass generated in the industry and the waste produced after a β-glucan extraction process from three different species (*Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*) for the production of biobased and biodegradable films. Initially, the composition of the starting material was characterized before film production. The results evidenced no significant compositional differences between the discarded mushrooms, proteins, and β-glucans being the main components in all varieties. In contrast, the residues were mainly composed of carbohydrates (glucans and chitin).

The films obtained from the residues presented a very rigid behaviour, with higher elastic modulus (ca. 2–4.5 GPa) and lower elongation values (ca. 1%) compared to their counterparts prepared with the mushroom's discards. The developed films outperformed benchmark biopolymers in terms of barrier properties with the additional advantages that they can be directly produced from fungal biomass (without plasticizers or any other additives) and they proved to be easily disintegrated according to the standard ISO 20200.

1. Introduction

The global mushroom cultivation market is considerably growing and is expected to reach $52 billion by 2026, posing an environmental challenge for the main industries that market these products worldwide (Fortune Business Insights, 2019). Mushroom waste is mainly composed of mushrooms with misshapen caps and/or stalks that do not meet the specifications set by retailers but have high nutritional value (Aguiló-Aguayo, Walton, Viñas, & Tiwari, 2017). Therefore, researchers are looking for ways to turn the significant amount of waste produced from mushroom cultivation into valuable products since they offer significant economic potential as fungal cell walls contain chitin, hemicelluloses, and, among the most interesting functional components, β-glucans (Muzzarelli et al., 2012; Synytsya & Novák, 2013). Their valorisation has been gaining attention not only because of the health-promoting effects of β-glucans but also due to the different potential industrial applications, their impact on the economy, and the support for sustainability. Several valorisation schemes have been proposed from mushroom' waste and by-products to obtain valuable compounds (Grimm & Wösten, 2018; Zhu, Du, & Xu, 2016). Of especial interest is the integrated value chains as one of the most promising pathways to achieve the zero-waste goal and to accelerate the transition of the mushroom industry to a circular bioeconomy. For instance, the use of the waste streams, generated after β-glucans extraction from mushrooms' residues and by-products, to develop biopolymers is an underexplored area but with a great potential since some of the components present in the native mushrooms (i.e. chitin and recalcitrant β-glucans), remain in the residue (Ifuku, Nomura, Morimoto, & Saimoto, 2011).

Over the last decades, plastics have been widely used with an alarmingly growing rate of global production (Payne, McKeown, & Jones, 2019), raising concerns related to their waste management and environmental contamination, thus urging to look for alternatives. In this context, it is worth mentioning the current open debate and activity around plastic packaging and its environmental impacts that have mainly focused on the grocery and food retail sectors. Packaging has become an essential element in the commercialization of food products, being fundamental to ensuring food quality and safety and facilitating purchasing and transportation activities throughout "the farm to fork chain", contributing to reducing food waste (Bhargava, Sharanagat,

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Feasibility of *Agaricus bisporus* waste biomass to develop biodegradable food packaging materials☆

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ABSTRACT

This work aimed to valorise *Agaricus bisporus* biomass residues to produce bio-based and biodegradable films. To this end, two of the most widely consumed varieties, *Agaricus bisporus brunnescens* (Abb) and *Agaricus bisporus hortensis* (Abh), were compositionally characterised and then, films were formed by extrusion and compression-moulding. Proteins and β-glucans were the major components in both varieties. Abb showed a higher protein content, whereas the amount of polysaccharides and lipids was higher in Abh. Thermally-processed Abh-films showed better mechanical and barrier performance than their counterparts prepared with Abb, mainly ascribed to the higher carbohydrate and lipid content. Thus, Abh was selected to optimize the processing conditions in terms of energy consumption (from 100 to 130 °C) and barrier properties. As a result, 110 °C was selected as the optimum processing temperature for Abh-films since the mechanical properties in terms of strength were not significantly modified and barrier properties were significantly improved. The resulting film was coated with a beeswax layer to reduce its hydrophilic nature. The coated films showed similar mechanical properties than their counterparts prepared without the beeswax coating but displayed significantly better water vapour barrier performance. Furthermore, both films were easily disintegrated under composting conditions according to ISO 20200.

1. Introduction

Nowadays, the consumption of mushrooms has remarkably increased due to their excellent nutritional and health benefits ascribed to the presence of fungal polysaccharides (especially β-glucans), proteins, vitamins, antioxidants, and minerals. More than thirty-five species of edible mushrooms are commercially cultivated throughout the world, being around twenty of them produced at industrial scale (Ramos et al., 2019). Amongst them, *Agaricus bisporus* (*A. bisporus*) is one of the most consumed mushrooms in the world (Kala et al., 2021). Specifically, the global white mushroom market (*A. bisporus hortensis*) accounted for USD 16.73 billion in 2020 and is expected to reach USD 27.39 billion by 2028 (Zion Market Research, 2021). Unfortunately, the sharp increase in industrial edible mushroom production has also resulted in the generation of large amounts of mushroom waste each year, including mushroom stems and misshapen mushrooms whose size or shape do not meet commercial standards. Therefore, recent academic and industrial interest in mushroom's waste is increasingly being focused towards its valorisation to fulfil the pursuit of circular economy and sustainability principles.

Discarded mushrooms and mushroom stems contain structural carbohydrates, such as β-glucans and chitin, very interesting for the development of biodegradable materials. This is a highly underexplored area with potential interest to shift the food packaging market towards more sustainable packaging solutions to develop bio-based and biodegradable polymers. In a recent work, biodegradable films were directly produced (without plasticizers or any other additives) by means of the solvent-casting method, either from discarded mushroom biomass generated in the industry or from the waste produced after a β-glucan extraction process from three different species (*Grifola frondosa*, *Lentinula edodes*, and *Pleurotus ostreatus*) (Feng et al., 2022; Pérez-Bassart, Martínez-Abad, Reyes, López-Rubio, & Fabra, 2023). However, the

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Annex B: list of additional publications

Polímeros naturales obtenidos de residuos agroindustriales y sus potenciales aplicaciones

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Resumen

En el contexto de la economía circular, en el que se busca un aprovechamiento integral de los recursos, la valorización de residuos generados en actividades agroindustriales para la obtención de materiales es un área de creciente interés. En concreto, la obtención de biopolímeros a partir de residuos es una opción muy atractiva, ya que permitiría, por un lado, abaratar el coste de materiales necesarios para que puedan competir con los plásticos convencionales, sobre todo en aplicaciones de menor coste añadido (como es el área del envasado alimentario) y, por otro lado, valorizar macromoléculas presentes en dichos residuos con potenciales aplicaciones en distintas áreas. En este artículo mostramos el potencial de diferentes residuos agroindustriales, concretamente los residuos del cultivo de setas, corteza de sandía y de algas marrones para la obtención de biopolímeros de interés industrial.

Palabras clave: pectinas, fucoidanos, beta-glucanos, alginatos, laminarina.

Abstract

In the context of circular economy, in which an integral exploitation of resources is sought, the valorisation of agro industrially generated residues for obtaining materials is an area of increased interest. Specifically, the extraction of biopolymers from residues is a very attractive option as it would allow, on one hand, to lower the price of materials needed to compete with conventional plastics, especially in low added-value applications (like in the case of food packaging) and, on the other hand, to valorise macromolecules present in these residues with potential applications in different areas. In this dissemination paper, we will show the potential of various residues, concretely residues from mushroom cultivation, watermelon rinds and residues from brown algae for obtaining biopolymers with industrial interest.

Keywords: pectins, fucoidans, beta-glucans, alginates, laminarin.