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Selection, Cultivation and Biochemical analysis of fungi with pharmacological properties.

Selezione, Coltivazione ed Analisi biochimiche di funghi con proprietà di interesse farmacologico

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Summary

The importance of medicinal mushrooms has rapidly increased in the last decades thanks to the numerous studies carried out on their beneficial properties and the isolation of bioactive compounds able to bring significant benefits to human health. Among the most studied are found in particular, the saprotrophic fungi *Lentinula edodes* and *Pleurotus ostreatus*, belonging to the phylum Basidiomycota. Their economic and commercial interest has rapidly grown thanks to new methods of cultivation on substrates made ad hoc that allow to obtain high yields and high-quality mushrooms. Their food interest also derives from the fact that they adapt very well to different types of diets thanks to the high presence of nutrients such as vitamins and proteins and have now been classified as functional food. The main purpose of this PhD thesis is to characterize the shiitake and oyster strains collected by different world banks, both commercial and research, comparing their activities and evaluating their beneficial potentials to create high-potential food products for the market of Italian and international functional food. In order to achieve this goal, different properties were evaluated, and two cultivation methods were compared, one on a commercial substrate based on sawdust and straw and the second on hardwood logs using a traditional method. The first comparison carried out concerns the growth, yield and general quality of the fruiting bodies produced by the two different cultivation methods, fundamental characteristics to be taken into consideration in a large-scale production, above all for the evaluation of costs. Comparing the different shiitake and oyster strains, on sawdust substrates or different hardwood logs, some interesting morphological characteristics in the fruiting bodies emerged, although with lower yields for log-grown mushrooms. However, comparing the aqueous extracts of different strains and evaluating their antioxidant properties, it has been observed that, both in shiitake and oyster, the activity is much higher in mushrooms grown on log than those from the substrate (even of the same strain). Regarding the antibacterial properties of these fungi, their extracts were tested on two microorganisms such as *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* MSSA, but only for the shiitake mushroom was shown an effect against the two pathogens, greater in mushrooms grown on log with respect to those from a substrate. The antibacterial effect was then further characterized

by identifying a specific serine protease inhibitor that confers this activity. We performed a proteomic analysis to obtain an overall picture of the post-harvesting expressed proteins in fruiting bodies of *Lentinula edodes* grown on sawdust substrate or on oak log. For analyses on two tumoral cell lines, the HT-29 and the HCT-116, both from colon carcinoma, and on primary fibroblasts, fruiting body extracts treated in such a way as to simulate human digestion at the level of the stomach were used. By contacting the treated extracts and the cells, a net antiproliferative activity has emerged only on the tumor cells, whereas on the primary cells both fungi do not express any cytotoxic effect. The last activity analysed concerns the effect of the extracts treated on microorganism normally present in the human intestine as *Escherichia coli*, using the C1a strain. Compared to the use of a nutrient-poor M9 medium, the addition of digested extracts leads to slight increase the grow rate underlining a possible prebiotic effect. This research project allows to select specific strains according to the type of activity considered, also evaluating production costs, yields and timing in order to create food products for the functional food market.

Riassunto

L'importanza dei funghi medicinali è rapidamente cresciuta negli ultimi decenni grazie ai numerosi studi compiuti sulle loro proprietà benefiche e all'isolamento di composti bioattivi in grado di portare notevoli benefici alla salute dell'uomo. Tra i più studiati si ritrovano nello specifico, i funghi saprotrofi *Lentinula edodes* e *Pleurotus ostreatus*, appartenenti al phylum Basidiomycota. Il loro interesse economico e commerciale è rapidamente cresciuto grazie a nuovi metodi di coltivazione su substrati realizzati *ad hoc* che permettono di ottenere rese elevate e funghi di qualità elevata. Il loro interesse alimentare deriva anche dal fatto che si adattano molto bene a diversi tipi di diete grazie all'elevata presenza di nutrienti come vitamine e proteine e sono stati ormai classificati come functional food. Lo scopo principale di questa tesi di dottorato è quello di caratterizzare dei ceppi di shiitake e pleurotus raccolti da diverse banche mondiali, sia commerciali che di ricerca, confrontando le loro attività e valutandone le potenzialità benefiche per realizzare di prodotti alimentari ad alto potenziale per il mercato del functional food italiano ed internazionale. Per poter raggiungere questo obiettivo diverse proprietà sono state valutate e sono stati confrontati due metodi di coltivazione, uno su un substrato commerciale a base di segatura e paglia ed il secondo su log di legno seguendo un metodo più tradizionale. Il primo confronto effettuato riguarda la crescita, la resa e la qualità generale dei corpi fruttiferi prodotti dai due diversi metodi di coltivazione, caratteristiche fondamentali da prendere in considerazione qualora si voglia effettuare una produzione su larga scala, soprattutto per la valutazione dei costi. Confrontando i diversi ceppi di shiitake e quelli di pleurotus non sono emerse differenze a livello morfologico dei corpi fruttiferi, ma ciò che differenzia i metodi di coltivazione sono le rese, più basse e sporadiche nella coltivazione tradizionale, più elevate e rapide nella coltivazione su un substrato commerciale. Confrontando però gli estratti acquosi di diversi ceppi e valutandone le proprietà antiossidanti è stato osservato che, sia nel fungo shiitake che nel fungo pleurotus, l'attività risulta molto più ingente nei funghi cresciuti su legno rispetto a quelli da substrato (anche dello stesso ceppo). Per quanto riguarda le proprietà antibatteriche di questi funghi, sono stati sperimentati i loro estratti su due microorganismi come *Pseudomonas aeruginosa* PAO1 e *Staphylococcus aureus* MSSA,

ma solo per il fungo shiitake è stata evidenziata un'attività contro i due patogeni, maggiore nei funghi cresciuti su log rispetto a quelli da substrato. L'attività antibatterica è stata poi maggiormente caratterizzata realizzando prima un'analisi proteomica completa del fungo shiitake per poi individuare il gruppo di proteine specifiche che conferiscono questa attività, quantificandole inoltre nei due diversi metodi di coltivazione e confrontando i dati con quelli ottenuti dall'analisi in HPLC/MS-MS degli estratti da corpo fruttifero precipitati in ammonio solfato e separati tramite elettroforesi. Per le analisi su due linee cellulari tumorali, le HT-29 e le HCT-116, entrambe da carcinoma di colon, e su fibroblasti primari sono stati utilizzati degli estratti da corpo fruttifero trattati in modo tale da simulare la digestione umana a livello dello stomaco. Mettendo a contatto gli estratti trattati e le cellule è emersa una netta attività antiproliferativa solo sulle cellule tumorali, mentre su cellule primarie entrambi i funghi non esprimono nessun effetto citotossico. Questo dato rappresenta una base su cui sperimentazioni future verranno strutturate per approfondire l'effetto citotossico degli estratti su cellule tumorali. L'ultima attività analizzata riguarda l'effetto prebiotico degli estratti trattati su microrganismi normalmente presenti nell'intestino umano come *Escherichia coli*, utilizzando il ceppo C1a. Rispetto all'utilizzo di un terreno M9 povero di nutrienti, l'aggiunta degli estratti digeriti porta ad un aumento della velocità di crescita sottolineando un effetto prebiotico su questi microrganismi. Questo progetto di ricerca consente di selezionare ceppi specifici a seconda del tipo di attività considerata, tenendo conto anche di costi, rese e tempistiche di produzione al fine di realizzare prodotti alimentari per il mercato del functional food.

Sommario

Introduction

Basidiomycetes: classification, macroscopic characteristics and nutritional strategies

Mushrooms are eukaryotic organisms, chemoheterotrophs that feed by absorption [1, 2]. They are either unicellular or organized in multinucleated structures. The heterotrophic nutrition is implemented through different lifestyles: they implement saprotrophic nutrition or different forms of mutualistic, pathos and commensal symbiosis [3]. Today about 120,000 species have been described, but the total estimated number of species is thought to be around 1.5 million. These data highlight how the Fungi Kingdom is one of the least explored kingdoms of our planet [4-6]. The evolutionary success of mushrooms is evidenced by the high number of species and by the variety of ecological niches and occupied habitats. They are the main colonizers of soil and air and recent studies have highlighted the massive presence of fungi also in the aquatic (both freshwater and marine) environment where they can also establish symbiosis with plants and animals and adapt to survive in restrictive conditions that are extreme for most other organisms[2, 3, 7]. From the cellular point of view, fungi present a thick wall that acts as an exoskeleton, protecting their cells from external environmental factors. The wall is directly involved in the growth process; it also confers resistance to physical stress and is essential for interaction with other cells. Ultrastructure analysis revealed that the wall is mainly composed of chitin and other polysaccharides such as glucans and mannoproteins, organized in a complex fibrillary network with also associated different proteins and glycoproteins[8]. The main nutritional requirements of macronutrients concern carbon sources, nitrogen, phosphorus, sulphur, potassium and magnesium, while for micronutrients the most important elements are copper, iron, manganese and zinc. Some mushrooms are oligotrophic and are able to grow up with limited nutrient availability and survive by recovering scarce amounts of volatile organic compounds directly from the atmosphere [8]. Since they are not nitrogen-fixing organisms they recover the nitrogen needed for their growth up both from inorganic compounds (such as ammonium salts) and from organic compounds (such as amino acids)[2, 8]. On the other hand, phosphorus is the main source

from which the cell produces nucleic acids, phospholipids, ATP and glycophosphates; the vacuoles inside the cells serve as storage sites for phosphates, stored in the form of polyphosphates. The sources of both nitrogen and phosphorus can be very limited in nature, so the filamentous fungi have developed different morphological and biochemical strategies to recover sufficient quantities of them from the environment.

Nutrient absorption is an energy-dependent process: sugars, amino acids, nitrates, ammonium ions and sulphates are absorbed by proton simulation [2, 8, 12].

Mushrooms can be classified according to nutritional strategies in three main ecological categories:

• Saprotrophic mushrooms: they are able to degrade cellulose, hemicellulose and lignin using lignocellulose enzymes (cellulase, laccase, pectinase, peroxidase) and hydrolytic enzymes [13].

• Pathological symbiont mushrooms: they are able to attack living organisms, behaving like parasites. When they induce a disease in the host they are called pathogenic. Equipped with mechanical, chemical and / or enzymatic structures, they are able to behave like necrophagous or biotrophic parasites. For example, many of them possess chitinase enzymes that break down chitin polymers in the cell wall of other fungi, insects or soil organisms [2].

• Mutual symbiotic mushrooms: they can interact with other organisms obtaining a mutual benefit. The symbiosis can be established with particular plant species thus creating a mutual-root-fungus association, called mycorrhiza, which leads to significant benefits for all the organisms involved [14].

Saprotrophic mushrooms: cultivation strategies and worldwide market

Mushrooms are in prime focus in the food industry for their multi-functional benefits. They are gaining popularity owing to their high nutritional values and are gradually approaching a “super food” status. Mushrooms are widely accepted in most of the regions of the world. Increase in the consumption of processed food across the world is one of the major driving factors of the mushroom market[15]. Being a promising and profitable

220 business, mushroom cultivation is widely adopted by growers. Factors such as R&D and
221 innovations to enhance the acceptability and continuous improving technologies to
222 increase mushroom shelf-life are also projected to drive the mushroom market in the next
223 five years. As reported by the company MarketandMarkets, the global market for
224 mushrooms was valued at $29,427.92 million in 2013. The mushroom market is
225 projected to grow at a CAGR of 9.5% between 2014 and 2019. The market was
226 dominated by Europe in 2013, which accounted for around 45% of the total market.
227 Cultivated mushrooms have become popular with over 200 genera of useful macro fungi
228 in the world. The most common ones that are produced and consumed are button
229 mushrooms (*Agaricus bisporus*), shiitake mushrooms (*Lentinula edodes*), and oyster
230 mushrooms (*Pleurotus* spp.) and accounted for nearly 76% of the global mushroom
231 market size in 2013 [16]. Button mushrooms are projected to have the largest market,
232 globally; however, the shiitake mushrooms market is projected to be the fastest growing
233 from 2014 to 2019.The market for mushroom is characterized by intense competition due
234 to the presence of many both large- and small-scale firms. Expansions, acquisitions,
235 agreements, and new product launches are the key strategies adopted by market players
236 to ensure their own growth. Players such as Costa Pty Ltd. (Australia), Phillips
237 Mushroom Farms, Inc. (U.S.), Shanghai FINC Bio-Tech Inc. (China), and Bonduelle
238 Fresh Europe (France) dominate the market. The total number of edible and medicinal
239 fungi is over 2,300 species. One of the most expensive steps of mushrooms production is
240 the realization of the cultivation substrates. The cultivation of lignocellulosic mushrooms
241 such as *Lentinula edodes* and *Pleurotus ostreatus* concern two different methods. The
242 traditional method concerns the use of hardwood logs of a specific kind of wood and
243 dimension depending on the mushroom species cultivated. Mushroom's grain spawn is
244 inoculated inside holes along the entire surface of logs and, generally, fruiting bodies will
245 take approximately one year before their appearing[17, 18]. Logs can be incubated in sprayed
246 greenhouses or directly in an environment protected from wind and direct sunlight. This
247 practice has for long represented an important improvement of forest's waste resources
248 and today is called "Forest farming". The advantages of log production are the high
249 quality of fruiting bodies harvested and the use of cheap substrates derived from other
250 forest activities. This method has also important disadvantages such as the low

production yields and long incubation times of substrates before producing fruiting bodies[19]. Nowadays, mushroom farms need a cheaper process of production that can guarantee high yields in a shorter time. The cultivation is based on the use of sterile substrates, derived from poor raw materials such as sawdust and straw, and takes about two-three month since the inoculum to the harvest of fruiting bodies. This cultivation strategy seems to be the smartest solution for massive productions, beeing able to satisfy the worldwide demand of edible mushrooms [17, 18, 20, 21].

Mushrooms as functional food

Mushrooms can be considered a valuable dietary component thanks to their high content of fibers, proteins and vitamins, but also, they are well known as organisms with relevant medicinal properties. Nowadays, they can be considered a functional food with therapeutic effect against specific chronic diseases[22, 23]. The typology of activity depends on which species of mushroom attention has been focused. For example, *Lentinula edodes* and *Pleurotus ostreatus* are two of the most important and consumed medicinal mushroom of the world. The first is well known for his antimicrobial and antioxidant activity and for its anticancer effect thanks to the beta-glucan lentinan. Conversely, the popularity of *Pleurotus ostreatus* raises thanks to its anti-cholesterolemic activity in addition to a high antioxidant effect [22, 24]. Nowadays medicinal mushrooms can be consumed as food, dried or fresh, and most of them have the organic certification, but for the treatment of pathologies or as dietary supplement the most effective method is the integrator consumption. Integrators contains high levels of medicinal compounds derived from fruiting bodies extraction of different species from genera *Auricolaria, Flammulina, Ganoderma, Grifola, Hericium, Lentinus, Pleurotus, Trametes, Schizophyllum* and *Tremella* [24, 25]. Scientific and medical studies have established that many compounds are commonly used as immune-modulators or as biological response modifier. It is also important to consider that the enhancing or suppressing of the immune response depends on different factors such as the site of activity, the administered dose or the mechanism of action [25].

Lentinula edodes (Shiitake)

Shiitake is the common Japanese name for the *Lentinula edodes* species; it is one of the most cultivated edible mushrooms in the world [21]. Thanks to the high content of nutritional and medicinal compounds its popularity has grown worldwide both in the food market and in medical research. Concerning its nutritional values, raw fruiting bodies are rich in carbohydrates and proteins, in particular beta-glucans with a complex three-dimensional structure [26]. One of the most studied is the beta-glucan lentinan. Many studies concerning its antitumoral activity, have shown a complete regress of two different kind of tumors such as Sarcoma 180 and methylchloranthrene-induced fibrosarcoma. The interaction of beta-glucan lentinan with specific lymphocytes surface proteins leads to subsequently activation of the immune system cells with the production of antibodies, interleukins and interferons. (The carcinogenic effect of lentinan results from an activation of the host's immune system[24-27]). Recent studies about shiitake and its antiviral and antibacterial activity show also an important protection against HIV virus. The beta-glucan lentinan in combination with AZT (antiretroviral 3'-azido-3'-deoxythymidine) suppressed the in vitro expression of surface antigens of HIV more than a normal treatment with only use of AZT and also increase its antiretroviral effect on virus replication[28]. This mushroom species has also a specific compound involved in the lowering of the blood cholesterol; this molecule is known as eritadenine and it is a purine alkaloid involved as an inhibitor of S-adenosyl-L-homocysteine hydrolase. Differently from statins, this compound does not inhibit the biosynthesis of cholesterol inside liver cells but it enhances removal of blood cholesterol[29]. The total amount of eritadenine in shiitake fruiting bodies is between 40-70 mg/100 dry weight with a greater accumulation inside the cap[30]. The characteristic smell of shiitake, of dried fruiting bodies, derives from a specific molecule commonly known as lanthionine. This cyclic 5-sulfur compound is a volatile molecule has different healthy benefits such as an inhibitory activity against platelet aggregation[31] and bactericidal activity with inhibitory effects against *Staphylococcus aureus*, Bacillus subtilis and *Escherichia coli*[28]. Ergothioneine and polyphenols are important substances involved in antioxidant processes. Free radicals can be responsible or play a major role in many diseases such as cancer, Alzheimer

diseaes, atherosclerosis and the aging process. To fight the accumulation of these kinds of substances, polyphenols and ergothioneine play a crucial role as reducing agents. In particular, in vivo studies have been demonstrated that ergothioneine is a cellular protector against oxidative damages but its physiological role is still unknown [32].

Pleurotus ostreatus (oyster mushroom)

This mushroom is also known with the common name oyster fungus and similar to shiitake is a primary decomposer of vegetal residues. *Pleurotus ostreatus* is one of the most appreciate edible mushroom thanks to its high content of proteins, vitamins (niacin, thiamine and riboflavin) and minerals (iron, calcium, phosphorus) [33]. Since the 1986 this mushroom has been studied and many medicinal activities have been attributed to it such as the hypotensive activity in mouse model and anti-tumoral activity as showed by Nanba in his study[22, 34]. Fruiting bodies also possesses many medicinal properties such as ribonuclease activity, anti-inflammatory activity or immunostimulatory activity[22, 24, 35, 36]. *Pleurotus ostreatus* contains different biologically active compounds similarly, once more, to shiitake mushroom. For example ubiquitin proteins isolated from fruiting bodies showed a particular anti-viral activity or its ribonuclease have the potentiality to neutralize HIV virus [37]. Concerning the antitumor activity, Choi and his collaborators have reported a positive effect of Pleurotus extracts on lung and cervical carcinoma. Many reports show a strong in vitro activity of *Pleurotus ostreatus* extracts against cancer cells HL-60, and this cytotoxic effect could be due to a high content of flavonoids present inside fruiting bodies that can induce the cells to apoptosis [38-40]. Furthermore, different studies describe the anti-inflammatory activity of *Pleurotus ostreatus* fruiting body extracts both on acute and chronic inflammation[41-44]. The hepatoprotective effect of *Pleurotus ostreatus* mushrooms is exerted through the lipid peroxidation inhibition and an increasing in levels of aminotransferase enzymes in animals[45-47]. Many compounds such as glucans, vitamins and phenols increase the activity of antioxidant enzymes and this fact could lead to a conspicuous reduction of hepatic cell necrosis[48, 49]. One of the most appreciated properties of this species of mushroom is the presence of a natural statin

339 known as lovastatin, used as drug since 1987 and approved by FDA. Its activity is exerted
340 through a reduction of the arterial pressure and a lower blood cholesterol levels,
341 concerning a diet rich in oyster mushroom fruiting bodies and compared with a normal
342 diet in rabbits and rats[50-52]. Using dried fruiting bodies in the diet of experimental animals
343 lead to an accelerator of HDL (high density lipoprotein) and to a reduction of the
344 production of VLDL (very low density lipoproteins), LDL (low density lipoprotein),
345 cholesterol absorption and HMG-CoA reductase activity in the liver [53, 54]. *Pleurotus*
346 *ostreatus* fruiting bodies show high content of antioxidants than other commercial
347 mushrooms[55, 56]. Their content of antioxidant enzymes (superoxide dismutase, peroxidase
348 and catalase) contribute to reducing the oxidative stress in humans. Their capacity to
349 prevent diseases and to neutralize the free radical activity make these mushrooms
350 particularly appreciated in many types of food diets [57]. *Pleurotus ostreatus* fruiting bodies
351 have showed an antihypertensive activity. This capacity is related to a high content of
352 carbohydrates such as D-mannitol that are able to lower blood pressure[58].

353

Aim of the thesis

My PhD project has been financed by the Lombardy Region and the biotechnological department of the private company PreventPCB srl, Società agricola IoBoscoVivo srl. In the last 3 years this private company has focused on medicinal mushroom, in particular on their cultivation and marketing. The main aim of the company is to achieve new nutraceutical products using two edible mushrooms with known medicinal properties, *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster mushroom). In my thesis, different strains of these two mushrooms species have been screened considering their cultivation yields and their medicinal and biochemical properties.

Novel food products for the high-end market require high quality mushrooms both in morphological characteristics and in beneficial properties for human health.

To this purpose, fruiting bodies from two different cultivation methods, on hardwood log and sawdust substrate, were compared and their quality is evaluated (in **chapter 1)**. The hardwood logs are mostly used in Asian mushrooms cultivation, in particular in China, for the export all over the world, including Europe. The cultivation on log has also important forest farming applications and for this reason many investments are performed by the company PreventPCB in woods restoration. Different strains of *Lentinula edodes* and *Pleurotus ostreatus* were collected both from commercial and research strain banks. *In vitro* growth tests were carried out on agarized medium to screen the strains suitable for log cultivation. This study had also the important objective of evaluating the costs of production on logs and which kind of wood species gave the best fruiting yields. One strain of each species was cultivated also on sawdust substrate and used as reference strains during all the experiments.

In **chapter 2**, the characterization of the different strains of *Lentinula edodes* and *Pleurotus ostreatus* strains was carried out analyzing the antioxidant activity of mushroom's water extracts. Using different in vitro assays, we compared antioxidant activity levels in fruiting bodies obtained from different cultivation methods. A deeper characterization of strains was performed through the study of the antibacterial activity against different nosocomial microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

In **chapter 3**, the effect of *Lentinula edodes* and *Pleurotus ostreatus* aqueous extracts has been analyzed on intestinal cell line HT29 and HCT-116 and on primary fibroblasts. The aim of this work was the evaluation of a possible protection effect against oxidative stress on the intestinal cells operated by the mushroom extracts and an antitumoral activity. To simulate the digestive process, the mushroom extracts were treated with HCl (pH 1-2) and pepsin before the execution of the experiments on cells. Digested extracts were also used to analyze growth performance on *Escherichia coli* C1α strain in order to evaluate the prebiotics activity of *L. edodes* mushroom on intestinal bacteria species after the digestion process inside the stomach.

In **chapter 4,** the proteomic analysis of shiitake mushroom, comparing fruiting bodies grown on oak logs and on commercial sawdust substrate was performed and the attention was focused on the detection of *Lentinula edodes* compounds responsible of the antimicrobial activity and a characterization of the proteins involved was performed.

Chapter 1

Evaluation of productivity of a strain collection of *Pleurotus ostreatus* (oyster) and *Lentinula edodes* (shiitake) mushrooms on different hardwood logs

Introduction

Over the centuries, the cultivation of saprotrophic mushrooms such as shiitake and oyster has been diversified; different method and techniques have been used that can affect the general quality and the external aspect of fruiting bodies [21, 59-61]. The cultivation on hardwood logs is a traditional method based on the use of ligneous substrates rich in important nutrients for the metabolism of mushrooms [60, 62-64]. Generally, log-cultivated mushrooms show a higher quality than the same species grown on artificial substrates. In particular, the content of health-promoting polysaccharides in log-grown shiitake is about twice than the same mushroom grown indoor or on artificial substrates [8]. As regards the economy of the forest logs used for mushroom production are worth at least five times more than the same log sold as firewood. Moreover, once a log may continue to produce mushrooms for several years in a row. The aim of this study is to compare the productivity and the morphological characteristics of fruiting bodies of several strains of *Pleurotus ostreatus* and *Lentinula edodes* grown on different hardwood logs or on a commercial sawdust substrate.

Materials and methods

Fungal strains

All strains of *Lentinula edodes* (Berk) Pegler, 1976 and *Pleurotus ostreatus* (Jacq.) P. Kumm., 1871, were collected from different strain banks in Europe (CBS, Mycelia, CNC, BCCM, Società agricola IoBoscoVivo srl) and USA (ACB).

Bank	Strain	IoBoscoVivo Code	Origin
CBS (CBS-KNAW Fungal biodiversity centre)	134.85	KCS0157	China
CBS (CBS-KNAW Fungal biodiversity centre)	225.51	KCS0158	Japan
CBS (CBS-KNAW Fungal biodiversity centre)	833.87	KCS0159	Germany
BCCM (Belgian collection of micro-organisms)	28773	KCS0138	Belgium
BCCM (Belgian collection of micro-organisms)	29756	KCS0139	China
Mycelia	M3710	KCS0141	Far east
Mycelia	M3770	KCS0142	Far east
Mycelia	M3790	KCS0143	Far east
Aloha colture bank	Jumbo	KCS0144	Ukraine
Società agricola IoBoscoVivo	KCS0140	KCS0140	China
Società agricola IoBoscoVivo	KCS0127	KCS0127	China
Società agricola IoBoscoVivo	KCS0128	KCS0128	China
CBS (CBS-KNAW Fungal biodiversity centre)	145.22	KCS0153	Germany
CBS (CBS-KNAW Fungal biodiversity centre)	291.47	KCS0154	France
CBS (CBS-KNAW Fungal biodiversity centre)	342.69	KCS0155	Netherland
BCCM (Belgian collection of micro-organisms)	28511	KCS0146	Belgium
Mycelia	M2181	KCS0160	Europe
Mycelia	M2191	KCS0148	Europe
Mycelia	M2153	KCS0149	Europe
Aloha colture bank	JB	KCS0150	USA
Società agricola IoBoscoVivo	KCS0147	KCS0147	Italy
Società agricola IoBoscoVivo	KCS0050	KCS0050	Italy
Società agricola IoBoscoVivo	KCS0152	KCS0152	Italy

Table I: selected strains used in this project and reference international banks of strains origin

Reference strains

KCS0140 for shiitake and KCS0147 for oyster strains grown on sawdust substrates at 20°C and 85% humidity in sprayed greenhouses, were used as reference for all the analysis and the comparison with log cultivated mushrooms.

Sawdust substrate cultivation

Reference strains KCS0140 and KCS0147 were cultivated on commercial sawdust substrates in sprayed greenhouse. The composition of substrates is property of Società Agricola IoBoscoVivo srl (via Sempione 26, Vergiate, Varese, Italy). Standard conditions of temperature and humidity were used inside the greenhouse; temperature was set at 20°C and the tolerance at ± 2°; humidity was set between 80% to 85% for maintaining the right level of moisture of substrates.

Log cultivation

Grain spawn method was used for mycelia cultivations. Spawns of different strains of *Lentinula edodes* were inoculated in fresh woods of *Quercus robur* (oak), *Robinia pseudoacacia* (robinia), and *Fagus sylvatica* (beech). For *Pleurotus ostreatus* logs of *Populus tremula* (poplar), *Salix alba* (willow), *Tilia platyphyllos* (tilia) and *Robinia pseudoacacia* (robinia) were used. For each mushroom strain 20 logs of each wood species were used, resulting in 1040 logs for *Lentinula edodes* and 1040 logs for *Pleurotus ostreatus*. Spawn was driven into the holes of each wood species by means of an inoculator gun. The holes were then covered with plastic foam plugs and logs were stored in sprayed greenhouses for four months to achieve the complete colonization of the mycelia. Finally, logs were put outside to produce the fruiting bodies. Fruiting bodies were harvested within 12 months.

Sample water extraction

The extraction method reported by N.M. Tonucci *et al.* (2015) [65] was modified. In brief, 5 g of the fruiting bodies that underwent thermal treatment were powdered and extracted for 72 h at 4°C in 100 ml of water. The final water extracts were centrifuged and the supernatants were filtered on 0.2 μm nitrocellulose Millipore membranes and freeze dried. After lyophilization, all the samples were conserved at -20°C.

Microelements analysis

Water extracts of shiitake KCS0140 fruiting bodies harvested from sawdust substrate, oak and beech logs and oyster KCS0147, from sawdust substrate, and from willow, poplar and robinia logs, were used for the analysis of the microelements content. Every sample was divided in two aliquot of 300 mg each with 5 ml of HNO_3 70% and 3 ml of H_2O_2 (Sigma Aldrich) and mineralized with microwave oven Ethos One (Milestone). The atomic absorption spectrometer Solaar M6 (Thermo scientific) was used for the analysis of different microelements. Ca, Cu, Fe, Mg and Mn were analysed using FAAS (Flame Atomic Absorption Spectroscopy) technique and a deuterium lamp for background correction. As, Cd, Cr and Pb were analysed using the Graphite Furnace Atomic Absorption technique. Background corrections were carried out exploiting the Zeeman effect. For each element were implemented calibration curves with standard solutions.

Results

Mycelium growth and spawn preparation

All *Lentinula edodes* and *Pleurotus ostreatus* strains were successfully maintained on artificial PDA medium and used for the subsequently analysis. A comparison of myceliar growth on petri dishes showed a good colonization level of all strains after 10 days of incubation at 23°C, but it highlighted a clear difference of vitality between the strains selected. Shiitake strains (Fig.1) showed different myceliar growth: the strains with a vigorous growth were the KCS0127 (e), KCS0128 (h), KCS0143 (k) and KCS0144 (l). All the other strains have thin hyphae and, as regards KCS0158 (b) and KCS0159 (f), a non-complete colonization of the petri dish. All *Pleurotus ostreatus* strains (Fig.2) showed a high vitality and a complete colonization of the petri dishes. In particular, the strains KCS0050 (g), KCS0148 (k) and KCS0147 (b) showed primordia formation at the edge of the petri dishes after the incubation period.

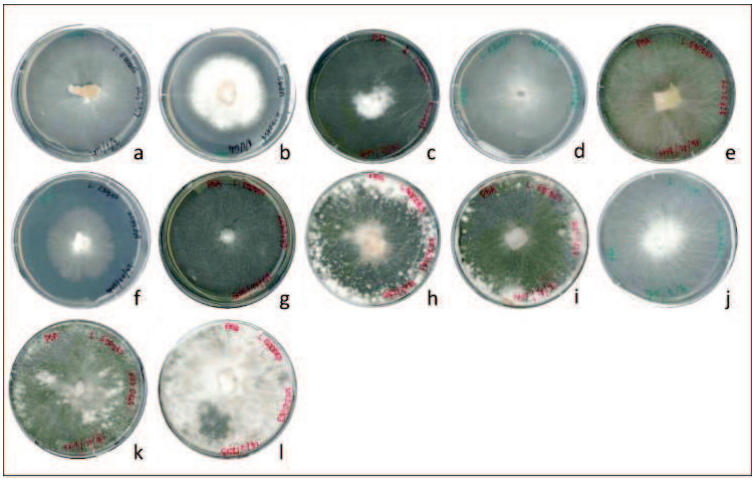

Fig.1: comparison of myceliar growth of the strains of Lentinula edodes. a. KCS0157, b. KCS0158, c. KCS0138, d. KCS0140, e. KCS0128, f. KCS0159, g. KCS0139, h. KCS0141, i. KCS0127, j. KCS0142, k. KCS0144, l. KCS0143.

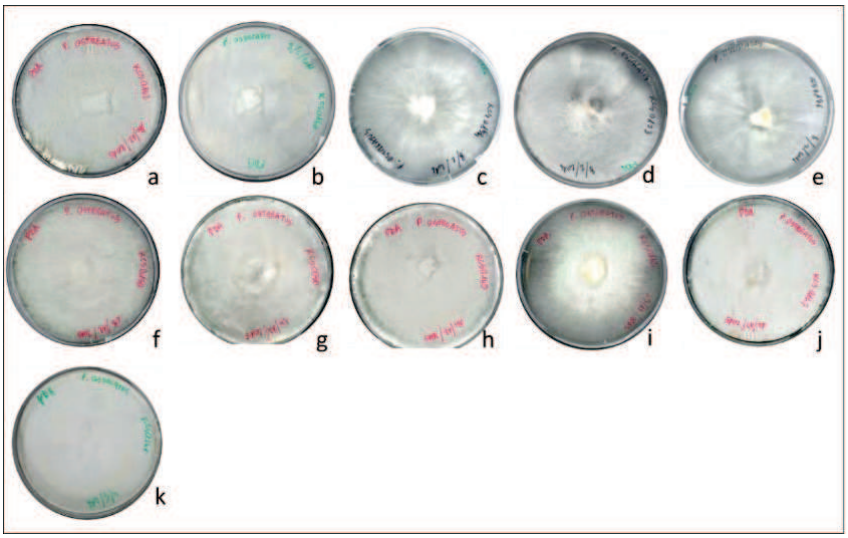

Fig.2: comparison of myceliar growth of the strains of Pleurotus ostreatus: a. KCS0152, b. KCS0160, c. KCS0154, d. KCS0153, e. KCS0156, f. KCS0150, g. KCS0050, h. KCS0149, i. KCS0146, j. KCS0147, k. KCS0148.

All strains with a vigorous growth *in vitro* were cultivated on sterilized wheat grains for the next cultivation step on logs.

Greenhouse trials

Here are reported the productions of fruiting bodies of *Lentinula edodes* and *Pleurotus ostreatus* cultivated on hardwood logs after 2 years from the inoculum. Production levels are indicated using + symbol and the average amount of fruiting bodies per log is expressed as gram/ log.

Lentinula edodes

Shiitake strain	Oak	Robinia	Beech
KCS0128	+ (75 g/ Log)	-	-
KCS0138	+ (56 g /Log)	-	-
KCS0139	+ (44 g /Log)	-	+ (35g/Log)
KCS0140	+ (54 g/ Log)	-	+ (42g/ Log)
KCS0141	+ (61 g/ Log)	-	-
KCS0142	+ (77 g/ Log)	-	-

KCS0144	+ (58g /Log)	-	+ (61g /Log)

Table I: log production of shiitake strains. Symbol – refers to a no production, + refers to a low productivity, ++ refers to a medium productivity and +++ refers to a high productivity related to the total number of logs.

Seven out of twelve inoculated shiitake strains were able to grow on Oak log although with a low productivity (Table I). The two best strains are the KCS0128 and KCS0142 that give an average production per log of 75-77 g. Only three strains (KCS0139, KCS0140, KCS0144) of shiitake showed a low-level production on beech logs. As for robinia logs, no strain gave a fruiting bodies production.

Pleurotus ostreatus

Oyster strain	Willow	Poplar	Robinia	Tilia
KCS0050		+++ (170g/Log)	++ (125g/Log)	
KCS0146		+++ (195 g/Log)	++ (110 g/Log)	
KCS0147	++ (180 g/ Log)	+++ (160 g/Log)	+ (95 g/Log)	-
KCS0148	++ (120g/Log)	+++ (230 g/Log)	-	-
KCS0150	+ (110 g/Log)	-	-	++ (130 g/Log)
KCS0152	++ (100 g/Log)	+++ (155 g/Log)	+ (80 g /Log)	-

Table II: log production of oyster strains. Symbol – refers to a no production, + refers to a low productivity, ++ refers to a medium productivity and +++ refers to a high productivity related to the total number of logs.

Concerning *Pleurotus ostreatus,* only six out of eleven strain tested have produced fruiting bodies on different logs (table II). In particular, poplar is the best log species: five strains reached the fruiting stage with an average production of 155-230 g per log. Good results were also showed by strains KCS0147, KCS0148, KCS150 and KCS0152 on willow log with an average production of 100-180 g per log. Only four strains produced fruiting bodies on robinia logs and the average production was lower than poplar and willow species (80-125 g per log). The strain KCS0150 was the only one able to produce fruiting bodies on tilia logs. Remarkably, the strains KCS0152 and KCS0147 can reach the fruiting stage on three different log species (poplar, willow and robinia).

Fruiting bodies comparison

Macroscopic analysis

Fig.3: Shiitake fruiting bodies comparison. a. Strain KCS0144 harvested from oak and beech logs. b. Strain KCS0140 harvested from sawdust substrate, oak and beech logs. c. Strains KCS0141 and KCS0142 harvested from oak logs. d. Strain KCS0139 harvested from oak and beech logs. e Strains KCS0128 and KCS0138 harvested from oak logs.

Fig. 3 shows a comparison of different shiitake fruiting bodies obtained from log and sawdust substrate. Log grown mushrooms display a lower average size of cap compared to the sawdust substrate mushrooms (KCS0140). All the strains show optimal characteristics for fresh market sale.

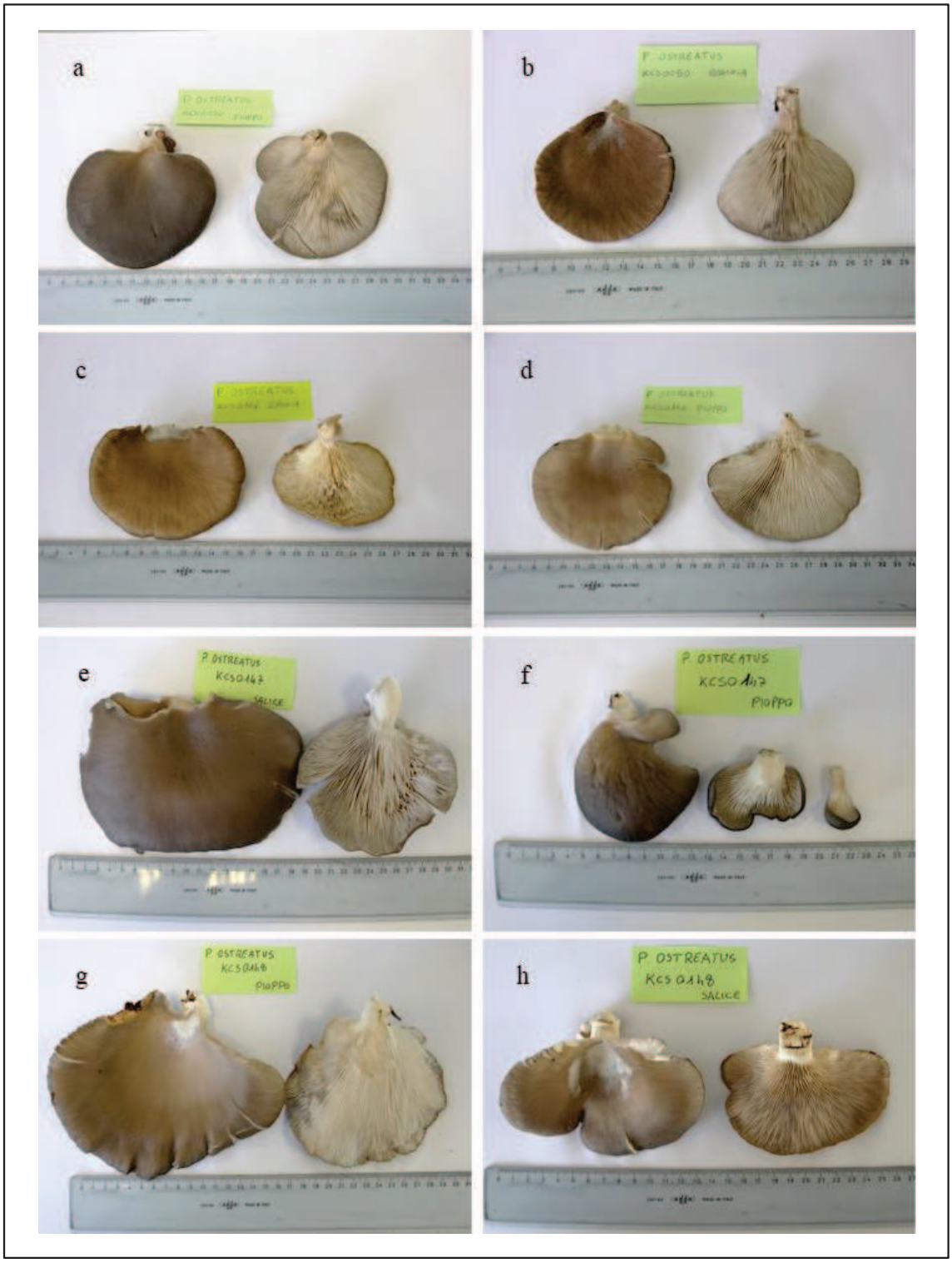

Fig.4: fruiting bodies comparison of Pleurotus ostreatus. a. Strain KCS0050 harvested from poplar logs. b. Strain KCS0050 harvested from robinia logs. c. Strain KCS0146 harvested from robinia logs. d. Strain KCS0146 harvested from poplar logs. e. Strain KCS0147 harvested from willow logs. f. Strain KCS0147 harvested from poplar logs. g. Strain KCS0148 harvested from poplar logs. h. Strain KCS0148 harvested from willow logs. i. Strain KCS0150 harvested from poplar logs. l. Strain KCS0150 harvested from tilia logs. m. Strain KCS0152 harvested from robinia logs. n. Strain KCS0152 harvested from willow logs. o. Strain KCS0152 harvested from poplar logs. p. Strain KCS0147 grown on sawdust substrate.

Fig. 4 shows a comparison of different oyster fruiting bodies obtained from log and sawdust substrate. Log grown mushrooms display a higher average size and a darker colour of cap as compared to the strain KCS0147 grown on sawdust substrate. Robinia grown fruiting bodies are smaller and drier than the other strains. This fact could be

related to a non-optimal log species used. All the strains show optimal characteristics for fresh market sale.

Microelements analysis

We also analysed the levels of different micronutrients and toxic elements comparing fruiting bodies grown on sawdust substrates and different logs. As showed in table III, in general the concentrations of microelements tested are lower in log grown mushrooms (in particular on oak), with respect to sawdust substrate.
Similar results have been obtained for heavy metal concentrations (table IV).

	Mg (µg/g DW)	Ca (µg/g DW)	Fe (µg/g DW)	Mn (µg/g DW)	Cu (µg/g DW)
Pleurotus KCS0147 Willow	787.3 ± 2.8	101.7 ± 5.4	26.6 ± 0.2	4.7 ± 0	4.1 ± 0
Pleurotus KCS0147 Robinia	1040.7 ± 1.0	167.5 ± 11.6	27 ± 0	7.8 ± 0	4.5 ± 0
Pleurotus KCS0147 Poplar	1023.2 ± 1.0	63.3 ± 10.2	27.1 ± 0	4.9 ± 0	6.8 ± 0.1
Pleurotus KCS0147 Sawdust substrate	1014.1 ± 2.2	87.1 ± 7.1	36 ± 0.1	6.4 ± 0	12.2 ± 0
Shiitake KCS0140 Oak	586.7 ± 1.4	464.5 ± 25.4	9.2 ± 0	4.6 ± 0	2.9 ± 0
Shiitake KCS0140 Beech	858.4 ± 0.6	322.2 ± 14.4	11.3 ± 0	26.5 ± 0	9.1 ± 0
Shiitake KCS0140 Sawdust substrate	994.6 ± 3.8	152.9 10.8	21.7 ± 0.1	19.2 ± 0	8.1 ± 0

Table III. Microelements in fruiting bodies of shiitake and oyster

	Cd (µg/g DW)	Cr (ng/g DW)	Pb (ng/g DW)	As (ng/g DW)
Pleurotus KCS0147 Willow	1.5 ± 0	233.9 ± 7.7	44.5 ± 1.3	nd
Pleurotus KCS0147 Robinia	1.7 ± 0	335.3 ± 0.3	33.2 ± 1	nd
Pleurotus KCS0147 Poplar	7 ± 0	158 ± 6.9	59.9 ± 0.3	nd
Pleurotus KCS0147 Sawdust substrate	0.8 ± 0	151.1 ± 0.5	55.7 ± 0.7	nd
Shiitake KCS0140 Oak	0.3 ± 0	157.1 ± 1.1	2.5 ± 0.1	nd
Shiitake KCS0140 Beech	2.4 ± 0	161 ± 2.0	61.6 ± 3.9	nd
Shiitake KCS0140 Sawdust substrate	2.4 ± 0	149.1 ± 1.3	26.9 ± 2.1	nd

Table IV. Toxic elements in fruiting bodies of shiitake and oyster

Discussion

The first growing trial on Petri dishes proved to be an important step to understand whether the mushroom strain is able to grow and reach the fruiting stage on logs. In particular, oyster strains KCS0050, KCS0148 and KCS0147 that exhibited primordia on the edge of the Petri dishes also showed a remarkable attitude to fruiting on logs. On the other hand, *L.edodes* strains were unable to reach a complete colonization after 10 days

563 of incubation on PDA medium and showed a lower productivity on logs. This different
564 behaviour can be linked to a lower saprotrophic activity of these strains. Making a first
565 growth trial is important because log cultivation is a costly and laborious long process.
566 The cultivations on hardwood logs are widespread in Asian countries, in particular in
567 China, for the export all over the world, including Europe. The fruiting body yields from
568 hardwood log cultivation have been compared with those obtained from sawdust
569 substrate. Our results confirmed that: oak log is the best substrate for shiitake strains, as
570 previously reported by Delmas[66], Chang[21] and Sanchez[67], whereas there is
571 incompatibility between robinia wood and shiitake. Even though the data previously
572 published were not positive, we used robinia wood also because one of the main
573 environmental objectives of the Società Agricola IoBoscoVivo company is to remove
574 this non-native species from the Italian forests. Finally, shiitake strains produced fruiting
575 bodies also on beech logs with yields similar to oak logs, but only after the second year
576 of incubation. This delay could be due to a less efficient saprotrophic activity of shiitake
577 on beech wood. Concerning oyster strains, the higher growth performance shown on Petri
578 dishes has been confirmed by the fruiting bodies growth on logs after only six months of
579 incubation. Moreover, oyster production yields are higher than shiitake and many strains
580 are able to produce on different log species such as poplar, willow and robinia. However,
581 tilia logs seem not to be a good substrate for oyster mushroom; only one strain produces
582 fruiting bodies on them. The study also indicates that *P. ostreatus* and *L. edodes* can be
583 successfully grown on different log species. The cultivation process influence costs and
584 yields of mushroom production. For these reasons choose a specific strain with known
585 characteristics and able to grow on different kind of substrates can be useful to produce
586 high quality mushrooms with limited costs. Concerning the morphological and
587 macroscopic characteristics of fruiting bodies of shiitake mushrooms, no differences
588 appear between sawdust substrate and log cultivation. What differentiates a sawdust
589 substrate cultivated mushroom from a log-cultivated shiitake is the size of the
590 sporophores. The average size of a log-cultivated mushroom is lower than a sawdust
591 substrate shiitake, but the flesh is stronger and harder. This is one of the most appreciated
592 characteristics for mushrooms intended for the worldwide market.

593 Oyster fruiting bodies differ for pigmentation, shape and size but all show good
594 characteristics for the worldwide market except the strain KCS0152 from robinia logs
595 where they appear small and drier compared to the other strains. The literature
596 demonstrates that substrates can facilitate higher accumulation of trace elements such as
597 micronutrients or toxic elements. Various investigations have dealt with metal
598 concentrations of mushrooms, especially edible ones, and many species are known to
599 accumulate high levels of heavy metals [68-70]. We analysed fruiting bodies micronutrients
600 content and the content of toxic elements were assessed. We also used those data to assess
601 whether mushroom consumption poses any risks to humans. As shown in Table III, as
602 regards important micronutrients for human health such as Mg, Ca, Fe, Mn and Cu, oyster
603 mushrooms do not show differences between the two methods of cultivation except for
604 iron and copper microelements. Shiitake fruiting bodies from logs show higher content
605 of 3 different micronutrients (Ca, Fe and Mn) compared to the same strain grown on
606 sawdust substrate. From the nutritional point of view, log-cultivated shiitake show the
607 best characteristics and given the same strain, the cultivation method and in particular the
608 composition of the substrate, can affect the content of micronutrients. Concerning oyster
609 mushrooms, the substrate composition seems to affect only the iron and copper content
610 and this fact could be relevant if correlated to particular food request or intolerance. To
611 evaluate the quality of mushrooms it is also necessary to analyse the content of toxic
612 microelements such as As, Cd, Pb, and Cr (Table IV). From the data collected it is
613 possible to see that there is no direct correlation between the method of cultivation and
614 the heavy metal content; differences could be due to a different exposition to pollutants
615 derived from agricultural machines or to the presence of these elements inside the water
616 used for sprayed greenhouses. The detected amounts of these elements are not over
617 quantity tolerated by law. Therefore, there is no risk for human health related to the
618 consumption of these mushrooms.

619

Chapter 2

Research Article
Open Access

Lentinula edodes and *Pleurotus ostreatus*: functional food with antioxidant - antimicrobial activity and an important source of Vitamin D and medicinal compounds

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ABSTRACT:

Background: Mushrooms produce a large number of medicinal compounds, and are also an optimal source of fibres, proteins, vitamins (like groups B and D), and other

649 micronutrients including potassium, magnesium, etc. Consequently, mushrooms are
650 commonly considered to be functional foods. Many works report the high biological
651 potentials of medicinal mushrooms involving their antibacterial, hypoglycaemic,
652 anticholesterolemic, radical scavenging, and anti-inflammatory effects.
653
654 **Context and purpose of this study:** First off, this work aimed to find strains of *Lentinula*
655 *edodes* and *Pleurotus ostreatus* from a bank of edible mushrooms bought from
656 international strain banks (Table I) that could possess health benefit related properties,
657 such as a radical scavenging activity (antioxidant effect), antibacterial effects against
658 common pathogenic bacteria, and being able to produce interesting nutrients and
659 secondary metabolites. As the fungal bank comprises of 20 strains of *L. edodes* and 20
660 strains of *P. ostreatus,* a first screening was made by the selection of 13 strains for each
661 mushroom able to grow in multiple wood types or that were particularly productive and
662 had proved good growth reproducibility over the last 5 years. This work also studied the
663 correlation between culture conditions and mushroom quality in terms of the previously
664 reported properties. Comparison among the selected strains was operated by the
665 assessment of antioxidant and antimicrobial activities after different sample treatments.
666 Furthermore, an initial optimization of the analytic techniques was produced for the direct
667 estimation of important secondary metabolites and nutrients by means of HPLC-MS/MS
668 technique. Further research will encompass an evaluation of transformation processes
669 (drying, freezing, rehydration, cooking, etc.) impact on radical scavenging, antibacterial
670 activity, and possible degradation/loss of nutraceutically important substances such as
671 vitamin D2, ergothioneine, eritadenine, lovastatin, lentinan, and lenthionine.
672
673 **Results:** 13 strains of each mushroom species have been cultivated on different wood
674 logs. Seven strains of shiitake and six strains of oyster mushroom were able to produce
675 sporocarps. Antioxidant levels in water extracts from dried mushrooms produced
676 significatively different results on the basis of strains and of wood. Both mushrooms
677 demonstrated higher radical scavenging activity in log cultivation than substrates
678 cultivation, which was subsequently used as reference. Furthermore, all strains of *P.*
679 *ostreatus* demonstrated the lowest level of antioxidant activity at 4°C, a significant

increase towards 50°C and a limited decrease towards 80°C. The same trend was observed for shiitake extracts. Concerning the shiitake mushroom only, crude water extracts showed an interesting antibacterial activity against the model microorganisms *Pseudomonas aeruginosa* and *Staphylococcus aureus*. A comparison was also performed between the best performing strain extract and the commercial antibiotic Ceftriaxone against *P. aeruginosa*, assessing that 20 mg of crude extract corresponds to 0.2 mg of the pure antibiotic when studied by means of disk diffusion assay.

Conclusions: The results suggested that the cultivation of both shiitake and oyster mushrooms on logs could enhance the content of antioxidant and antibacterial activities, compared to the cultivation of mushrooms on sawdust substrates. Radical scavenging and antibacterial activity depends both on *L. edodes* strain and the log type. The bacteriostatic/bactericidal activity of the best performer strain may depend on a pH and solvent treatment sensitive substance. Secondary metabolites such as ergothioneine and vitamin D2 from both shiitake and oyster were released just after water extraction: this suggests that the transformation/cooking processes may produce a loss of characteristic mushroom biological properties in water. Further evaluation of biologically relevant compounds content and loss during different food transformation and cooking processes will be assessed.

Key words: Shiitake, Oyster, *Pleurotus ostreatus, Lentinula edodes, Pseudomonas aeruginosa, Staphylococcus aureus*, Log grown mushroom, antioxidant, radical scavenging, total phenolic content, DPPH, ABTS, Folin-Ciocalteu, antibacterial, fruiting bodies, sporocarps.

Introduction

Throughout history, mushrooms are well-known organisms with relevant medicinal properties in addition to the consumption of common food [1]. Edible mushrooms are valuable dietary components thanks to the high content of nutritionally relevant

710 compounds, in addition to their taste and organoleptic properties. Nutritional value was
711 found in many works due to their high content of proteins, fibres, vitamins, and mineral
712 salts, in addition to their low-fat level [2-4], and high content of micronutrients [5-6].
713 *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster) are among the most
714 cultivated mushrooms in the world [7]. These can be grown using a wide range of
715 methods, conditions, and substrates. Moreover, the choice of the cultivation technique
716 was previously found both to affect the fruiting yield and the production of secondary
717 metabolites. Oyster and shiitake are characterized by their short growing time compared
718 to other edible species, and feasible production during all the year. Accordingly, their
719 cultivation and experimentation, and the knowledge of their nutritional and medical value
720 has increased over the years [8], [9], [10], [11]. Shiitake and oyster fruit bodies have
721 actually been demonstrated to hold effective antioxidant activity due to a high phenolic
722 compounds content [12], [13], [14], [15]. Additionally, shiitake extracts demonstrated
723 antibacterial activity against *Staphylococus aureus, Bacillus subtilis* and *Pseudomonas*
724 *aeruginosa* in previous research [16], [17], [18], [19], [20], [21]. Many studies regarding
725 the medicinal properties of these mushrooms have been conducted, which have also
726 allowed the recovery of interesting secondary metabolites. In the shiitake mushroom, one
727 of the most relevant is lentinan, a β-glucan compound demonstarting several activities
728 that comprise the antitumor effect due to induced stimulation of the host immune system
729 [6]. Other interesting metabolites are ergothioneine, showing antioxidant activity and
730 eritadenine, which holds an hypocholesterolemic effect [22], [23], [24]. Moreover,
731 *Pleurotus ostreatus* also contains a large number of medicinal compounds. In particular,
732 lovastatin is a secondary metabolite which is capable of lowering blood cholesterol levels
733 and has been therefore suggested to prevent cardiovascular diseases [25], [26].
734 In this work, 13 strains of *L. edodes* and *P. ostreatus* were selected on the basis of their
735 good and reproducible growth yield over the last five years. All the mushrooms were
736 evaluated for antimicrobial activity against two bacterial strains, *Pseudomonas*
737 *aeruginosa* and *Staphylococcus aureus*. Radical scavenging activity was studied by
738 means of ABTS and DPPH assay, and total phenolic content was also measured by means
739 of a Folin-Ciocalteu assay. Currently, there are not enough studies regarding temperature
740 and transformation/cooking processes effects on the nutrients content and the biological

activity of characteristic compounds contained in mushrooms [27]. Accordingly, we started the optimization of mass spectrometry procedures to allow the analysis of crude water extracts for the direct evaluation of nutrient and metabolites loss during transformation and mushroom cooking processes involving water contact (i.e. extraction) and thermal treatments.

Materials and methods

Fungal strains

All strains of *Lentinula edodes* (Berk) Pegler, 1976 and *Pleurotus ostreatus* (Jacq.) P. Kumm., 1871, were collected from different strain banks in Europe (CBS, Mycelia, CNC, BCCM), USA (ACB) and Italy (Società agricola IoBoscoVivo srl).

***Lentinula edodes* strains**

Bank	Strain	IoBoscoVivo Code	Origin
CBS (CBS-KNAW Fungal biodiversity centre)	134.85	KCS0157	China
CBS (CBS-KNAW Fungal biodiversity centre)	225.51	KCS0158	Japan
CBS (CBS-KNAW Fungal biodiversity centre)	833.87	KCS0159	Germany
BCCM (Belgian collection of micro-organisms)	28773	KCS0138	Belgium
BCCM (Belgian collection of micro-organisms)	29756	KCS0139	China
Mycelia	M3710	KCS0141	Far east
Mycelia	M3770	KCS0142	Far east
Mycelia	M3790	KCS0143	Far east
Aloha colture bank	Jumbo	KCS0144	Ukraine
Società agricola IoBoscoVivo	KCS0140	KCS0140	China
Società agricola IoBoscoVivo	KCS0127	KCS0127	China
Società agricola IoBoscoVivo	KCS0128	KCS0128	China

***Pleurotus ostreatus* strains**

Bank	Strain	IoBoscoVivo Code	Origin
CBS (CBS-KNAW Fungal biodiversity centre)	145.22	KCS0153	Germany
CBS (CBS-KNAW Fungal biodiversity centre)	291.47	KCS0154	France
CBS (CBS-KNAW Fungal biodiversity centre)	342.69	KCS0155	Netherland
BCCM (Belgian collection of micro-organisms)	28511	KCS0146	Belgium
Mycelia	M2181	KCS0160	Europe
Mycelia	M2191	KCS0148	Europe
Mycelia	M2153	KCS0149	Europe
Aloha colture bank	JB	KCS0150	USA
Società agricola IoBoscoVivo	KCS0160	KCS0147	Italy
Società agricola IoBoscoVivo	KCS0050	KCS0050	Italy
Società agricola IoBoscoVivo	KCS0152	KCS0152	Italy

Table I: Selected strains used in this project and reference international banks of strains origin

Bacterial strains and growth conditions

Bacterial strains used in this study includes Gram-positive *Staphylococcus aureus* MSSA (ATCC29213) and Gram-negative *Pseudomonas aeruginosa* PAO1, both grown in Luria Bertani (LB) broth under aerobic conditions at 37°C for 24 h [28].

Standard compounds

Standards for HPLC-MS analysis of lentinan and eritadenine were bought from Carbomer Incorporation (San Diego) and Alpha Chemistry, Holtsville, NY 11742, USA. Vitamin D2, Lovastatin, and Ergothioneine were obtained from Sigma-Aldrich, Darmstadt, Germany. Antibiotic Ceftriaxone disodium salt was purchased from Sigma Aldrich.

Reference strains

KCS0140 for shiitake and KCS0147 for oyster strains grown on sawdust substrate, were used as reference for antioxidant and antibacterial analysis. Substrates composition were the property of Società Agricola IoBoscoVivo srl (via Sempione 26, Vergiate, Varese, Italy).

Log cultivation

779 The grain spawn method was used for mycelia cultivations [11], [29], [30]. Spawns of
780 different strains of *Lentinula edodes* were inoculated in fresh woods of *Quercus robur*
781 (oak), *Robinia pseudoacacia* (robinia), and *Fagus sylvatica* (beech). For *Pleurotus*
782 *ostreatus* logs of *Populus tremula* (poplar), *Salix alba* (willow), *Tilia platyphyllos* (tilia),
783 and *Robinia pseudoacacia* (robinia) were used. For each mushroom strain, 20 logs of
784 each wood species were used, resulting in 1040 logs for *Lentinula edodes* and 1040 logs
785 for *Pleurotus ostreatus*. Spawn was driven into holes in each wood species by means of
786 an inoculator gun. Afterwards, holes were covered with plastic foam plugs and logs were
787 stored in sprayed greenhouses for four months to achieve the complete colonization of
788 the mycelia. Finally, logs were put outside in order to obtain the production of fruiting
789 bodies [29]. Within 12 months, every harvest of fruiting bodies was performed.
790
791 **Sample thermal treatments**
792 Each mushroom strain was dried at 37°C for 2 days, subsequently 5g aliquots of each
793 substrate grown strain (KCS0140 and KCS0147) were subjected to thermal treatments
794 either at 4°C or 37°C or 50°C or 80°C for 2h in dark conditions. After treatment, materials
795 were powdered and immediately subjected to extraction.
796
797 **Sample water extraction**
798 The extraction method reported by N.M. Tonucci *et al.* (2015) was modified. Briefly, 5
799 g of all fruiting bodies that underwent thermal treatment were powdered and extracted
800 for 72 h at 4°C in 100 ml of water [31]. The final water extracts were centrifuged and the
801 supernatants were filtered on 0.2 µm nitrocellulose Millipore membranes and freeze
802 dried. After lyophilization, all the samples (coded **W1**-*strain-log*) were conserved at -
803 20°C.
804 This procedure was repeated more than 5 times during the assessments. Extraction yields
805 were reported as the average yield values.
806
807 **Fractionation of KCS0140 strain crude water extract**
808 **Solvent counter-extraction:** The crude water extract W1-kcs0140-beech was subjected
809 to counter-extraction with an increasing polarity series of solvents, starting from

ciclohexane (kcs0140-CHE), then dichloromethane (kcs0140-DCM), ethanol (kcs0140-EtOH), methanol (kcs0140-MeOH), and finally water (kcs0140-WR). An aliquot of 1 g of the freeze dried extract was resuspended in 10 ml of milliQ water and extracted with 20 ml of the solvents. Every solution was centrifuged at RT for 30 min at 4000 rpm in Falcon tubes on an Eppendorf 5810R centrifuge, then filtered on paper disks, and finally evaporated under vacuum conditions at 30°C. The last water fraction was freeze dried. Each solid was resuspended in the same extraction solvent at 250 mg/ml. The ethanol/water 2:1 solution resulting from ethanol extraction was evaporated, freeze dried, and resuspended in water at the same concentration of 250 mg/ml as the other fractions.

Acid-basic separation: An aqueous solution of 1g of the extract in 10 ml was prepared and adjusted to pH 3 with HCl 2.5N, allowed to precipitate at RT for 3h, and then centrifuged at 4000 rpm in Falcon tubes. The supernatant (kcs0140-pH3) was recovered and filtered on paper disks, and the precipitate was extracted with 10 ml of water adjusted to pH 9 by the addition of NaOH 2.5N.

After precipitation of 3 h at RT, supernatant (kcs0140-pH9) was recovered by centrifugation and filtration on paper disks. The last precipitate was extracted again with 10 ml of water and pH 5, producing a suspension (kcs0140-pH5). After the centrifugation of this fraction, the last insoluble material was used as a suspension (kcs0140-pellet) at 500 mg/ml. All the solutions were adjusted to pH 5 for the subsequent assays.

Oversaturation-based separation: An aliquot of 1 g of the freeze dried extract was resuspended in 1 ml of water and vigorously shook for 30 min at RT, before being allowed to precipitate at 4°C for 24 h. The suspension was centrifuged at 13000 rpm at 4°C in Eppendorf centrifuge 581 0R.

The supernatant (kcs0140-S) was transferred and the precipitate (kcs0140-P) freeze dried, weighed, and resuspended in 100 µl of milliQ water.

ABTS radical scavenging activity

Antioxidant activity of the water extracts was performed by ABTS radical cation decolorization assay [32]. Freeze dried extracts were resuspended in water at a concentration of 0.1 mg/ml, allowing a complete dissolution. In a cuvette, 500 µl of the

sample solution and 500 µl of $ABTS^{\circ+}$ was added for spectrophotometric analysis. After 15 minutes, the OD_{734nm} has been measured and was compared with the Trolox titration curve and reported on a Trolox equivalents scale.

DPPH scavenging activity

Radical scavenging activity was performed on all W1 extracts following the method of Villano et al. (2007), which was modified by Baba and Malik (2014) and adapted to our samples [33], [34]. Briefly, 100 µl of each extract ranging from 100 to 800 µg/ml were mixed with 1.9 ml of DPPH reagent to a final concentration of 80 µM (Sigma) and incubated in a dark chamber for 1 h. Solutions absorbance was measured at 517 nm. Ascorbic acid was used as positive control. IC_{50} values were calculated from each sample scavenging – concentration curve.

The percentage of DPPH scavenging activity was determined following the formula:

DPPH scavenging % = [(Control OD – Sample OD) / Control OD] x 100

Total phenolic content estimation

Total phenolic content of each W1 extract was assessed by means of Folin-Ciocalteu assay (Kaur et al. 2002, Baba and Malik. 2014) adapted to our samples. Extracts were prepared in methanol at a concentration of 100 µg/ml. Sodium carbonate was prepared as a 20% (w/v) stock solution. 20 µl of sample solution was mixed with 1 ml of milliQ water and 100 µl of Folin-Ciocalteu reagent (Sigma). After 8 min of incubation in a dark chamber at RT, the solution was added with 400 µl of sodium carbonate and 490 µl of milliQ water, and then incubated for 1 h. The absorbance at 750 nm of the solution was then measured. Gallic acid was used as a reference compound and the results were expressed as its equivalents [34], [35].

Antibacterial assays

Disk diffusion assay

Antibacterial activity of crude water extracts was assessed by a modified Kirby-Bauer agar disk-diffusion assay. An inoculum of the selected bacterial strain grown overnight in LB was diluted to approximately 10^6 cfu/ml and seeded in LB-agar plates, using a

cotton swab. Freeze dried extracts were suspended in water at a concentration of 500 mg/ml, allowing complete dissolution. Each paper disk (BD, Blank Paper Disks) was loaded with 100 µl of sample solution and allowed to dry for 20 min. Dried disks were placed onto the LB-agar layer and allowed to incubate for 24 h before reading the results [36]. Ceftriaxone was used as antibiotic control: ceftriaxone powder was resuspended in water solutions at 4, 2, 1, or 0.5 mg/ml. Inhibition zone (halo) diameter was reported as the average of measurements in three directions of the inhibition zone diameter subtracted by disk diameter.

Minimal inhibitory concentration (MIC)

The MIC of each water extract stored at 4°C (i.e. control condition) was established according to the official CLSI (Clinical and Laboratory Standards Institute) protocol for each model microorganism (*S. aureus*, *P. aeruginosa*). A 96-wells microplate was loaded with 20µl of a twofold dilution of 10 mg/ml mother solution of each water extract up to 5 µg/ml [37]. Next, each well was inoculated with 80 µl of 10^6 cfu/ml MH broth suspension of the appropriate bacterial culture. Control samples were set up loading the wells with sterile growth medium or the crude extracts or the bacterial inoculum. The lower extract concentration which demonstrated no visible growth was considered the MIC [37].

Viable counts

Viable counts (expressed as colony forming units per mL, CFU ml^{-1}) were estimated by a plate count technique: a volume (0.01 ml) of undiluted or serially diluted samples was plated on LB Agar plates and incubated for 24 h at 37°C. Detection limit < 100 cfu/ml.

Time-kill assay

A time-kill assay was performed for the extract W1-kcs0140-beech against *P. aeruginosa* bacterial strain. A diluted inoculum of the appropriate bacterial culture was prepared to 10^6 cfu/ml in MH broth. An aliquot of 5 ml was treated with 25 mg/ml of powdered extract. The bacterial biomass, expressed as OD_{600}, and cellular concentration (cfu/ml), were checked after 2h, 4h, and 6h treatment with gentle shaking at 37°C. Samples,

collected after 2 and 24 h treatment, were observed by means of phase-contrast microscopy (100x magnification). At least four images were acquired for each sample. A detail of each capture field is reported for comparison purpose.

HPLC-MS/MS analysis

Freeze dried extracts obtained from dried samples treated at 4°C (W1) were resuspended in water at 100 mg/ml and added with acetonitrile 1:1 v/v, the solutions were allowed to precipitate and centrifuged at 13000 rpm in 1.5 ml vials for 20 min. Each supernatant recovered was diluted 1:50 for MS analysis. HPLC-MS/MS analyses of each sample were performed on a Perkin Elmer UHPLC system with an OD-300 Aquapore column. The elution gradient used was based on (A) Water and (B) Acetonitrile. Starting from A 80% for 5 min, then linear gradient to A 10% in 15 min. Isocratic to 25 min. MRM scan was used for the quantification of lovastatina (MW 405; F1 199, F2 225), vitamin D2 (MW 397; F1 379, F2 309), ergothioneine (MW 230, F1 143, F2 127), eritadenina (MW 254; F1 178, F2 136), and lenthionine (MW 191, F1 168, F2 150) [22].

Statistical analysis

Data was compared on the basis of significance levels obtained by one-way ANOVA test followed by Tukey HSD post-hoc test. Probability levels of 0.05 were marked with a single sign (*) or different letters [38]. All the extractions and assessments were repeated and reported as the average (n = 3) with standard deviation.

Results

Water extraction of dried mushrooms yield

Water extracts of selected strains of *L. edodes* produced from 0.7 g to 1.8 g of raw material after freeze drying process. Regarding *P. ostreatus*, extracts from 0.9 to 3.1 grams were obtained (Table1).

Fruiting bodies production from log cultivation

Shiitake strains KCS0128, KCS0138, KCS0139, KCS0141, KCS0142, and KCS0144, showed the production of fruiting bodies after one year from the inoculum and only on oak, while KCS140 was able to grow on substrate, oak, and beech logs (Table 1).

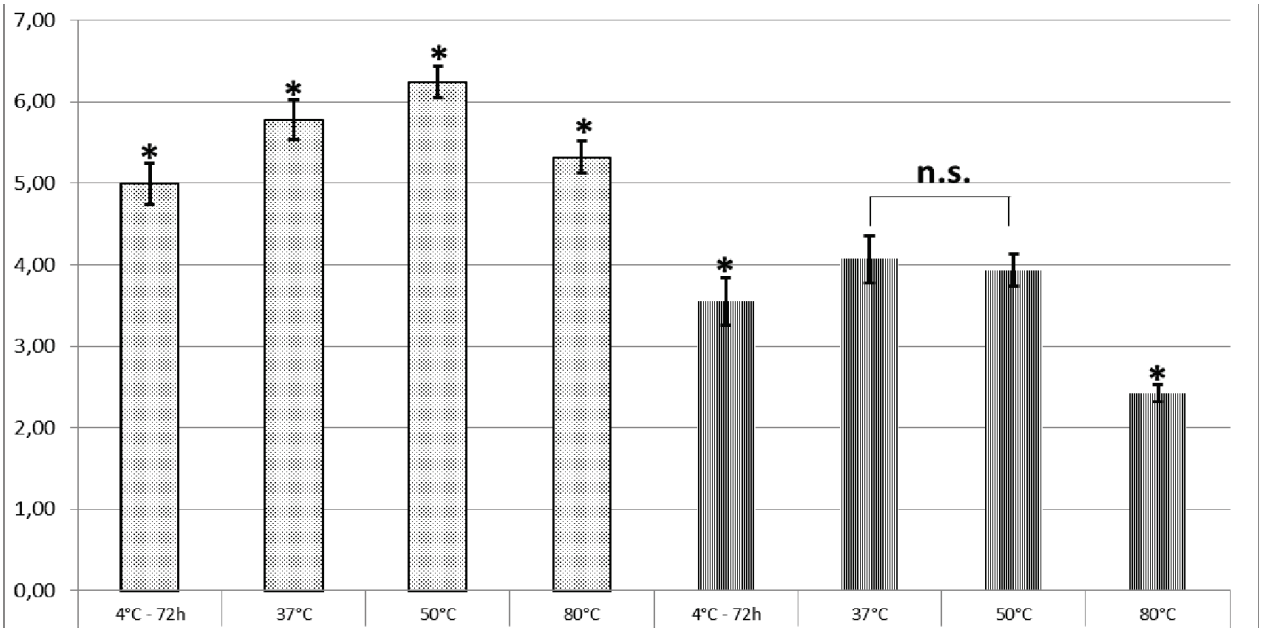
Oyster strains KCS0050, KCS0146, KCS0147, KCS0148, KCS0150 and KCS0152, showed the production of fruiting bodies after six-month from the inoculum. All the oyster strains produced fruiting bodies on poplar logs. Strains KCS0050, KCS0146, and KCS0152 showed fruiting bodies on robinia logs. Strains KCS0147, KCS0148, KCS0150, and KCS0152 showed fruiting bodies production on willow logs. Only the strain KCS0150 showed fruiting bodies production on tilia logs (Table 1).

Antioxidant activity of W1 extracts of shiitake and oyster

Water extracts from thermal treated substrate grown *L. edodes* KCS0140 and *P. ostreatus* KCS0147 were assessed for heat induced alterations in the antioxidant activity. This aspect was investigated by means of the ABTS radical scavenging assay using the extracts of shiitake and oyster at a concentration of 100 μg/ml, as this produced the most repeatable results. As it concerns shiitake, this strain showed the highest activity both at 37°C and 50°C, a significatively ($p < 0.05$) lower activity was observed either at 4°C and 80°C (Figure 1). Regarding the oyster strain, the highest activity was recorded at 50°C and a significatively ($p < 0.05$) lower activity was observed either at 4°C, 37°C, or 80°C (Figure 1). When *P. ostreatus* ABTS radical scavenging activity was assessed, substrate grown strain KCS0160 was found to possess the lowest efficacy, while KCS0147 showed the most evident activity when grown on poplar wood, with a slight less efficiency when cultivated on willow logs. Log species produced significant alteration in the activity of strain KCS0146, that was most effective when grown on robinia than on poplar. KCS0050 resulted more active when cultivated on poplar than on robinia. KCS0148 was more performing when grown on willow than on robinia logs (Table 1, Figure 2).

Reference strain	Thermal treatment	Extract Weight (g ± sd)	Trolox eq. (mM)
***P. ostreatus* KCS0147 on substrate**	4°C	3.13 ± 0.43	4.99
	37°C	1.54 ± 0.43	5.78
	50°C	1.74 ± 0.33	6.24
	80°C	1.24 ± 0.35	5.32
***L. edodes* KCS0140 on substrate**	4°C	1.50 ± 0.50	3.55
	37°C	1.50 ± 0.51	4.07
	50°C	1.52 ± 0.35	3.93
	80°C	1.47 ± 0.19	2.43

A



B

P. ostreatus KCS0147 *L. edodes* KCS0140

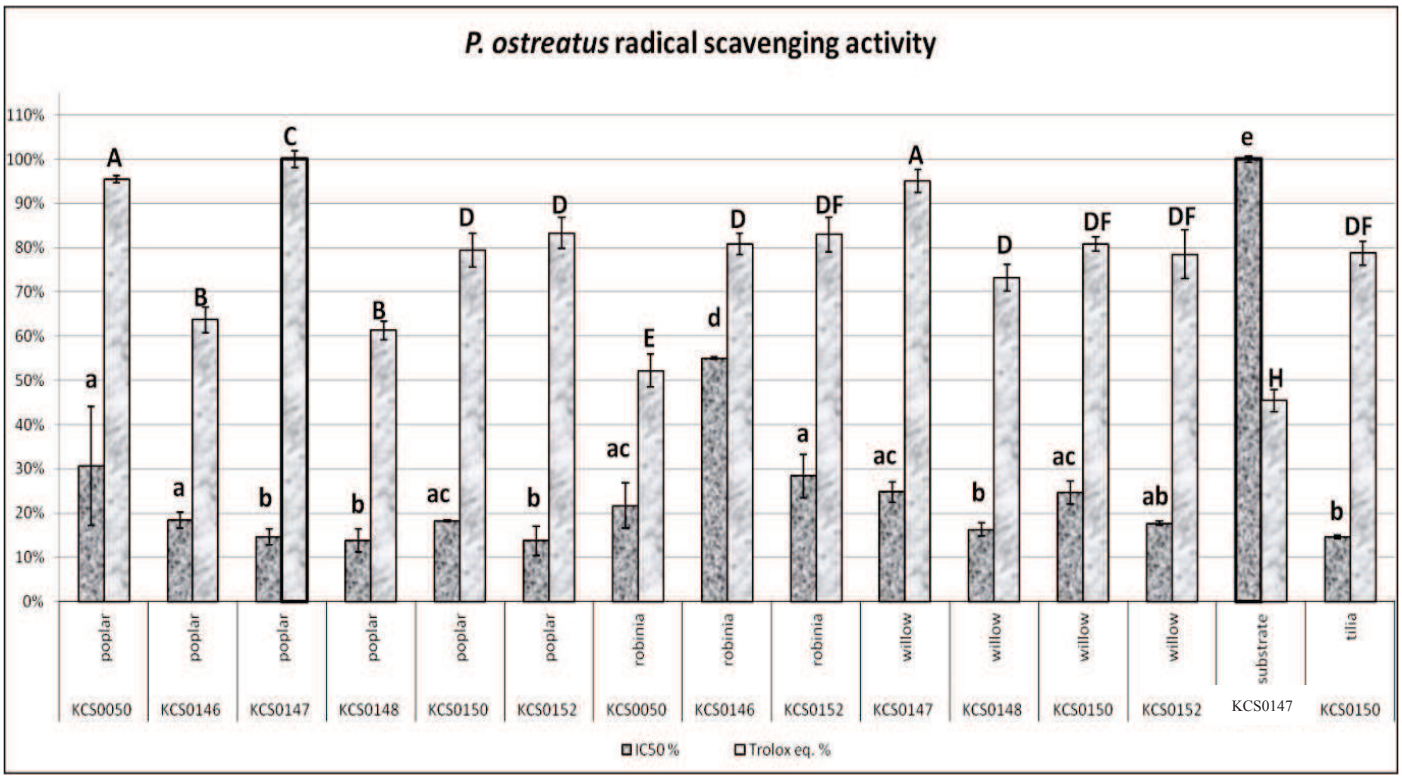
Figure 1: *Antioxidant levels (Trolox equivalents) in dried, thermal treated samples of shiitake and oyster (0.1 mg/ml water extracts W1) grown on substrates and used as reference (A). Levels comparison of antioxidant activity (ABTS radical scavenging) (B). Significant samples are indicated by single sign ($p < 0.05$).*

As DPPH radical scavenging activity was concerned, the same strains demonstrated a similar trend. The lowest IC_{50} (i.e. highest efficacy) was recorded for KCS0147 grown on poplar and significatively higher IC_{50} was observed when cultivated on willow wood. KCS0146 resulted in an about 30% lower IC_{50} when grown on poplar in respect to willow

985 (Table 1, Figure 2). KCS0152 was more effective on poplar, demonstrating a lower IC_{50}
986 when compared to the cultivation on robinia (Table 1, Figure 2). KCS0148 instead
987 showed the same IC_{50} as the previous strain whether grown on poplar or willow logs. A
988 higher value in respect to the previous mentioned strains was observed for KCS0050 and
989 also this strain did not yield significant differences either on poplar or robinia. KCS0150
990 yielded a slightly lower IC_{50} on robinia comparing to the cultivation on poplar and
991 willow.
992 In regard to *L. edodes* ABTS radical scavenging activity, substrate grown KCS0140 had
993 the lowest efficacy. The same strain grown on oak wood revealed the second highest
994 activity, a lower efficacy was detected when it was cultivated on beech. Strain KCS0144
995 allowed the observation of the highest activity among shiitake strains. KCS0139
996 demonstrated an activity less than 5 % lower than oak grown KCS0140. All the other
997 strains (KCS0128, KCS0138, KCS0141, and KCS0142) resulted in a similar and lower
998 activity as compared with the previously mentioned strains (Table 1, Figure 3).
999 As DPPH assay was concerned, the most efficient were KCS0140 and KCS0144 both
1000 grown on oak logs that demonstrated the lowest IC_{50}. Substrate grown KCS0140 showed
1001 the lowest activity, resulting in the highest IC_{50}. When KCS0140 grown on beech and
1002 oak were considered, significatively different results were observed, with the former
1003 showing an IC_{50} about 60% higher than the latter (Table 1, Figure 3).
1004
1005

Strain	Growth substrate	W1 extract weight (g)	IC_{50} (mg/ml)	Trolox eq. (mM)
P. ostreatus KCS0050	Poplar	1.75	2.061 ± 0.151	10.48 ± 0.277
	Robinia	2.5	1.458 ± 0.156	5.73 ± 0.173
P. ostreatus KCS0146	Poplar	2.36	1.236 ± 0.103	7.00 ± 0.329
	Robinia	2.17	3.695 ± 0.390	8.88 ± 0.485
P. ostreatus KCS0147	Poplar	1.17	0.980 ± 0.177	10.98 ± 0.173
	Willow	2.56	1.663 ± 0.035	10.44 ± 0.624
P. ostreatus KCS0148	Poplar	1.23	0.926 ± 0.032	6.73 ± 0.606
	Willow	2.26	1.094 ± 0.019	8.03 ± 0.225
P. ostreatus KCS0150	Poplar	1.65	1.225 ± 0.050	8.72 ± 0.277
	Tilia	2.13	0.978 ± 0.137	8.65 ± 0.502
	Willow	0.92	1.657 ± 0.422	8.88 ± 0.433
P. ostreatus KCS0152	Poplar	1.16	0.923 ± 0.026	9.15 ± 0.294
	Robinia	2.5	1.907 ± 0.210	9.11 ± 0.208
	Willow	1.72	1.188 ± 0.210	8.62 ± 0.346
P. ostreatus KCS0147	Sawdust	3.13	6.718 ± 0.633	4.99 ± 0.433
L. edodes KCS0128	Oak	1.98	1.127 ± 0.093	6.40 ± 0.381
L. edodes KCS0138	Oak	1.02	1.070 ± 0.093	5.76 ± 0.242
L. edodes KCS0139	Oak	1.08	0.981 ± 0.128	8.80 ± 0.277
L. edodes KCS0140	Oak	1.04	0.231 ± 0.009	9.83 ± 0.485
	Beech	1.65	3.375 ± 0.669	6.50 ± 0.104
	Sawdust	1.5	4.994 ± 0.246	3.55 ± 0.502
L. edodes KCS0141	Oak	1.87	0.648 ± 0.163	5.71 ± 0.468
L. edodes KCS0142	Oak	1.17	1.275 ± 0.257	5.51 ± 0.485
L. edodes KCS0144	Oak	0.73	0.254 ± 0.016	13.01 ± 0.312

Table 1: *Water extracts (W1-) dry weights yielded from 5 g of dried mushrooms. IC_{50} and Trolox equivalents in 0.1 mg/ml water extract solutions (reported with standard deviations) from log/substrate grown strains; reference strain for each mushroom species is underlined*



1010 ***Figure 2:*** *Comparison between P. ostreatus strains antioxidant levels either substrate or log grown.*
1011 *Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant*
1012 *differences ($p < 0.05$) between strains and log species are indicated with different letters on top of each bar.*
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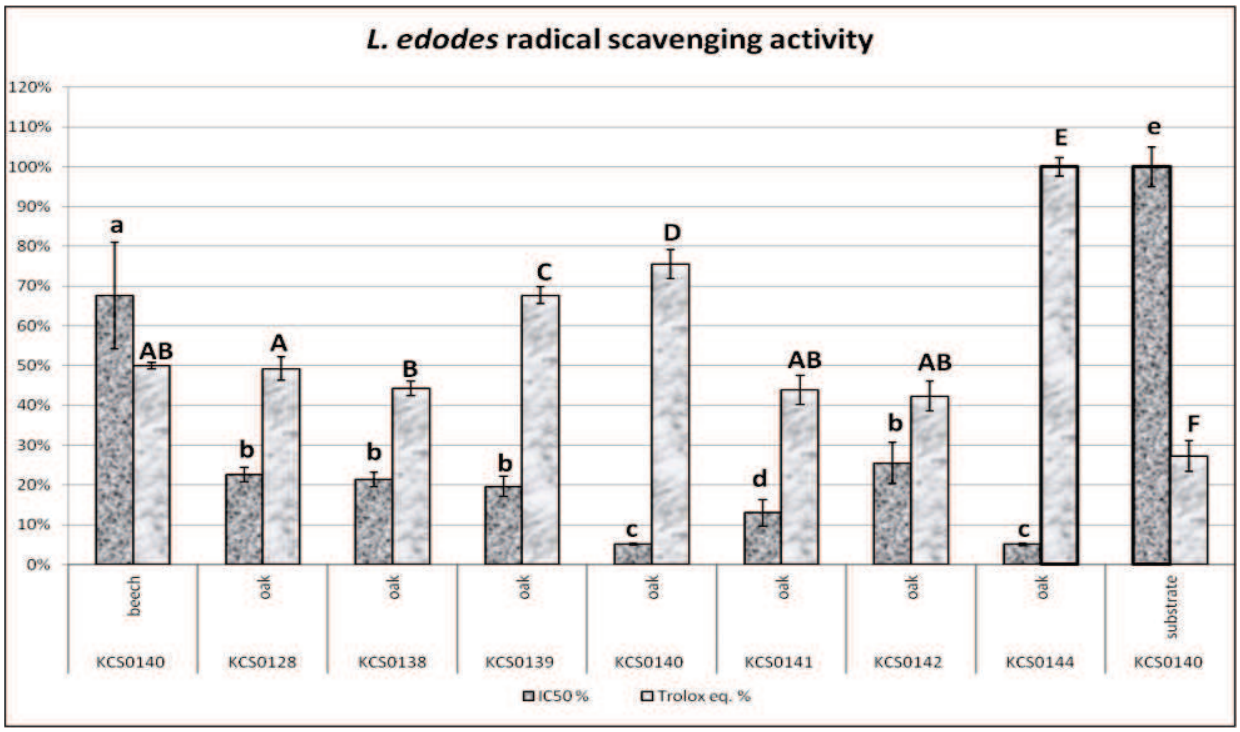

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***Figure 3**: Comparison between L. edodes strains antioxidant levels either substrate or log grown. Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant differences ($p < 0.05$) between strains and log species are indicated with different letters on top of each bar.*

Total phenolic content

The Folin-Ciocalteu reagent was used to assess the total phenolic content in all the mushroom strains. Different *P. ostreatus* strains were found to change in phenolic content when cultivated on different wood species. Poplar wood resulted in the highest phenolic content for strains KCS0146 and KCS0147, robinia allowed the production of the highest content in KCS0152 only, while willow allowed a higher content for the strain KCS0148. Tilia allowed the highest content of phenols among all the samples and only for KCS0150 strain (Figure 4).

Shiitake	growth substrate	Gallic eq. (mM)
KCS0140	beech	0.746 ± 0.023
KCS0128	oak	1.159 ± 0.010
KCS0138	oak	0.958 ± 0.013
KCS0139	oak	1.401 ± 0.029
KCS0140	oak	1.013 ± 0.008
KCS0141	oak	0.682 ± 0.032
KCS0142	oak	0.780 ± 0.019
KCS0144	oak	1.541 ± 0.022
KCS0140	sawdust	0.583 ± 0.047

Oyster	growth substrate	Gallic eq. (mM)
KCS0146	poplar	0.872 ± 0.010
KCS0147	poplar	0.995 ± 0.016
KCS0148	poplar	0.583 ± 0.010
KCS0150	poplar	0.855 ± 0.010
KCS0152	poplar	0.694 ± 0.017
KCS0050	robinia	0.945 ± 0.008
KCS0146	robinia	0.291 ± 0.010
KCS0152	robinia	1.000 ± 0.013
KCS0147	willow	0.933 ± 0.036
KCS0148	willow	1.021 ± 0.005
KCS0150	willow	0.779 ± 0.026
KCS0152	willow	0.795 ± 0.008
KCS0147	sawdust	1.025 ± 0.010
KCS0150	tilia	1.561 ± 0.025

Table 2: *Folin-Ciocalteu assay gallic equivalents measures in 0.1 mg/ml water solution of the W1 extracts for P. ostreatus (left) and L. edodes (right).*

Concerning *L. edodes*, different strains on the same oak logs produced significantly different phenolic content. KCS0144 grown on tilia logs allowed the observation of highest content. KCS0140 has showed a higher content when grown on oak with significantly lower levels when cultivated on beech. The lowest content was observed for the same strain grown on sawdust substrate (Figure 5).

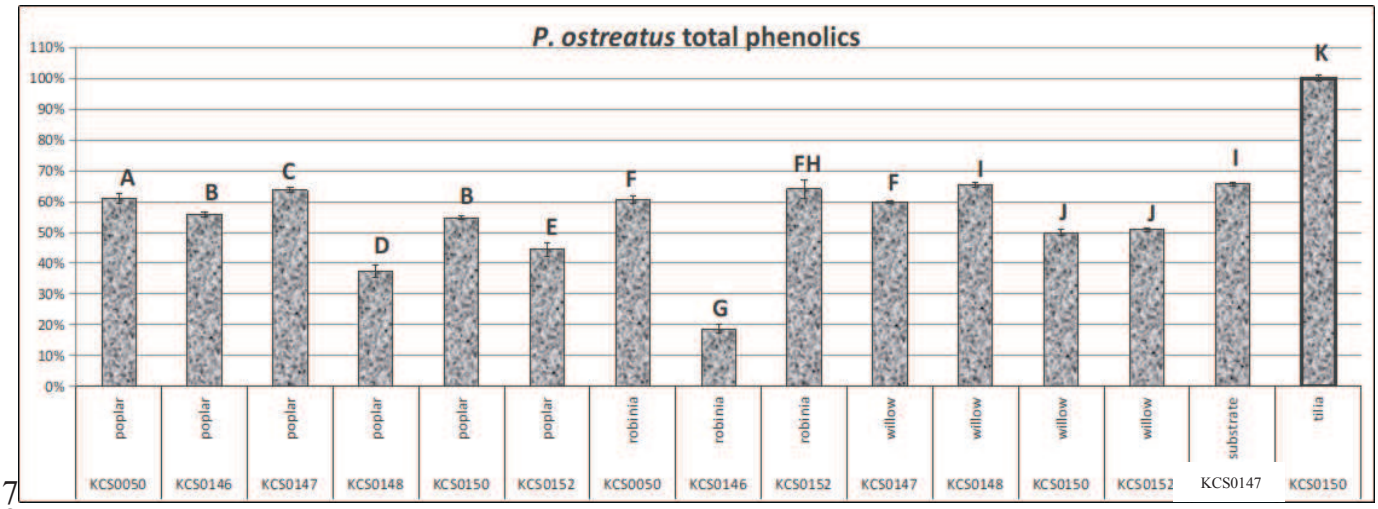

Figure 4: *Phenolic content in P. ostreatus strains grown on different log types and substrate. Values reported are normalized to the highest content observed (KCS0150 on tilia) and indicated as the respective percentages.*

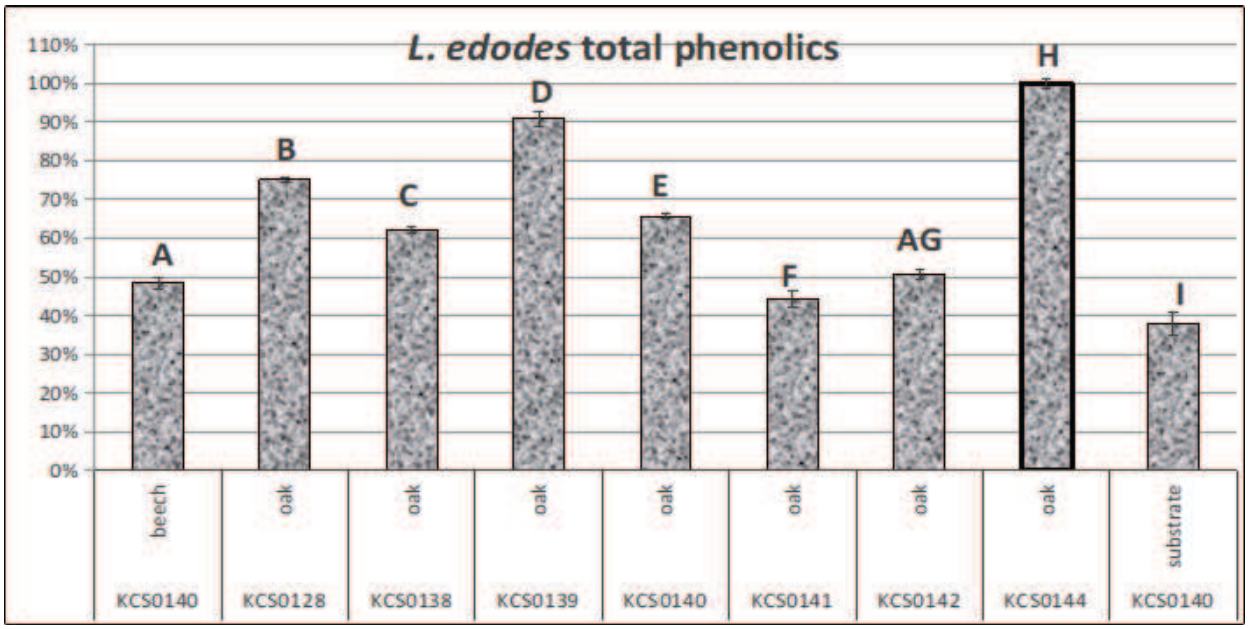

Figure 5: *Phenolic content in L. edodes strains grown on different log types and substrate. Values reported are normalized to the highest content observed (KCS0144 on oak) and indicated as the respective percentages.*

Counter-extractions of W1-kcs0140-beech

In concerns to the increasing polarity solvents counter-extraction of the water extract of KCS0140 grown on beech (W1-kcs0140-beech), it yielded five fractions with different

weight and consistence, as reported in Table 2. All the extracts were dried and dissolved back into the respective extraction solvent not producing any precipitate. The last pellet that was produced in the extraction (after methanol) did not completely dissolve back into water, thereby used as a suspension (kcs0140-WR) for the subsequent assays.

The extract W1-kcs0140-beech resuspended showed pH 5 and was subjected to pH guided counter-extraction using acid (pH 3) and basic (pH 9) conditions. This yielded four fractions of different weight as reported in Table 3.

Fraction	**Weight (mg/g_{crude})**	**Consistence**
kcs0140-CHE	60	Oily
kcs0140-DCM	51	Oily
kcs0140-EtOH	287	Powder
kcs0140-MeOH	115	Powder
kcs0140-WR	421	Powder
kcs0140-pH3	615	Powder – sticky
kcs0140-pH9	173	Powder
kcs0140-pH5	92	Powder – sticky
kcs0140-pellet	106	Powder
kcs0140-S	675	Brown solution
kcs0140-P	325	Brown-white powder

Table 3: *W1-kcs0140-beech water extract 1 g yields after solvent and pH based counter-extractions. The last two extracts (kcs0140-S and kcs0140-P) were reported as the weights obtained from 100 mg of the crude extract.*

Antibacterial activity

Antibacterial activity of shiitake water extracts obtained both from log and substrate was assessed by means of agar disk diffusion assay. All strains were able to induce an inhibition zone (halo) in both the tested model microorganisms.

When the water extracts (W1) from *L. edodes* strains were investigated for their antimicrobial activity against the chosen model microorganisms, *S. aureus* and *P. aeruginosa*, the extracts showed significantly different activities. Shiitake extracts can be divided in three groups with increasing activities: the less active (KCS0128 and KCS0140 grown on substrate), the intermediate active (KCS0138, KCS0142, KCS0139 and KCS0144), and the most active (KCS0140 grown on oak and beech, and KCS0141). KCS0141 and KCS0140 cultivated on beech logs showed the highest antibacterial activities both against *S. aureus* and *P. aeruginosa*. In particular, KCS0140 showed a higher antimicrobial activity when grown on oak and beech logs respect with substrate, showing an inhibition zone against *S. aureus* six and seven fold larger than substrate, respectively. A similar antibacterial activity was observed in *P. aeruginosa* (Figure 6): the inhibition halos of oak and beech were six and eight-fold larger than substrate, respectively (Figure 6). The low antimicrobial activity of KCS0140 substrate grown was comparable to that of KCS0128.

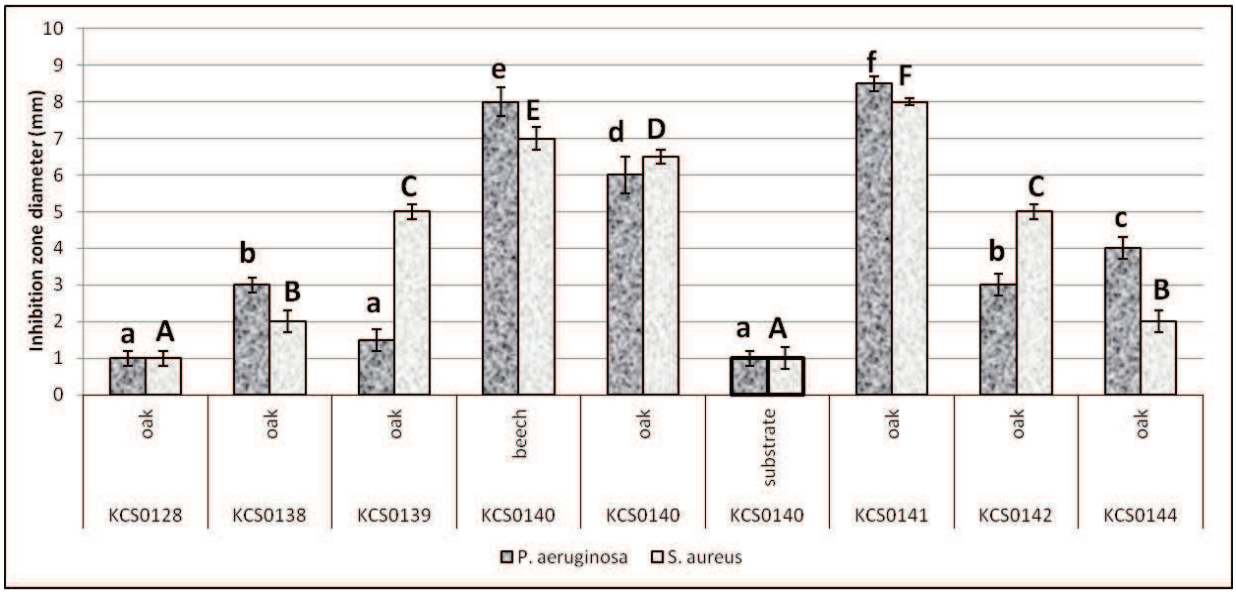

Figure 6: *Inhibition zones diameter of water extracts from the shiitake strains grown on logs or substrate. The same strain KCS0140 cultivated on substrate is highlighted. Significance ($p < 0.05$) reported with different letters.*

When the minimal inhibitory concentration (MIC) was assessed, *P. aeruginosa* was revealed to be more tolerant than *S. aureus* to the shiitake extracts tested (Table 4).

KCS0141 and KCS0140 cultivated on beech produced the only extracts that were found active against both microorganisms. All the other strains extracts showed activity only against *S. aureus* in a concentration range between 3.3 and 6.7 mg/ml. The strains KCS0141 and beech grown KCS0140 showed the best antibacterial activity against *S. aureus*. A significantly lower activity was observed for KCS0142, KCS0139, and KCS0128. The lowest activity was found for KCS0140 oak grown and KCS0128 strains. However, no activity was observed in KCS0140 and KCS0138 strains up to 10 mg/ml of crude W1- water extracts. Concerning *P. aeruginosa*, the only active strains with antimicrobial activity was KCS0141 and KCS0140 cultivated on beech logs (Table 4). Oyster mushroom did not show any antibacterial activity.

Strain	**Growth medium**	**MIC *S. aureus* (mg/ml)**	**Significance (*S. aureus*)**	**MIC *P.aeruginosa* (mg/ml)**	**Significance *(P. aeruginosa*)**
***L. edodes* KCS0141**	oak	3.3 ± 1.4	A	2.5 ± 0	a
***L. edodes* KCS0142**	oak	5.0 ± 0	B	> 10	d
***L. edodes* KCS0139**	oak	4.2 ± 1.4	B	> 10	d
***L. edodes* KCS0140**	beech	3.3 ± 1.4	A	2.5 ± 0	a
	oak	6.7 ± 2.8	C	> 10	d
	sawdust	> 10	D	> 10	d
***L. edodes* KCS0128**	oak	5.0 ± 0	B	> 10	d
***L. edodes* KCS0138**	oak	> 10	D	> 10	d
***L. edodes* KCS0144**	oak	6.7 ± 2.8	C	> 10	d

Table 4: *MIC values (reported with standard deviations) of log/substrate grown shiitake strains against P. aeruginosa and S. aureus. Different uppercase letters indicate significantly differences in the activities against S. aureus. Different lowercase letters indicates significantly differences in the activities against P.aeruginosa.*

The extract W1 from KCS0140 grown on beech was subjected to solvent counter-extraction with increasing polarity. CHE, DCM, and MeOH yielded fractions depleted of antibacterial activity in a 50 mg/disk diffusion assay against *P.aeruginosa* (Table 4). EtOH and WR fraction demonstrated a very low antibacterial activity when tested at 50 mg/disk against *P. aeruginosa* (Table 5). When the pH counter-extraction was concerned, it was observed a complete depletion of the antibacterial activity against *P. aeruginosa* (Table 5).

Fraction	diameter (mm)
kcs0140-CHE	0
kcs0140-DCM	0
kcs0140-EtOH	2.0 ± 0.8
kcs0140-MeOH	0
kcs0140-WR	2.5 ± 0.5
kcs0140-pH3	0
kcs0140-pH9	0
kcs0140-pH5	0
kcs0140-pellet	0
kcs0140-S	5.2 ± 1.2
kcs0140-P	6.5 ± 1.1

Table 5: *inhibition zone diameter (with standard deviation) in disk diffusion assay for 50 mg/disk of the fractions obtained by different counter-extraction of the crude extract W1-kcs0140-beech.*

A time-kill assay showed that KCS0140 grown on beech decreased *P. aeruginosa* inoculum of 6 x 10^5 cfu/ml of one log unit upon 10 minutes incubation, and four log unit upon two hours incubation reaching the detection limit (< 10^2 cfu/ml). Phase-contrast microscopy images showed the detrimental effect of KCS0140 after 24-hour treatment on *P. aeruginosa* cells (Figure 7). Furthermore, 20 mg of W1-kcs0140-beech produced an inhibition zone comparable with Ceftriaxone 0.2 mg, antibiotic that was chosen as the control (Table 6).

Sample	Concentration (mg)	Inhibition zone diameter (mm)
W1-kcs0140-beech	5	2.1 ± 0.4
W1-kcs0140-beech	10	4.7 ± 0.6
W1-kcs0140-beech	20	7.7 ± 0.8
W1-kcs0140-beech	40	8.1 ± 0.8
Ceftriaxone	0.05	3.5 ± 0.4
Ceftriaxone	0.1	6.7 ± 0.7
Ceftriaxone	0.2	8.2 ± 1.1
Ceftriaxone	0.4	14.3 ± 1.7

Table 6: *Comparison of inhibition zone diameters in disk diffusion assay between the water extract of beech grown KCS0140 and the antibiotic Ceftriaxone.*

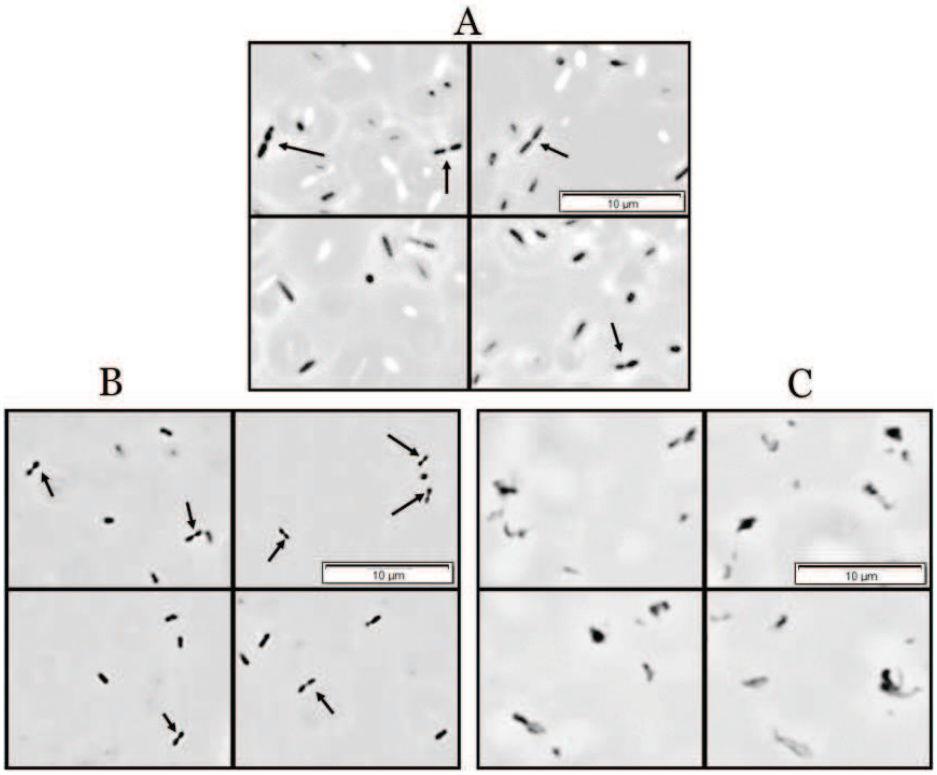

Figure 7: *(A) fresh inoculum of P. aeruginosa 30 min after dilution of an overnight LB inoculum to 10^6 cfu/ml; arrow indicates an example of cell under division. (B) bacteria after 2 h of incubation with 25 mg/ml of treatment; arrows indicate cell presumably under division. (C) treated inoculum after 24 h of incubation; arrows indicate aggregates and presumably degraded bacterial cells.*

Analyses of nutritional compounds loss

An early analysis of the medicinal compounds in water extracts was performed in all *Pleurotus* strains. Optimization of the HPLC-MS/MS analysis method was achieved to evaluate the concentration of vitamin D2 and ergothioneine in water extracts. Almost all mushrooms strains released vitamin D2 and ergothioneine in detectable concentrations. Release of vitamin D2 ranged from 12 to 46 µg/100g of dried mushrooms. Ergothioneine was found in a range from 20 to 80 µg/100g of dried material. In regard to eritadenine, lovastatin, and lenthionine, no extract showed a detectable concentration of any of these compounds.

Discussion

The same strain and culture condition produced reproducible extraction yields over the multiple water extraction performed. Extraction yield were found dependant on both the strain and the culture conditions. Thermal treatment of dry mushroom material before water extraction was used as a stress test, which allowed the assessment of alteration to important substances in the mushroom matrix, thereby simulating transformation and cooking processes [39]. As far as the thermal treatment effects on the antioxidant levels were concerned, temperatures of up to 80°C showed higher antioxidant activities than 4°C treatment in oyster. In contrast, shiitake revealed a decreased activity when the same temperatures were applied. Thus, it is conceivable that differences depended on thermal-induced modifications concerning some compounds in the dried sample [15].

When log grown strain of *P. ostreatus* and *L. edodes* were assessed after an exhaustive 72 h extraction, it was noticeable that most of the strains on each wood had significantly different levels of antioxidant activity. Furthermore, when both DPPH and ABTS radical scavenging assays were concerned, it was clear that all the strains of *P. ostreatus* and *L. edodes* performed more significantly when grown on logs than when cultivated on sawdust substrate, respectively. And with more in depth results, each strain resulted in performance levels from none to slightly correlated when tested either with the former or the latter assay. As the IC_{50} was not discovered to correlate to the activity as measured by ABTS, it is conceivable that multiple antioxidant compounds classes are present in

the extracts [40]. This also suggests there is a need for development of further purifications to reveal the most interesting substances owing the activity [30]. As the scavenging activity of various strain was found significantly dependant on log species, further studies will be necessary to better to investigate the interactions between selected strains and the different species of logs [41].

In regard to the assessment of total phenolic compounds, significant differences were observed when different logs were used for the cultivation of same strain; furthermore, there were significant differences among the strains for each log. It was noticeable that tilia logs show a concentration over the average in such compounds. On the other hand, only a strain was able to grow on tilia logs. Moreover, the Folin-Ciocalteu method that was extensively used to estimate phenols [42], [43], [44]. However, it was demonstrated this was possibly biased by the presence of some interfering compounds from the crude extract [45].

As the antibacterial activity of oyster and shiitake W1 extracts were tested by means of agar disk-diffusion assay, *P. aeruginosa* was discovered to be more sensitive than *S. aureus* to most of the extracts. All the log cultivated strains were more effective than the corresponding substrate cultivated strains. Log species was found to influence KCS0140 activity, suggesting that logs plays a critical role in the interaction with the mushroom, thereby resulting in different antibacterial activity yields. In consideration with the differentiation of antioxidant activity, this further suggests the need for a more thorough study focusing on the interaction that takes place at a proteomic and metabolic level [46], [47], [48].

As a subsequent MIC assay was concerned, it is noteworthy that sawdust grown shiitake strains did not show any efficacy, while most of the log grown strains were effective against *S. aureus*. On the other hand, only two strains demonstrated activity against *P. aeruginosa*. More in depth analyses will be performed to characterize the antibacterial activity, particularly against *P. aeruginosa*. W1-kcs0140-beech was chosen as it demonstrated the highest antibacterial activity against both *S. aureus* and *P. aeruginosa*. *P. aeruginosa* cells after 2 h treatment, seem to be compromised in cell division machinery as no complete duplication event was found to occur. Indeed, bacterial cells showed proper motion, as swimming and tumbling were detectable; however, cellular

viability was compromised as confirmed by viable counts. After 24 h treatment, no cells were observable, suggesting that this extract also has a bacteriolytic activity [49].

As counter-extractions were performed, the resulting fractions were depleted of antibacterial activity. It is significant that the EtOH and the WR last fraction demonstrated a low but still present activity while the MeOH fraction did not show any. This suggests that antibacterial compound/s are water soluble and particularly sensitive to the organic solvents. The same loss of activity was observed when the crude extract was subjected to acid and basic pH extractions. Furthermore, it conveys that antibacterial substances have complex structures and seem to require water as a solvent and specific pH values to exert its antibacterial effect.

Finally, a first evaluation of specific nutraceutical compounds in water extracts in both mushrooms showed that Vitamin D2 and ergothioneine are present in similar amounts in all strains of oyster and shiitake. No eritadenine in shiitake and lovastatin in oyster was noticed. Future studies will be carried out in order to achieve the best method to detect and quantify secondary metabolites of pharmacological interest and to research further how these metabolites are modified and/or degraded during food transformation processes.

Conclusions

Both shiitake and oyster demonstrated interesting radical scavenging (antioxidant) properties; additionally, shiitake was also able to produce antimicrobial metabolites. These data envisage their use as main ingredients for functional food formulation. Future studies will be needed in order to increase secondary metabolites of pharmacological interest production. In particular, a special effort will be made in selecting the best strains, formulating the best substrates and assessing the best parameters and conditions for mushroom incubation and fructification. Future researches will be focused both on the production of mushrooms with a high nutritional value and assess their possible use as food supplement and the purification of antimicrobial and antioxidant compounds for a more thorough and in-depth assessment of the interesting results obtained.

List of Abbreviations: *L. edodes, Lentinula edodes; P. ostreatus, Pleurotus ostreatus; CHE, ciclohexane; DCM, dichloromethane; EtOH, ethanol; MeOH, methanol; W, water*; LB, Luria Bertani; MH, Mueller-Hinton; MIC, Minimal inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

Author's Contribution: All authors contributed to this study.

Competing Interests: There are no conflicts of interest to declare.

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Chapter 3

In vitro evaluation of *L. edodes* and *P. ostreatus* fruiting bodies grown on different logs or commercial sawdust substrate, as potential dietary agents in colon cancer control

Introduction

Several natural substances have gained public attention to prevent/treat different dysfunctions or pathologies. In particular, antioxidant, immunomodulatory and antitumor properties have been intensively studied [71]. Nowadays, Medicinal mushrooms can be considered a functional food with therapeutic effects against specific chronic diseases [22, 23]. *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster) are two of the most important and consumed medicinal mushrooms in the world. Their therapeutic effects have been shown to be beneficial for serious diseases such as cancer and degenerative pathologies, thanks to their antioxidant, anti-inflammatory and anticancer activity [72], with minimal side effects. In the last 10 years, phenolic and methanol extracts of different mushrooms species were used to deepen the study of their effect during inflammation processes [73, 74]. These studies have provided evidence that these compounds stimulate the production of TNF-α, interleukins and cytokines both *in vitro* and *in vivo* in animal models [24, 73, 75]. As observed by Jedinak and Sliva (2008) [72], methanol crude extracts of *Pleurotus ostreatus* has cytotoxic effect on different cancer cell lines such as HT-29 from intestinal adenocarcinoma and MCF-7 breast cancer cells, but no cytotoxic effect was observed on non-tumorigenic MCF-10A cells. These results suggested a potential use of medicinal mushrooms as preventive and antiproliferative resources for breast and colon cancer [72]. The aim of this study was an *in vitro* evaluation of *L. edodes* and *P. ostreatus* fruiting bodies, cultivated on different logs or on a commercial sawdust substrate, as potential dietary agents in colon cancer control without adverse side effects. For this purpose, we tested the antiproliferative effects of aqueous extracts from the fruiting body of these mushrooms on colon cancer cell lines HT-29 and HCT-116. The protective

effects of extracts on H_2O_2 treated cells were investigated too. Moreover, to simulate the digestion process, we used mushroom extracts incubated with HCl and pepsin at 37°C. The wide range of doses tested has been chosen to reproduce possible physiological conditions.

Materials and methods

Cell culture

Human cell lines HT-29 and HCT-116, derived from colon cancer, were obtained from Molecular Biology laboratory of the DBSV of the University of Insubria. Human dermal fibroblasts from healthy adult donors (aged 35-45) were obtained from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases – NETWORK OF GENETIC BIOBANKS TELETHON" [76]. Fibroblasts were used for the experiments between the XI and the XIII passage.

HT-29 and fibroblasts were grown in RPMI 1640 medium (Carlo Erba) containing 10% fetal bovine serum (FBS) (Carlo Erba) and 1% L-Glutamine (Carlo Erba). HCT-166 cells were cultured in DMEM medium (Carlo Erba) containing 10% fetal bovine serum (FBS) (Carlo Erba) and 1% L-Glutamine (Carlo Erba). All cultures were maintained at 37°C in 5 % CO2 and 95% humidity.

Mushroom extract preparation

Shiitake strain KCS0140 and *P.ostreatus* KCS0160 and KCS0147 were selected to perform the extraction procedure. To simulate the digestive process after the ingestion of edible mushrooms in oral cavity, 500 mg of dried shiitake mushrooms were treated with 5 ml of water HCl pH 1.5 and 5000 UI of pepsin enzyme for 2.5 hours at 37°C in continuous agitation [77]. Sample's pH was neutralized using 1 M $CaCO_3$. Samples were filtered first on tissue paper and then on a nitrocellulose membrane 0,2 μm filter for sterilization and maintained at -20°C until use.

ABTS radical scavenging activity

Antioxidant activity of the water extracts was performed by ABTS radical cation decolorization assay [78]. Freeze dried extracts were resuspended in water at a

concentration of 0.1 mg/ml, allowing a complete dissolution. In a cuvette, 500 µl of the sample solution and 500 µl of ABTS°+ was added for spectrophotometric analysis. After 15 minutes, the OD734nm has been measured and was compared with the Trolox titration curve.

MTT assay method

To test the *in vitro* cytotoxic/beneficial effect of the mushroom digested extracts and the effect of these extracts in presence of H_2O_2, cell viability was determined by the MTT assay, following the protocol of Johan van Meerloo *et al.* [79]. Three concentrations of each extract were used in MTT experiments (560 µg/ml, 70 µg/ml and 2.3 µg/ml). This assay was performed in 96-well plates, seeding 12.500 HT-29 and HCT-116 cells and 30.000 fibroblasts cell per well with 200 µl of the appropriate culture medium. Controls are performed seeding 12.500 tumoral cells or 30.000 fibroblasts and 200 µl of the appropriate culture medium for each well. The formazan crystal formation was performed using Colorimetric cell viability kit IV (PromoKine) and 100 µl of the appropriate culture medium. After removing MTT reagent, dimethyl sulfoxide (DMSO) was added to each well and absorbance of formazan solution (590 nm) was evaluated by reading absorbance at 570 nm with a Tecan Infinite 200. Cell viability was then expressed as the percent (%) of viable cells relative to the control.

Bacterial strain

E. coli C1a strain were grow on M9 broth from Sigma Aldrich under aerobic conditions at 37°C.

Prebiotics analysis

E. coli cultures were grown overnight in Luria-Bertani (LB) at 37°C. The next day, 1 ml of the overnight culture was back diluted in 25 ml of fresh M9 Broth and grown at 37°C in continuous agitation on a shaker. Growth rates and bacterial concentrations were determined by measuring optical density (OD) at 600 nm every 30 min (OD_{600} of 0.1 corresponds to a concentration of 10^8 cells per ml). Mushroom's extracts were added to a final concentration of 1:125 from the stock solution (200 µl in 25 ml of M9 broth with

the inoculum of *E. coli* C1a). The OD at 600 nm was measured every hour to build the *E. coli* growth curve in presence or absence of mushroom extracts.

Statistical analysis

Data was compared based on significance levels obtained by one-way ANOVA test followed by Tukey HSD post-hoc test. Probability levels of 0.05 were indicated with different letters [80].

Results

Antiproliferative effects of mushroom extracts on colon cancer cell lines *in vitro*

We tested the antiproliferative and antioxidant effects of aqueous extracts from mature fruiting bodies of *P. ostreatus* grown on sawdust substrate (PS) or on different log species, willow (PW), poplar (PP) and robinia (PR) and of *L. edodes* grown on sawdust substrate (LS) or on different log species, oak (LO) and beech (LB). To simulate the digestion process, we used mushroom aqueous extracts incubated with HCl and pepsin at 37°C. The cell viability on colon cancer cell lines HT-29 and HCT-116 was evaluated by MTT assay. Antioxidant test were performed on extracts using ABTS assay.

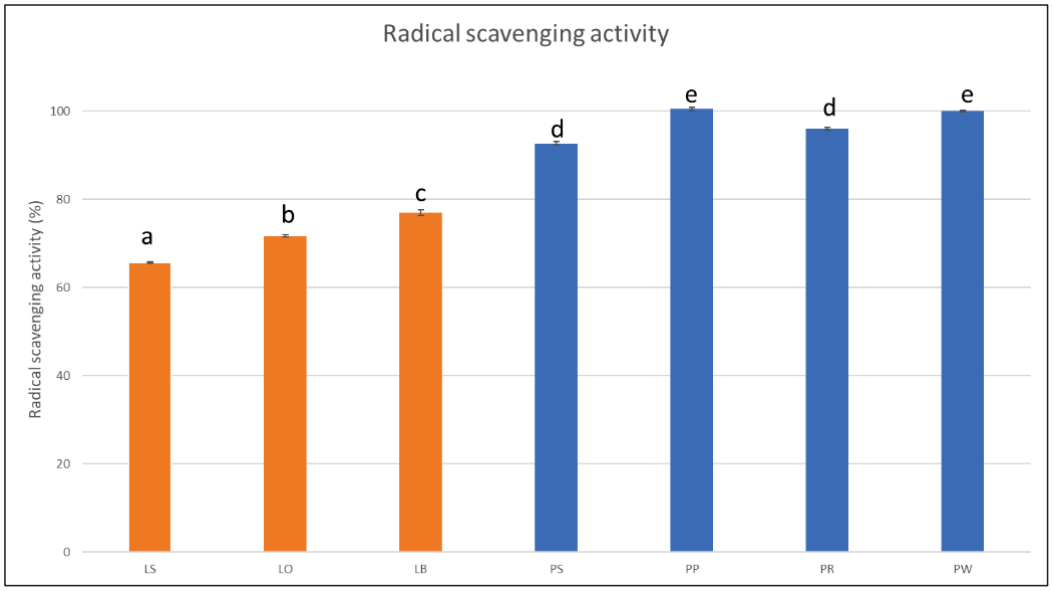

Figure 1: Comparison between shiitake (orange) and oyster (blue) mushrooms antioxidant levels either sawdust substrate or log grown. Activities are reported as normalized percentages to the highest one. Significance differences (p < 0,05) are indicated with different letters on top of each bar.

Effects of extracts on HT-29 cells

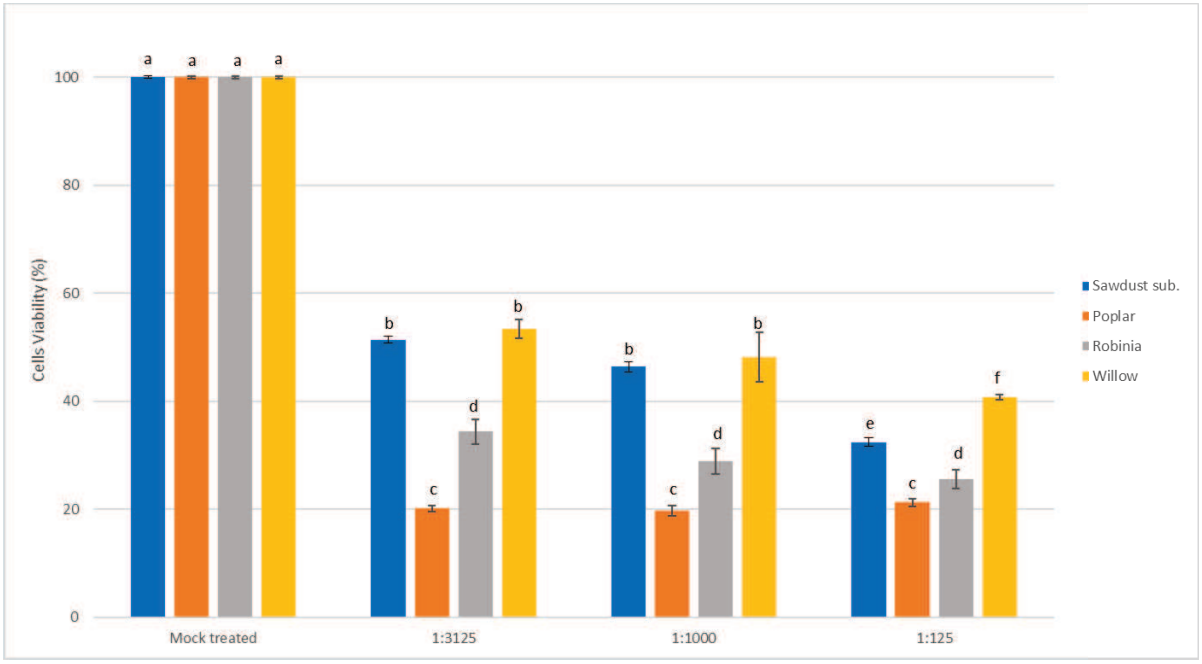

Figure 2: Effect of P. ostreatus extracts on cell viability. HT-29 cell line was cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

The effect of mushroom extracts on HT-29 cell viability was evident at all concentrations tested (Figure 2). A significant reduction in cell viability was already observed in HT-29 cells treated with the lowest concentration of PS (1:3125 dilution) and decreased up to 30% of control at the highest PS dose (1:125). Similar results were observed with PW extract. The PR and PP extracts were the most effective with a strong and gradual, but not dose-dependent reduction in cell vitality up to 80% in the highest PP dose (Figure 2).

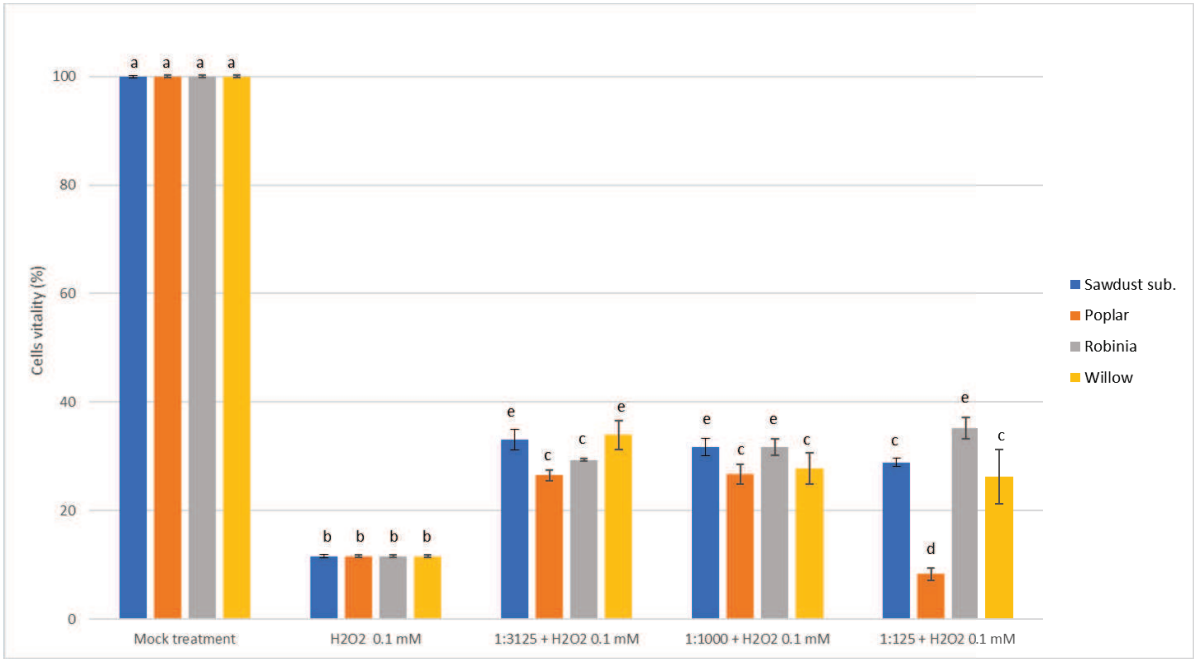

Figure 3: Effect of P. ostreatus extracts on cell viability after treatment with H_2O_2. HT-29 cell line was cultured with different concentrations of digested aqueous extracts for 44 h and other 4 h adding H_2O_2 and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

We previously showed that our mushroom extracts have phenolic content and display antioxidant activities [80]. To evaluate if their antioxidant activity had a protective role on colon cancer cells, we first treated HT-29 cells with mushroom extracts for 44 h, then we added 0,1 mM H_2O_2 and prolonged cell incubation for a further 4 hours. Residual cell viability of cells treated with both mushroom extracts was compared with cells treated only with 0,1 mM H_2O_2 for 4 hours or with untreated controls.

As shown in figure 3, a partial rescue of H_2O_2 induced toxicity was observed when cells were pretreated with all mushroom extracts, except with PP extracts at the highest dose. Indeed, a 90% reduction of HT-29 cell viability was observed after treatment with 0,1 mM H_2O_2 alone. Addition of mushroom extracts at the 1:3125 and 1:1000 dilutions before treatment with H_2O_2 diminished cell viability reduction to 65-70%. This fact points towards a protective effect of the extracts. Surprisingly, the PP extract showed a biphasic effect and inhibited cell proliferation (cellular viability <10%) at the highest dose, more than H_2O_2.

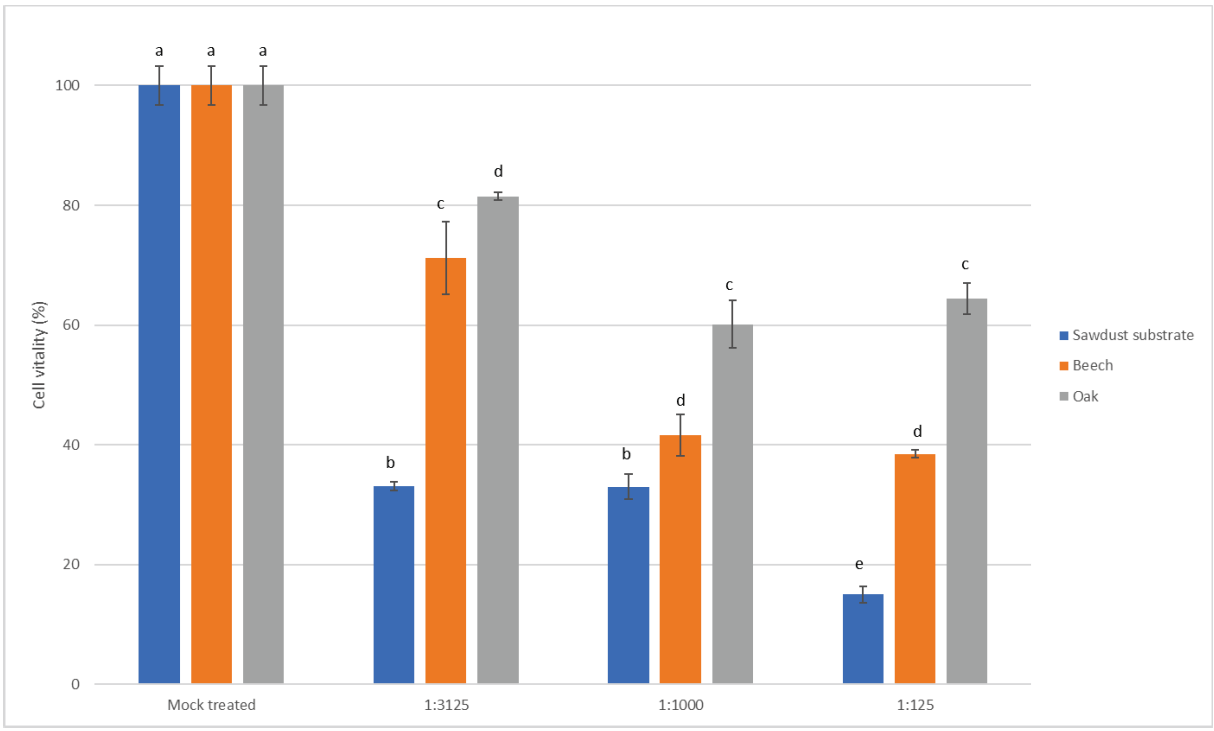

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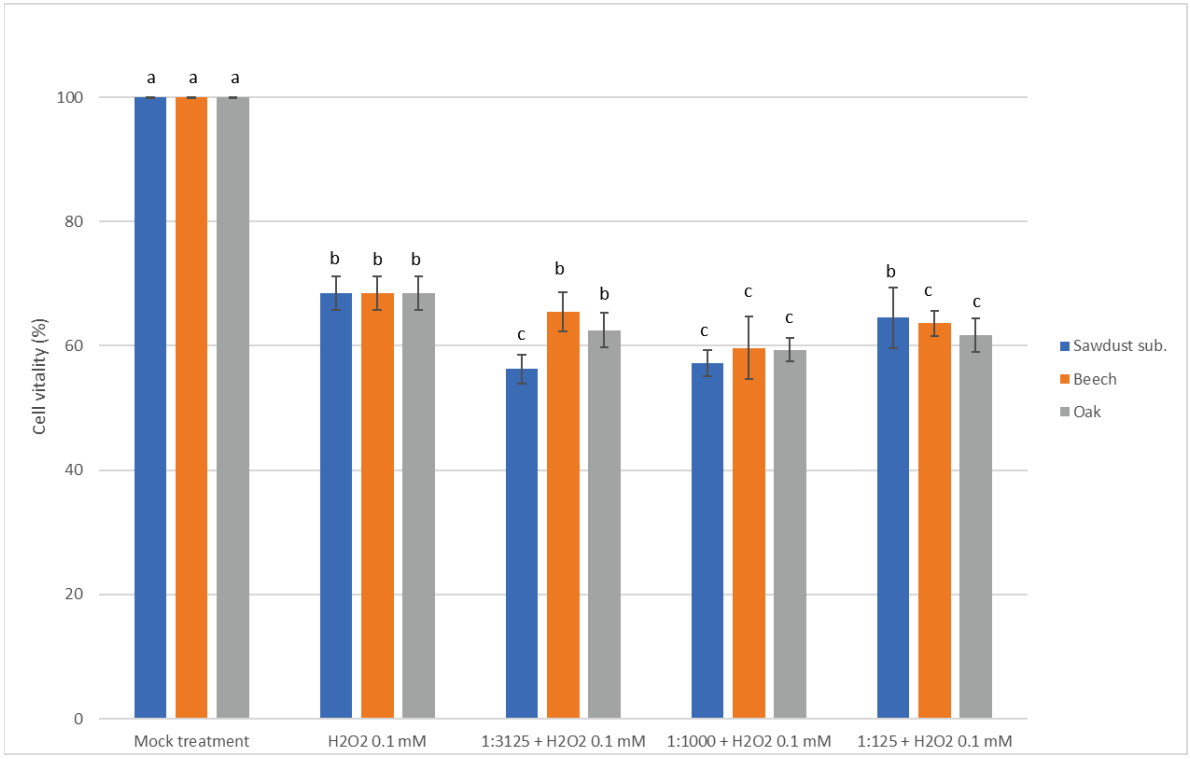
Figure 4: Effect of L. edodes extracts on cell viability. HT-29 cell line was cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).



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1519 *Figure 5: Effect of L. edodes extracts on cell viability after treatment with H_2O_2. HT-29 cell line was cultured with different concentrations of digested aqueous extracts for 44 h and other 4 h adding H_2O_2 and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).*

Regarding *L. edodes* extracts, we tested the aqueous extracts from mature fruiting bodies grown on sawdust substrate (LS), on beech (LB) or oak (LO) logs. As shown in Figure 4, also Shiitake extracts reduced HT-29 cell viability; LS was the most effective extracts, as a cell viability reduction up to 85% was observed at the highest LS concentration (1:125). The addition of LB or LO extracts induced a reduction of cell viability of 60% and 40%, respectively. No protective effect against H_2O_2 cytotoxicity was observed with extracts from this mushroom species (Figure 5).

Effects of extracts on HCT-116 cells

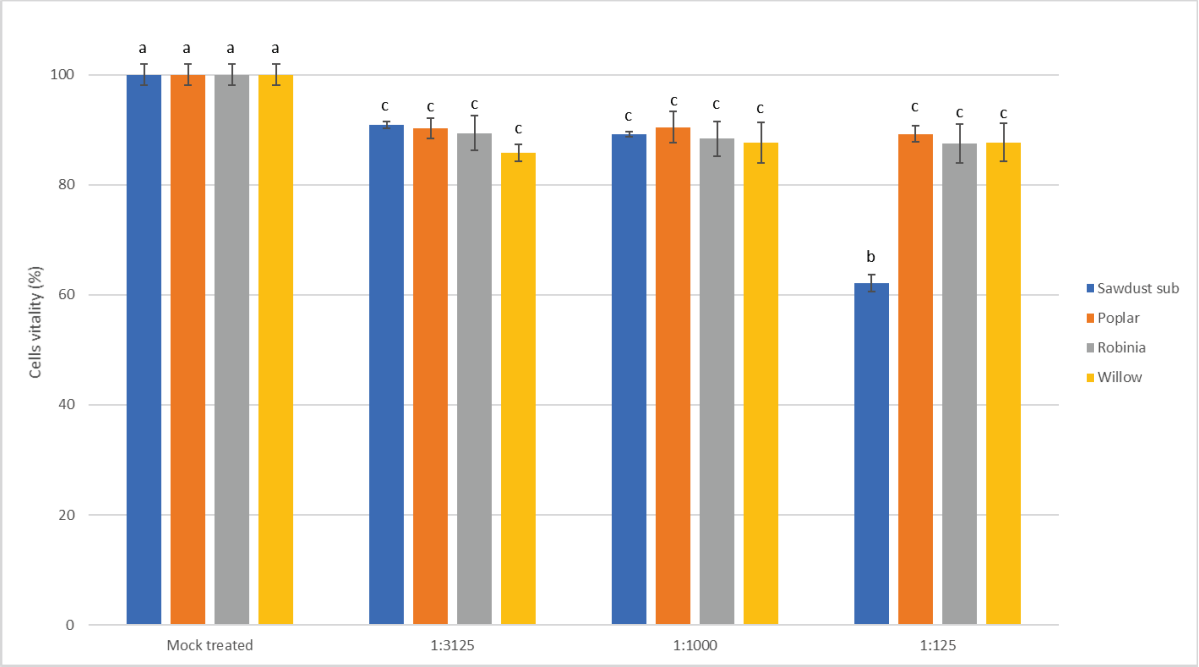


Figure 6: Effect of P. ostreatus extracts on cell viability. HCT-116 cell line was cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

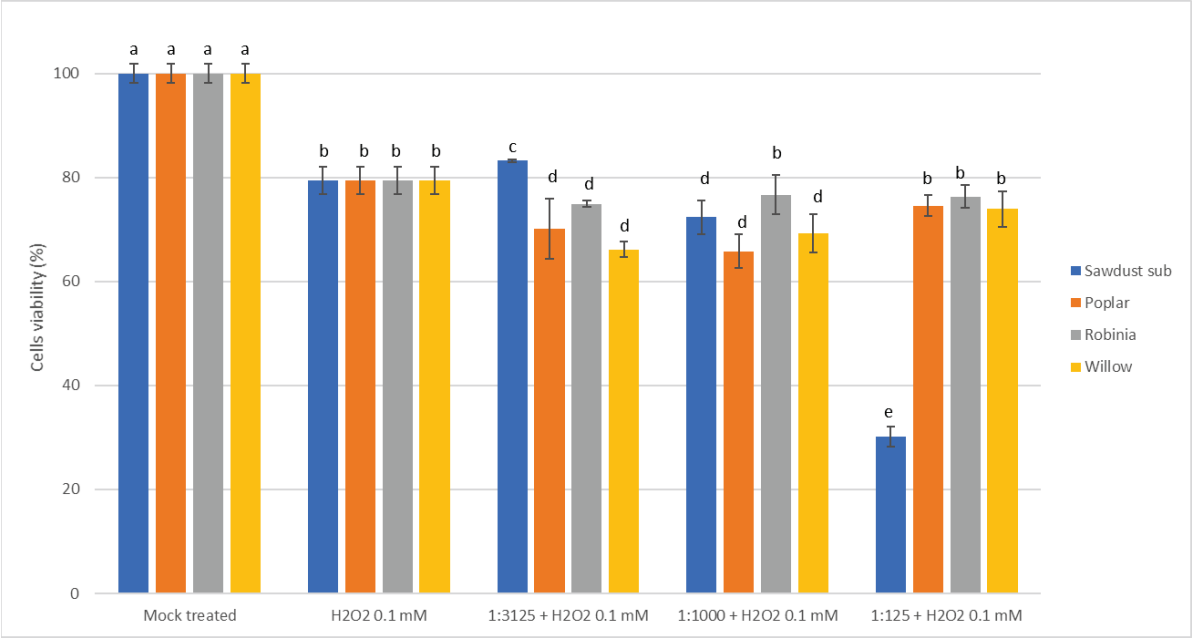


Figure 7: Effect of P. ostreatus extracts on cell viability after treatment with H_2O_2. HCT-116 cell line was cultured with different concentrations of digested aqueous extracts for 44 h and other 4 h adding H_2O_2 and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

Aqueous extracts from *P.ostreatus* grown on different substrates all showed lower cytotoxic effects on the HCT-116 line than on HT-29 cells, with a reduction in cell viability up to 15%. The only exception was the PS extract that reduced cell viability of 60% (Figure 6). Moreover, we did not observe any protection of these extracts against the H_2O_2 cytotoxic activity (Figure 7), but somehow, they seemed to have additive effects with H_2O_2.

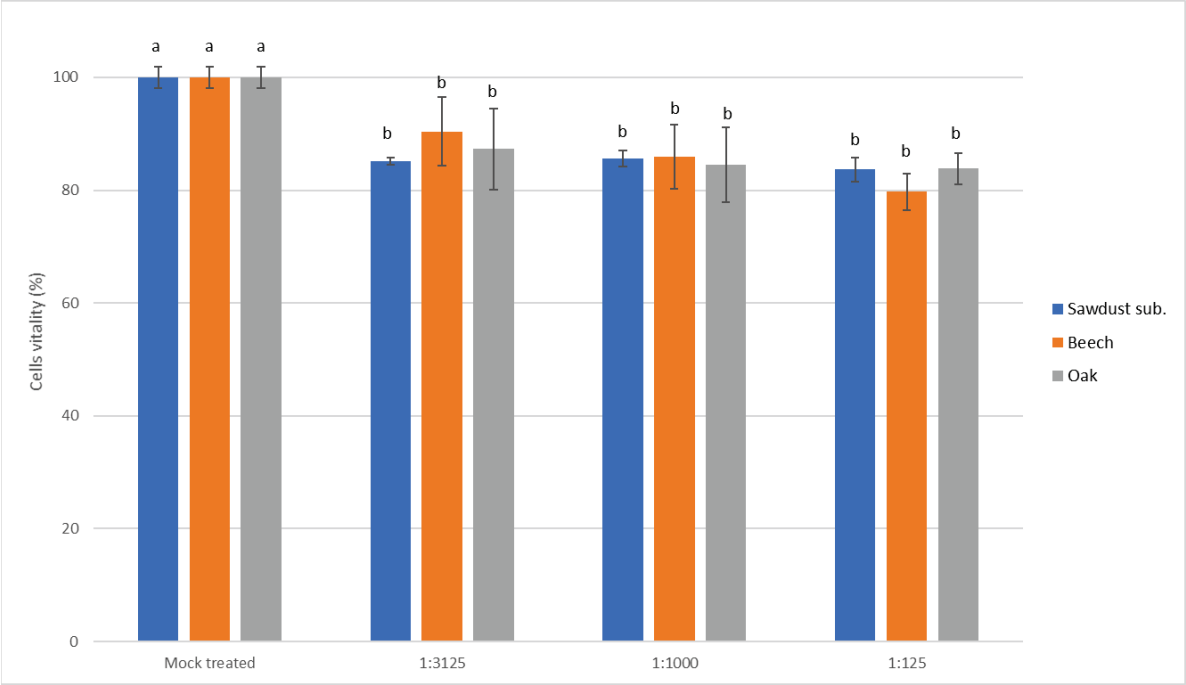
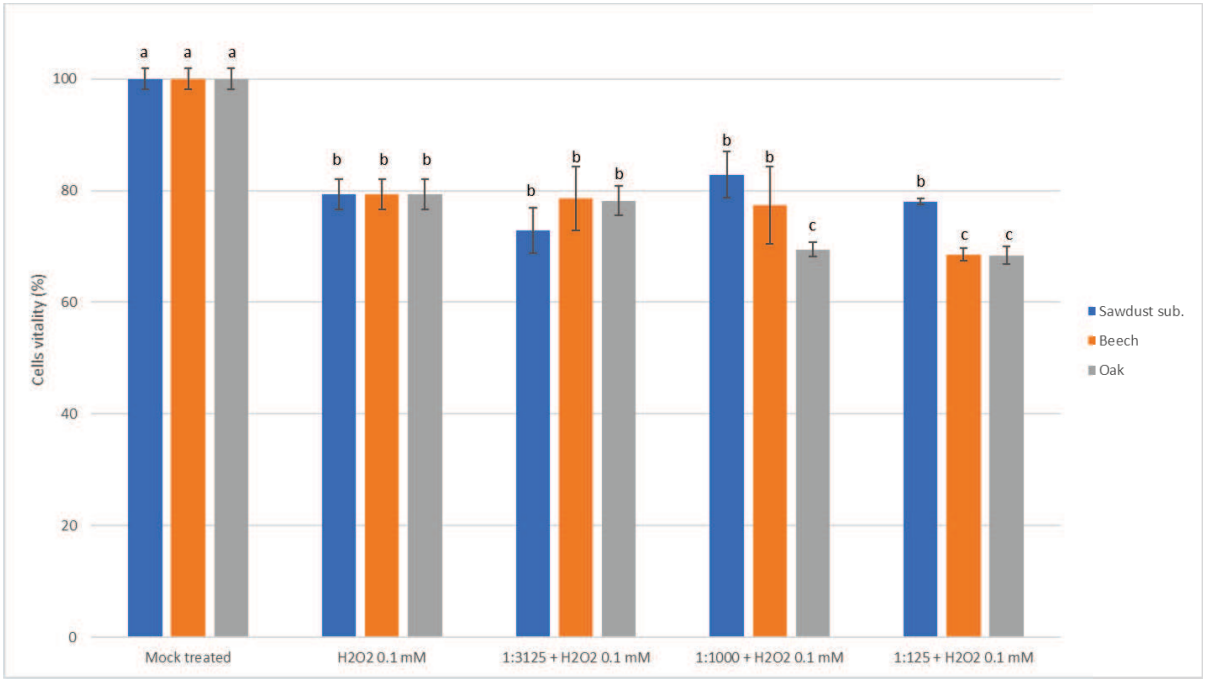


Figure 8: Effect of L. edodes extracts on cell viability. HCT-116 cell line was cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).



1546

Figure 9: Effect of L. edodes extracts on cell viability after treatment with H_2O_2. HCT-116 cell line was cultured with different concentrations of digested aqueous extracts for 44 h and other 4 h adding H_2O_2 and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

1547 The same small reduction in HCT-116 cell viability was observed with *L. edodes* extracts
1548 (Figure 8). These extracts had small or no effect on the cytotoxicity of H2O2 (Figure 9);
1549 this result could be due, at least in part, to the low antiproliferative effect of 0.1 mM H_2O_2
1550 on HCT-116 line. In fact, the reduction of cell viability after the treatment with H_2O_2
1551 alone was comparable to that observed using extracts alone.

1552

Analysis of side effects

Primary Fibroblasts

We then asked if mushroom extracts had the same effects on non-tumor, primary cells. We tested mushroom extracts on primary fibroblasts and found that they had no effect on proliferation of these cells except for PP extract at the highest concentration (Figures 10 and 11).

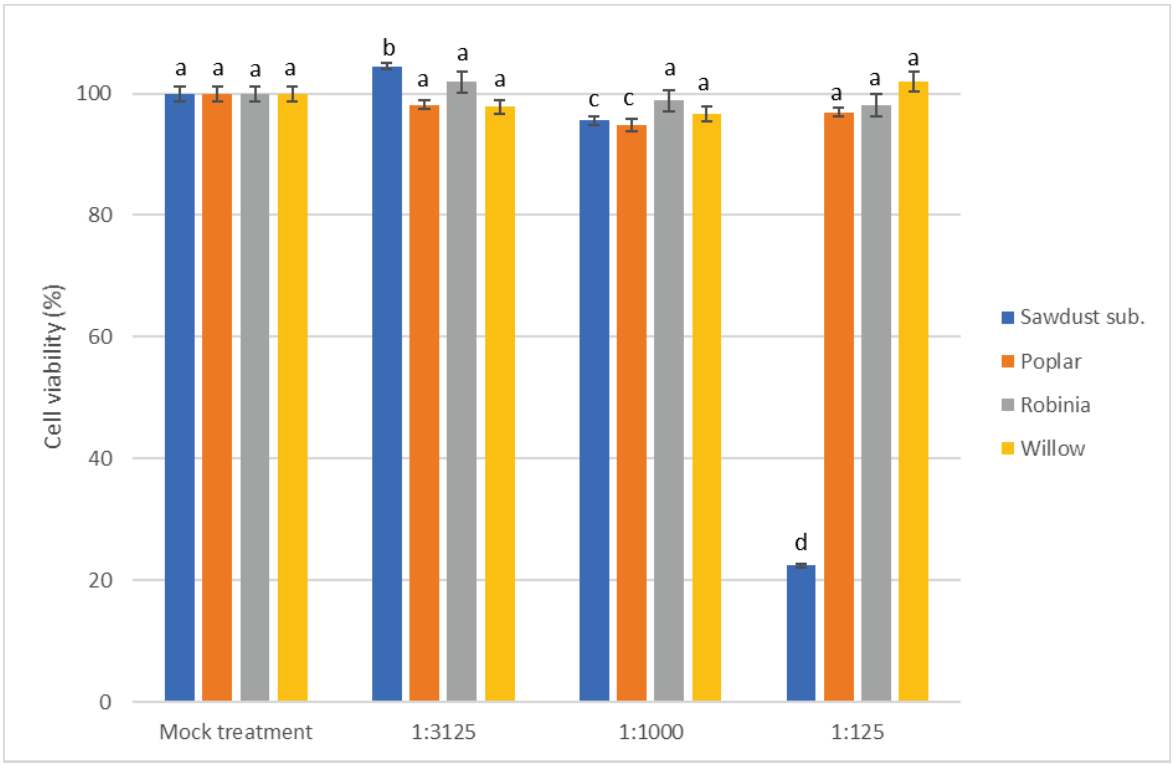


Figure 10: Effect of P. ostreatus extracts on cell viability. Primary fibroblast were cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

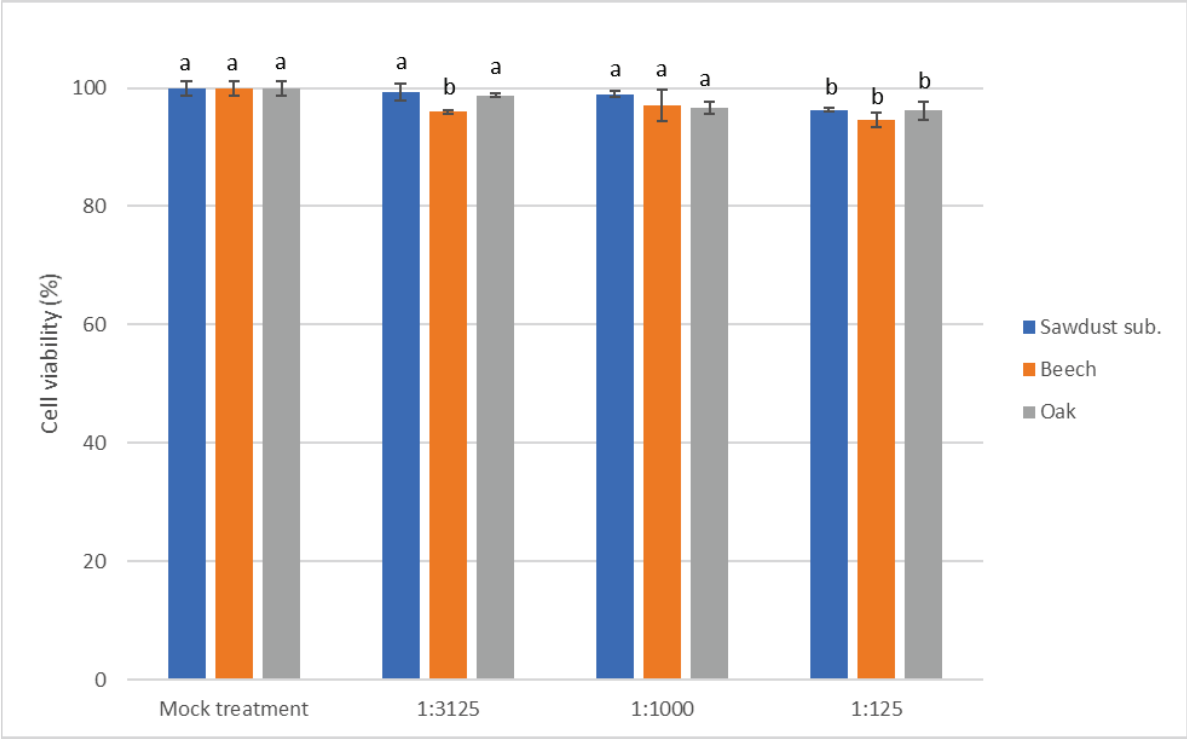


Figure 11: Effect of L. edodes extracts on cell viability. Primary fibroblast were cultured with different concentrations of digested aqueous extracts for 48 h and cell viability was assessed by the MTT assay as described in Materials and Methods. Cell viability was expressed as percent (%) of residual viability compared to mock treated control (100%). Differences in cell growth between control and all treated cells were statistically significant (P<0.05). All data are expressed as mean of three independent experiments (in each experiment each condition was analysed in triplicate) ± standard deviation (SD).

Prebiotics activity

We previously showed that aqueous *L. edodes* extracts had antibacterial activity[81]. To investigate a possible negative effect on gut microbiota, we tested our extracts on the growth of C1a *E. coli* strain, one of the most representative model microorganisms of human intestine. As shown in figure 9, the addition of 560µg/ml of LS or LO or LB induces a slight increase of growth rate, suggesting even a possible prebiotic effect (Figure 12).

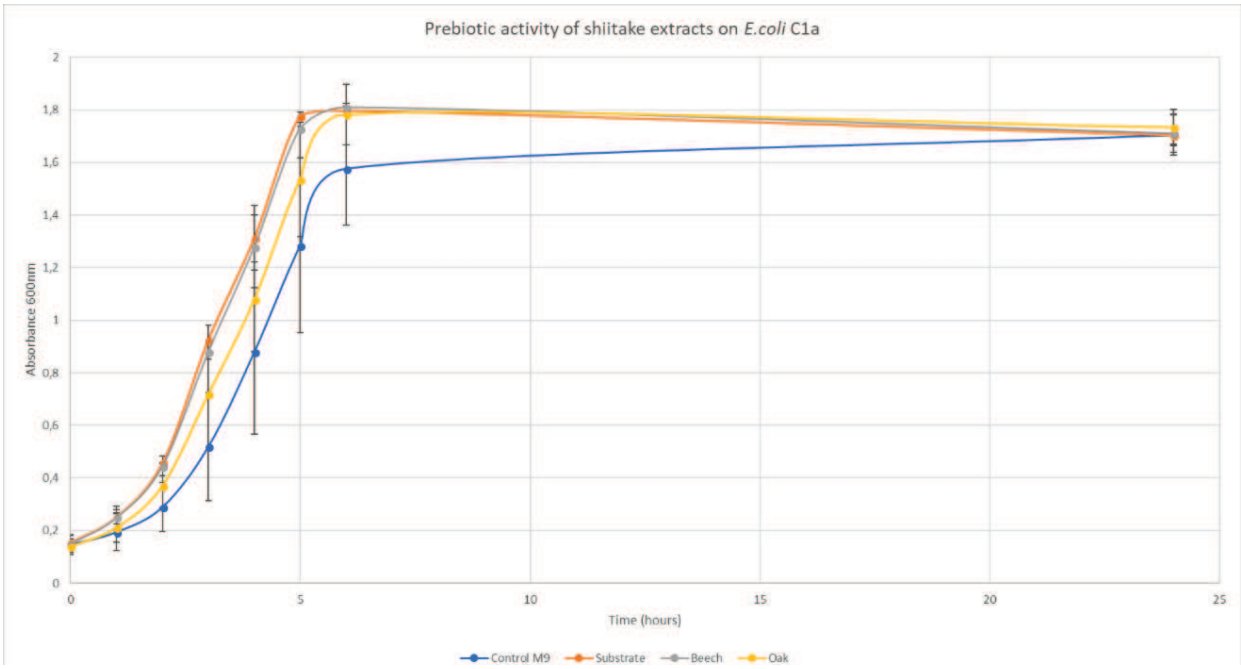


Figure 12: Prebiotics activity of shiitake mushroom extracts on E. coli C1a strain, a representative model microorganism of human intestine.

Discussion

In vitro studies of the effect of mushrooms extracts on cell growth have focused on non-aqueous extraction protocols. Here, we provide experimental evidence that a digestion simulation (incubation at 37°C with HCl and pepsin) of mature fruiting bodies is sufficient to observe an antiproliferative effect on different colon cancer cell lines, but not on normal fibroblasts. Concerning cytotoxic activities, *P. ostreatus* extracts were more effective than *L. edodes* extracts against colon cancer cell proliferation. Our observations confirm the results previously obtained by Finimundy and collaborators [82]. In particular, oyster extracts obtained from mushroom grown on poplar (PP) or willow (PW) logs displayed the highest antiproliferative activity on HT-29 cells and also a protective effect against H_2O_2–treated cells; both activities showed a dose-dependence. Our previous analyses revealed that aqueous extracts of *P.ostreatus* have higher antioxidant activity and higher polyphenolic content than *L. edodes,* in particular in oyster fruiting bodies grown on poplar and willow logs [80]. Therefore, the data presented here not only confirm our previous data, but suggest a positive correlation between the

presence of molecules with antioxidant activity and the inhibition of cancerous cell proliferation and suggest that phenolic compounds of aqueous extracts of *P. ostreatus* may, at least in part, influence the anticancer activity; however the mechanisms by which these activities take place have still to be elucidated [82]. *L. edodes* extracts displayed antiproliferative activity in spite of negligible antioxidant activity. This was particularly evident when mushroom was grown on sawdust (SS). As suggested by Lavi et al, 2006 [83], the antiproliferative activity of the *L. edodes* extracts could be related to an apoptosis induction operated by the polysaccharide fraction of extract. Lentinan is one of the most studied antitumoral compound of shiitake and the cytotoxic effect could be related to this beta- glucan, but the mechanisms involved are still unknown[73, 84]. The resistance to low pH and the fact that the proapoptotic activity is prevented by digestion with glycolytic enzymes supports this hypothesis. None of the tested extracts had a cytotoxic effect on primary fibroblasts, with the only exception of the highest dose of *P. ostreatus* extract grown on sawdust (PS); however, it is unlikely that this dose reaches any cells *in vivo*. The results obtained with primary fibroblasts suggest the specificity of the anti-tumor effect of mushroom extracts. Finally, the extracts used in this work slightly stimulate the growth of *E. coli* C1a strain, one of the most representative model microorganisms of human intestine. This result suggests the exclusion of a possible negative effect of our extracts on the gut microbiota, but rather a possible prebiotic effect. In conclusion our data indicate that *L. edodes* and *P. ostreatus* fruiting bodies, cultivated on logs or on a commercial sawdust substrate are potential dietary agents in colon cancer control without adverse side effects. Future work will focus on animal studies using dose-response data to test the effects of whole extracts and provide guidelines for incorporating mushrooms into diets to take advantage of their nutraceutical properties.

Chapter 4

Comparative proteomic analysis of postharvest fruiting bodies grown on sawdust substrate or hardwood log and isolation of antibacterial serine protease inhibitor.

Introduction

Since ancient times, mushrooms have been considered a valuable dietary component thanks to their high content in fibres, proteins and vitamins, but they are also well-known as organisms with relevant medicinal properties. Nowadays, they can be considered a functional food with therapeutic effect against specific chronic diseases[22, 23]. In particular the *Lentinula edodes* species, commonly named shiitake as in Japanese, is one of the most cultivated edible mushrooms all over the world [21]. Thanks to the high content in nutritional and medicinal compounds its popularity has grown both in the worldwide food market and in the medical research area. As regards its nutritional values, raw fruiting bodies are rich in carbohydrates and proteins, in particular in beta-glucans with a complex three-dimensional structure [26]. Its capacity to degrade lignin and cellulose makes this mushroom able to grow on different substrates such as dead trees or sawdust [85]. The increasing popularity of *L. edodes* has led to the construction of a de novo draft genome sequence [85-87]. This genomic information allowed to better investigate the metabolism of this mushrooms using modern analysis such as high-throughput transcriptomic, proteomic and metabolomic techniques [88-93]. As for the trascriptomic analysis, several studies have been reported focusing on the fruiting body development, the browning film formation on mycelial bags, the synthesis of secondary metabolites and the postharvest loss of quality [86, 94-96]. Maintaing the quality of the mushrooms after harvesting is a big problem from the economic point of view. In *Lentinula edodes*, a significant quality loss is caused by gill browning, fruiting body softening, or foul odour [86]. The transcriptomic analysis of Sakamoto et al. (2016) suggests that multiple novel cell wall enzymes, such as putative β-1,3-gluanases, β-1,6-gluanases and chitinases are upregulated after harvest

in *Lentinula edodes* providing insight for controlling postharvest freshness. At the proteomic level *Lentinula edodes* has been less investigated. Only a comparative study of light induced brown film formation in the vegetative mycelium [96] and the secretomes analysis of *L. edodes* grown on three different carbon sources [97] were reported in literature. The aim of this work is to combining technique gel-free proteomic analysis with the recent genome sequence of *L. edodes* [85] to obtain an overall picture of the post-harvesting expressed proteins in fruiting bodies of *Lentinula edodes* grown on sawdust substrate or on oak log. Our data provide new resources for a deeper characterization of this widely cultivated mushroom paying particular attention to different molecular processes induced by these two cultivation methods that affect the post- harvest fruiting body quality. Previous results have shown that shiitakes cultivated on hardwood log have higher antioxidant and antibacterial capacity than the ones grown on a sawdust substrate (Parola *et al.* (2018). Our data strongly support the antibacterial defensive role for a serine protease inhibitor that was strongly accumulated in post-harvest fruiting body grown on oak.

Materials and methods

Mushroom material

The strain KCS0140 of *L. edodes* was grown on the commercial sawdust substrate used by IoBoscoVivo srl company or cultivated on oak hardwood log substrate[80]. Shiitake is normally harvested when fruiting bodies grow large enough to harvest. Cropping time depends on which type of shiitake prefers [20, 98]. Middle or later stage of development is normally preferred as cropping time. In this study mushrooms cultivated on sawdust substrates were harvested at the later stage of development and oak log cultivated mushrooms were harvested in the middle developmental stage just before the veil break, as normally practiced by the company IoBoscoVivo srl that manage the shiitake production for the Italian market. Three biological replicates for each condition were have been set up. For post-harvest analysis, harvested fruiting bodies grown on sawdust substrate (LS sample) and grown oak log (FL sample) were cut in 6 mm slices, dried in

hot air stream at 37°C for 48 h and powdered in liquid nitrogen. Samples were immediately used for protein extraction.

Shotgun proteomic analysis

Protein extraction and digestion

Proteins were extracted separately from three biological replicates of powdered mushroom as described by Marsoni et al. [99]. After methanol precipitation proteins were suspended in SDS-buffer (SDS 4% w/v, 100 mM Tris-HCl pH 7.6 and 100 mM DTT) and quantified using a 2D Quant kit (GE Healthcare) using bovine serum albumin as a reference standard. Proteins (100 µg) were trypsinized using the FASP (Filter Aided Sample Preparation) method [100]. The obtained peptides were dried under vacuum and desalted using Zip-Tips (lC18; Millipore) prior to mass spectrometric analysis.

LC−MS/MS analysis and data processing.

Peptide samples were analyzed by liquid chromatography tandem mass spectrometry (LC- MS/MS) using an Q Exactive mass spectrometer as described by Garcia-Seco et al. [101]. MS/MS Raw data were searched with MaxQuant program (v. 1.5.3.3, http://www.coxdocs.org/doku.php?id=maxquant:start) against the protein database obtained from Shiitake Genome Database (Shiitake GDB, http://legdb.chenlianfu.com/index.html) and the MaxQuant contaminant list. The search criteria were as follows: trypsin digestion allowing two missed cleavages, fixed modification of cysteine (carbamidomethylation), variable modifications of methionine (oxidation), minimum peptide length of six amino acids, error tolerance was set to 4.5 ppm and 0.5 Da for the precursor and fragment ion respectively. Label Free Quantification (LFQ), "match between runs" (time window of 0.7 min) and target-decoy search strategy (revert mode) options were enabled. A false discovery rate (FDR) of 1% was accepted for peptide spectrum match (PSM) and protein identification. Protein identifications were accepted with at least two identified peptides. For the quantitative analysis protein abundance was calculated from LFQ intensities. They are based on the (raw) intensities and normalized on multiple levels to ensure that profiles of LFQ intensities across samples accurately reflect the relative amounts of the proteins [102].

The "ProteinGroups" files were processed using an in-house tool. Incorrect identifications ("Reverse", "One site", and "Contaminant" hits) and ambiguous identifications were filtered out: only proteins groups detected in at least two of three biological replicates in almost one analytical group (SW and OK sample) were considered to assess significant changes. Missing values were estimated from the dataset based on two criteria for each sample, depending on whether one or more missing values were observed for each entry: when two or three values were available, the missing value was set to a random value within an interval of 1/4 of the entire sample standard deviation centered on the entry average. When only one or no values were available, random values within an interval of 1/4 of the standard deviation of all sample values centered on the global minimum value of all samples in the dataset were imputed. The minimum dataset value and sample standard deviations were determined once before any imputation and applied to all subsequent imputations to avoid drift. Consequently, whole sample standard deviation and dataset minimum value only depended on the starting dataset for each entry calculation.

LFQ intensities of protein groups were Log_2 transformed and processed with the *Perseus* software (http://www.perseus-framework.org). To higlights the trends in our dataset we analysed the Log_2 LFQ intensities by the scatter plot tool using the Pearson correlation statistical parameter. Finally, protein abundance values were calculated from the Log_2 LFQ values of three biological replicates and data was subjected to an Anova based multiple sample test, using for truncation an FDR cutoff of 0.01 based on the Benjamini-Hochberg correction to assess the proteins changing in relative abundance.

Downstream bioinformatics analysis.

MaxQuant Output file hits were represented by a group of proteins (group of IDs) sharing the same set or a subset of peptides of the best-match leading protein. For bioinformatics analysis, only the leading protein was considered. Uniprot, KOG, KEGG and Gene Ontology (GO) functional annotation of leading proteins was obtained by Shiitake Genome Database resource page http://legdb.chenlianfu.com/page/download.html). The EuKaryotic Orthologous Groups (KOG) is a tool for characterize polipeptides identifying ortholog and paralog proteins. The KEGG database pathway (known simply as KEGG)

contains information about the metabolic pathways of the cell. GO is a computational resource that provides a set of defined and hierarchical controlled terms representing the attributes of a gene product at level of cellular component, molecular function and biological process. Carbohydrate-active enzymes (CAZymes) were identified using the automated annotation tool of db CAN meta server (http://cys.bios.niu.edu/dbCAN2/blast.php). CAZymes were classified separately by HMMER, DIAMOND and hotpep search tools (default cutoff threshold). Results were aggregate and for the best accuracy we kept candidates found by at least two methods. Among the CAZymes we identified Lignocellulolytic Genes as described by Chen et al. (2016) [103].

Characterization of a class of antibacterial proteins

Materials

A strain of *Lentinula edodes* that showed promising antibacterial activity was selected after a previous work [80]. The selected strain KCS0140 was grown on the commercial sawdust substrate used by IoBoscoVivo srl company or cultivated on oak hardwood log substrate[80]. Three biological replicates for each condition were have been set up. Shiitake mushroom was dried in hot air stream at 40°C to constant weight and powdered in liquid nitrogen. A dry powder weight of 20 g of the mushroom was extracted with 200ml of water for 24 h at 4 °C. The same procedure was used for mushrooms grown on sawdust (SW sample) and oak log (OK sample) substrate. After extraction, samples were centrifuged at 4000 rpm for 30 min, filtered on 0.2 μm membranes Millipore and freeze-dried.

P. aeruginosa PAO1 and *S.aureus MSSA* were routinely grown in Luria Bertani (LB) broth under aerobic conditions at 37°C. Mueller-Hinton (MH) broth was purchased from Sigma Aldrich [104].

Antibacterial protein characterization

Protein preparation

Freeze-dried extracts were resuspended in water to a final concentration of 100 mg/ml. Protein precipitation was obtained by addition of ammonium sulfate to a concentration of 20%. Aliquots (20 ml) were allowed to precipitate in ice-cooled tank for 1 h and centrifuged at 4000 rpm at 4°C for 2 h. Precipitates were recovered and resuspended in a volume of 1 ml of water. Each precipitated sample was loaded on 5 KDa 1 ml filter membranes (Millipore) and centrifuged for 30 min at 25°C. A volume of 1 ml of milliQ water was added to the filter and centrifuged another time in the same operating conditions. Samples were resuspended in 200 µl of water and transferred to a 30 KDa centrifugal unit. After 30 min of centrifugation at 25°C sample on membrane war recovered following addition of 200 µl water and filtrate was collected. The procedure yielded a low molecular weight fraction enriched in compounds with masses under 5 KDa (LMW), a medium molecular weight fraction, ranging from approx. 5 to approx. 30 KDa (MMW) and a high molecular weight fraction, higher than 30 KDa (HMW). After quantification with Bradford reagent (Sigma Aldrich), using bovine serum albumin (BSA) as standard, all the fractions were conserved at -20 °C.

Protein separation

Protein samples were first separated by a Native-PAGE. Proteins were mixed (4:1) with a non-reducing and non-denaturing 4x sample buffer (0.25M Tris/HCl, pH 6.8, 40% glycerol and 0.5% bromophenol blue) and separated onto a 12%polyacrylamide gel (Laemmli, 1970) [105]. For each sample, duplicate lanes were loaded to allow staining half of the gel and mark the approximate band position on the non-stained half. Resolved protein bands were visualized by Coomassie Blue R-250 staining (0,1% Coomassie G-250, 40% Ethanol, 10% Acetic acid) and resulting gels were digitalized using GS-800 densitometer (Bio-Rad). After visual comparison protein bands appeared in FL respect to the FS samples were excised from the corresponding non-stained half gel part. Proteins were extracted, crushing gel bands in 200 µl of PBS buffer and overnight shaking at 25°C. After the first extraction, the supernatant was collected and a second volume of 200 µl of PBS was added to the crushed gel and shacked overnight. After collection of the second volume, gel was discarded and protein concentration in total supernatant volume was measured. The supernatant was split in two and One-half served for MIC

analysis while the remaining proteins were separated by a denaturing SDS-PAGE. Protein supernatants were mixed (4:1) with the 4x sample buffer added with 8% SDS and 20% β-mercaptoethanol, boiled (95°C, 5 min) and separated according to Laemmli (1970) [105]. After Coomassie staining gels were digitalized and compared as previously described.

Protein digestion and identification by Mass Spectrometry analysis

Differentially SDS-PAGE gel lines from FL and FS were cut and digested enzymatically with trypsin as described by Marsoni et al. (2008) with some modifications. Briefly, the gel pieces were washed in H_2O HPLC-grade and subsequently in 50% acetonitrile (ACN) for 10 min. The gel fragments were incubated for 5 min in 100% ACN for 5 min; the liquid was discarded, and gel pieces were reduced in 25 mM Dithiothreitol (DTT) for 20 min at 56°C and subsequently alkylated for 20 min with 55 mM of iodacetamide (IAA) at room temperature in the dark. The gels pieces were washed in 20 volumes of H_2O in order to eliminate any residue of IAA and after in a solution 1:1 of ACN and NH_4HCO_3 100 mM for 15 min. The gel pieces were dried under vacuum on a centrifugal evaporator. For the protein digestion trypsin solution [Sequencing Grade Modified Trypsin V5111, Promega, Madison; 12.5 ng/µl in digestion buffer (25 mM NH4HCO3, 2.5 mM CaCl2)] was added and samples were incubated at 4°C. After 120 min, the supernatants (SN) were replaced with of digestion buffer and the proteins were digested O/N at 37°C. To extract the tryptic fragments, the gel pieces were sonicated for 5 min in a cool water bath and SN collected (fraction I). The gel pieces were incubated for 15 min at 37°C in 25 mM NH_4HCO_3 and, after the addition of one volume of ACN, were incubated for 10 min at room temperature, vortexing occasionally. The SN was collected and pooled with the fraction I and the samples were washed for 10 min in 5% formic acid and, after addition of one volume of ACN, incubated for further 10 min (vortexing occasionally). The SN were pooled again and DTT was added to give a final concentration of 1 mM. Finally, the samples were dried under vacuum on a centrifugal evaporator and the resulting tryptic fragments were dissolved in 0.1% formic acid analyzed by Liquid chromatography – Mass Spectrometry (LC-ESI MS/MS). The extracted tryptic fragments were resuspended and analyzed by LC-ESI-MS/MS. For all experiments, a Finningan LXQ Mass

spectrometer, equipped with a Finningan Surveyor Pump-plus HPLC system (Thermo Electron Corporation, California, USA), was used. Chromatography separations were conducted on Jupiter 4µm Proteo 90 A (15cmX 0.3 mm, Phenomenex), with a flow of 20 µl/min with a gradient from 5% A (0,1% formic acid in water) to 50% B (0,1% formic acid in acetonitrile). Acquisitions were performed in the data-dependent MS/MS scanning mode (full MS scan range of 400–2,000 *m/z* followed by full MS/MS scan for the most intense ion from the MS scan), thus enabling a dynamic exclusion window of 3 min. Protein identifications were conducted by correlation of uninterpreted tandem mass spectra to the entries of the most recent and complete *Lentinula edodes* protein database[106] using TurboSEQUEST Bioworks™ 3.2 software (Thermo Electron Corporation, California, USA). The software was set to allow two missed cleavages per peptide and considering cysteine carbamidomethylation and methionine oxidation. The precursor ion tolerance was set at 1.4 AMU. In order to identify the proteins, only the peptides with an X-correlation > 1.5 (+1 charge), 2.0 > (+2 charge), > 2.5 (+3 charge) and with a peptide probability < $1e^{-003}$ was considered.

Protein inactivation treatments

MMW fraction for each cultivation methods was subjected to heat treatment at 95°C for 5 min, yielding sample iMMW95. An aliquot of the MMW fraction for each cultivation methods was subjected to trypsin overnight treatment with the enzyme at 37 °C, yielding the tMMW37 fraction. The same amount of proteins of the MMW fraction for each cultivation methods were added with sodium chloride yielding the sMMW fraction.

Antibacterial activity determination

Agar disk diffusion assay

All samples were tested against *P. aeruginosa* PAO1 and *S.aureus* MSSA by means of agar disk-diffusion assay, following the procedure reported in a previous work [80]. Briefly, an aliquot of each sample (40 mg of crude extract powder or 400 µg of ammonium sulfate precipitated proteins) was prepared and reconstituted in 100 µl of sterile water. Samples were loaded on blank paper disks (BD BBL Sensi-discs 13 mm) and dried in air for 10 min. Disks were placed on the surface of an agar plate, freshly inoculated with the two

microorganisms. Three diameters were measured, and average crown radius was reported.

Determination of Minimum inhibitory concentration (MIC) of the crude extracts

Serial two-fold dilutions of samples were tested for growth inhibitory activity against *P. aeruginosa* and *S.aureus*. A fresh inoculum of 10^6 cfu/ml was prepared in Mueller-Hinton (MH) growth medium and added in a 96-wells plate to a final volume of 100 µl/well and 10^5 cfu/well. Samples volumes of 10 µl were added (to a final concentration of 25 mg/ml for the crude extracts or 20 µg/ml for extracted proteins) and a two-fold dilution of samples was produced. After overnight incubation at 37 °C, MIC value was recorded as the lowest concentration allowing no visible bacterial growth.

Statistical analysis

All experiments were repeated at least three times, the comparison between samples was performed following ANOVA, p = .95 was indicated with single *, whereas p= .99 was marked with **. StatSoft Statistica 10 software was used for data processing and elaboration.

Results

Proteomic analysis of post-harvest L. edodes mature fruiting bodies

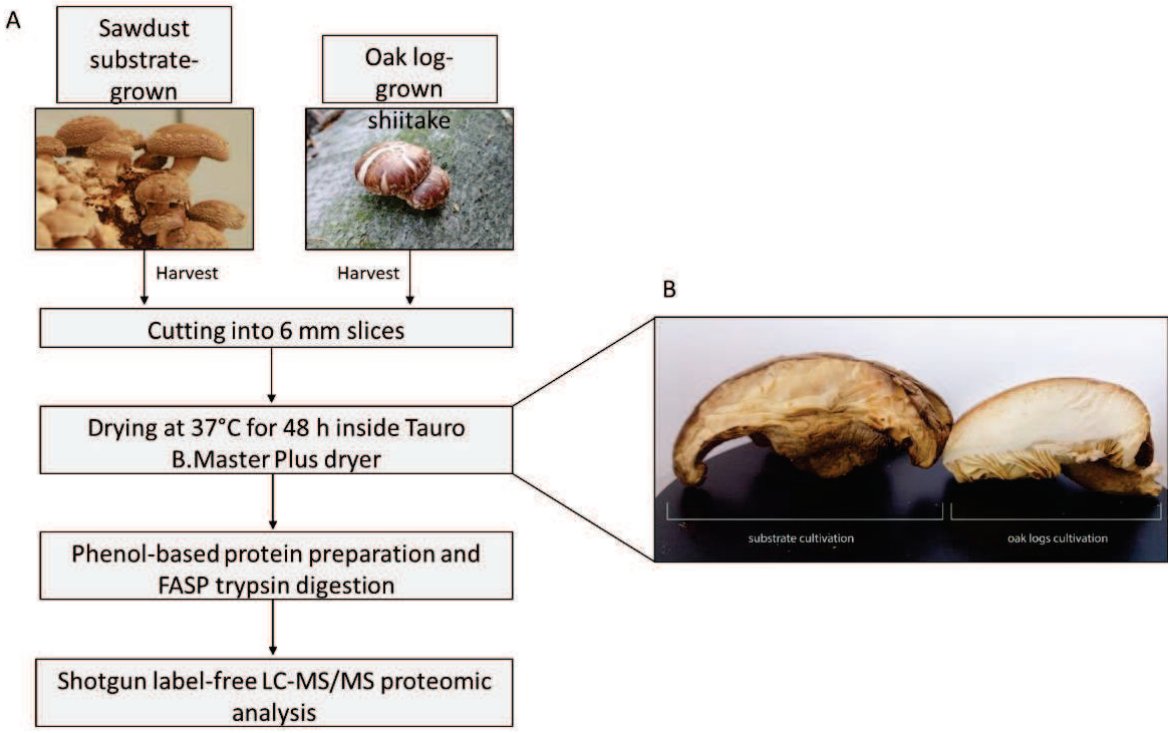


Figure 1: (A) Workflow from mushrooms harvested for the proteomics analysis. (B) Different gill browning effect on fruiting bodies grown on sawdust substrate (left) and on oak logs (right)

Proteins were prepared from post-harvest fruiting bodies grown on commercial sawdust (FS) or on oak log (FL) as described in Fig. 1. After tryptic digestion, the peptides were subjected to a label-free LC-MS/MS analysis. The acquired MS/MS spectra were searched against *L. edodes* protein sequences obtained by the sequencing of Shiitake genome by Chen et al (2016) [103]. 2915 protein groups were consistently identified and quantified in at least two biological replicates out of three and in almost one analytical group (Table S1). Among them, 2668 of the identified proteins (91.5 %) were produced under both conditions while 213 (7.3%) were exclusive to sawdust cultivation and 34 (1.2%) were exclusive to hardwood log cultivated mushroom (table S3 and S4).

We used the Perseus scatter plot tool to evaluate the correlation between samples. The biological replicates sharing the same treatment exhibited an average Pearson correlation of 0.96±0.009 for FS samples and 0.98±0.04 for FL samples (Fig. S1), indicating that the culture conditions, samples processing, and the LC-MS/MS analysis have robust reproducibility.

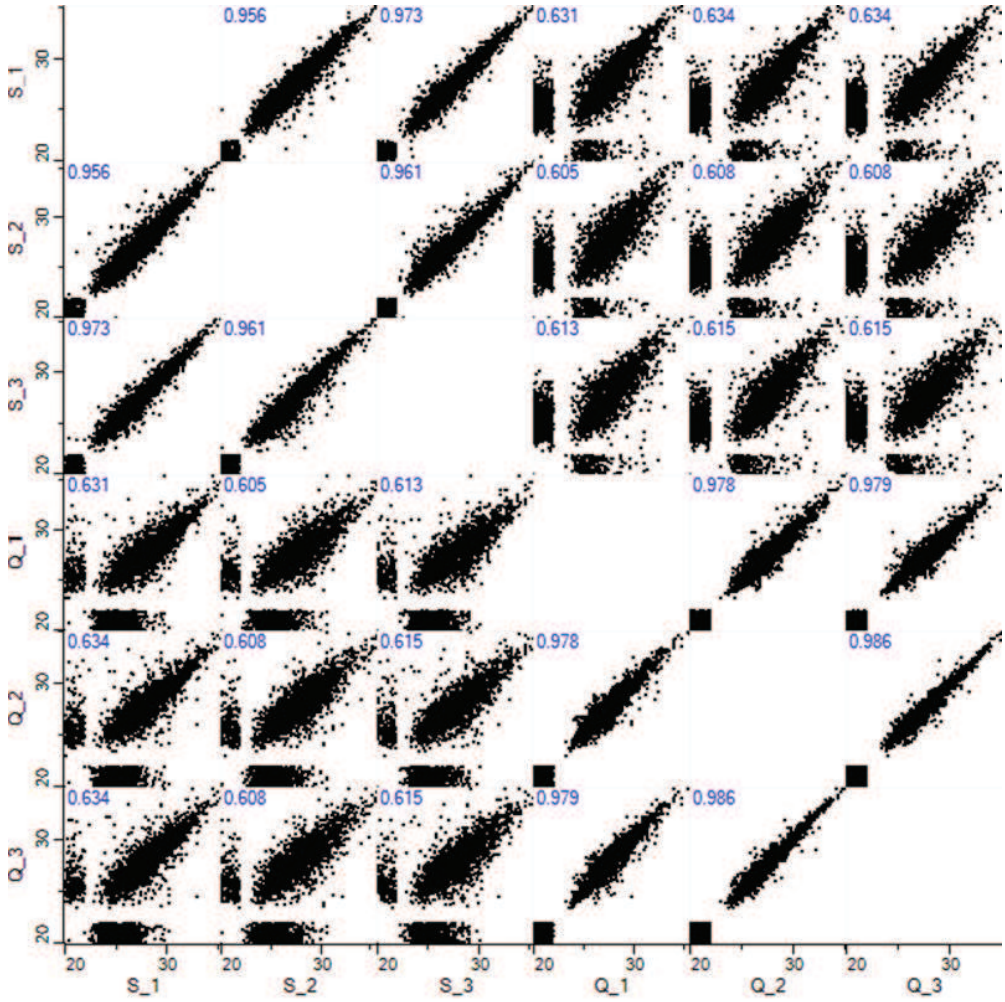


Figure S1. Scatter plot analysis of LFQ intensities of proteins expressed in post-harvest fruiting bodies grown on sawdust substrate (S_1, 2, 3 replicates) and on oak log (Q_1, 2, 3 replicates). For each pairwise comparison Pearson correlation parameter was showed.

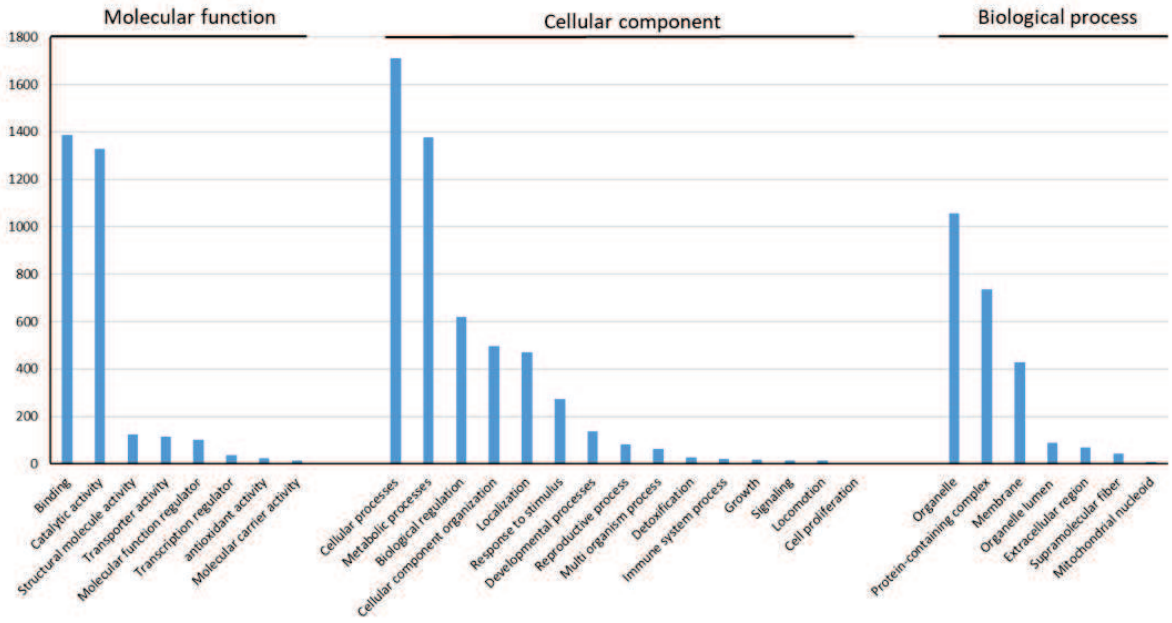


Figure 2: GO classification of proteins expressed in post-harvest fruiting bodies grown on sawdust substrate and on oak log (Table S1).

According to our data, shown in Fig. 2, 2498 (85.7%) protein groups were classified on functional groups based on the GO database (Table S2). The most representative terms in the molecular functions category were binding (1388 proteins, 60,1%) and catalytic activity (1330 proteins, 57,6%). In particular, the catalytic activity includes hydrolases (530), transferases (341) and oxidoreductases (323). The most represented proteins with binding function were ion binding proteins (880), drug binding (310) and protein binding (310). A lower number of entries were found for transporter activity (114) and molecular function regulator (101). In the category "cellular component" (1977 proteins) the most representative terms were organelle (1057), protein-containing complex (737), membrane (428) and extracellular proteins (69). Finally, among the biological processes (2016 proteins), the metabolic processes including primary metabolism (1138), metabolism of nitrogen compounds (985), biosynthetic process (580) and catalytic process (401) were dominants. In addition, biological regulation (619), cellular component organization (497), localization (472), response to stimuli (272), developmental process (137) and reproductive process (82) were well-represented processes in the proteome of post-harvest shiitake fruiting bodies.

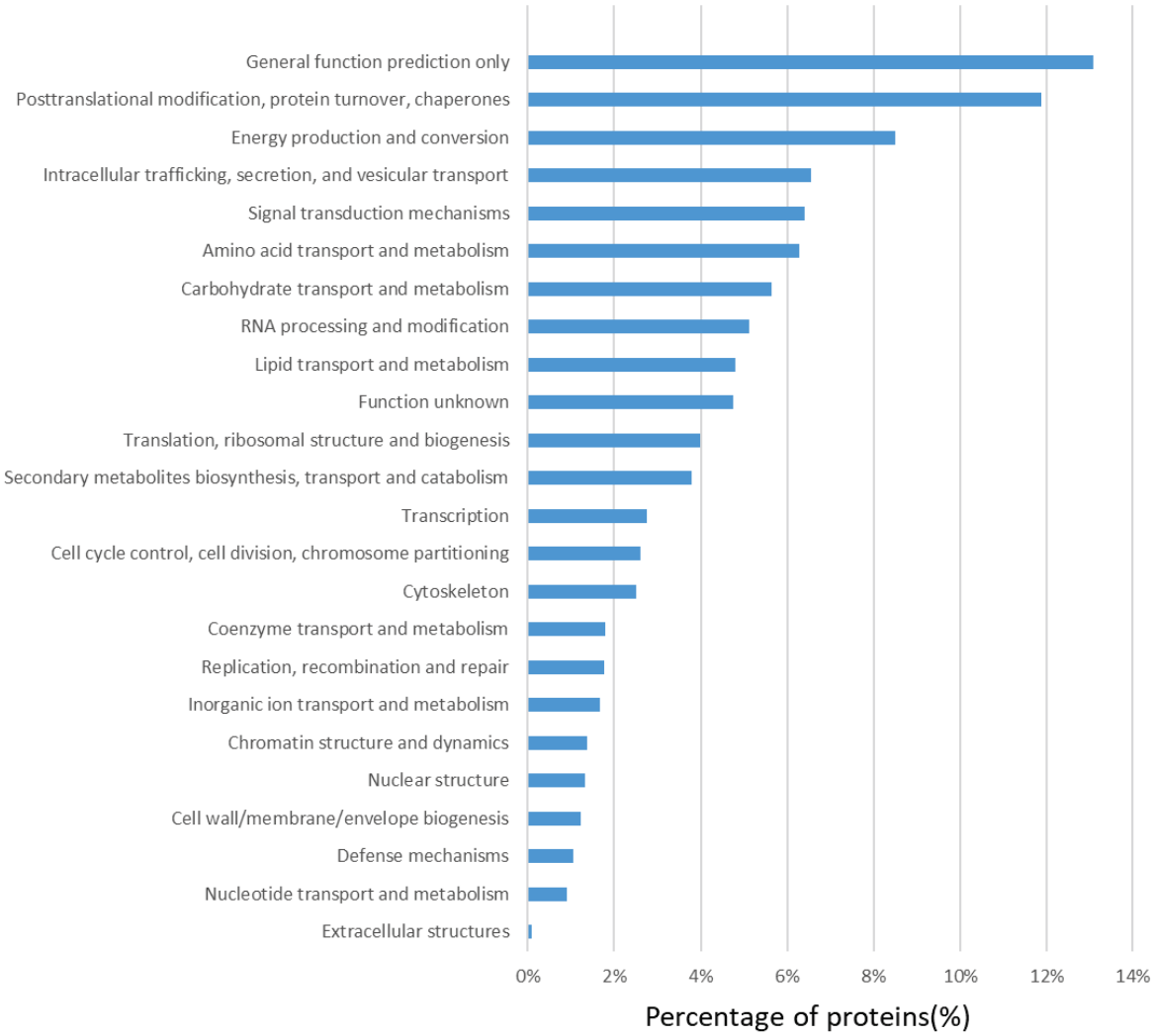


Figure 3. KOG classification of proteins expressed in post-harvest fruiting bodies grown on sawdust substrate and on oak log (Table S1).

To gain a more accurate functional classification we considered also the KOG and KEGG annotation (Table S2). As showed in Fig. 3, general function prediction only (14%) was the dominant KOG category, followed by post-translation modification, protein turnover, chaperones (10%), energy production and conversion (8%), intracellular trafficking, secretion and vesicular transport (6%), carbohydrate and aminoacid transport and metabolism (5% and 7%, respectively).

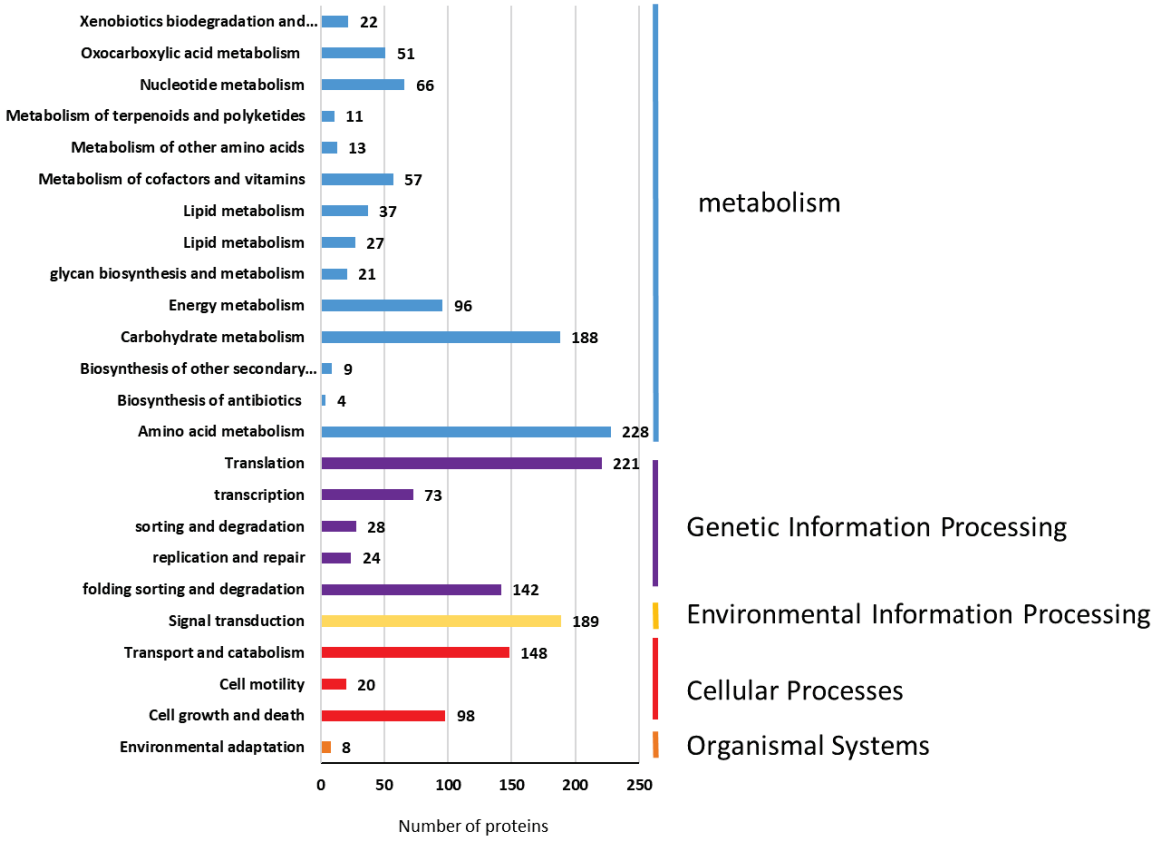


Figure 4. KEGG classification of proteins expressed in post-harvest fruiting bodies grown on sawdust substrate and on oak log (Table S1).

In Fig. 4 the KEGG pathways were divided into five categories: "cellular processes", "environmental information processing", "genetic information processing", "metabolism" and "organismal systems". The most represented pathways were "Amino acid metabolism" (228), "translation" (221), "signal transduction" (189), "carbohydrate metabolism" (188), "Transport and catabolism" (148), "Folding, sorting and degradation" (142), "Cell growth and death" (98), "Energy metabolism" (96) and "Transcription" (73).

All these data confirmed an active metabolism in the 2-day harvested fruiting bodies that was centered on carbohydrate and protein metabolism.

Differentially expressed proteins (DEPs)

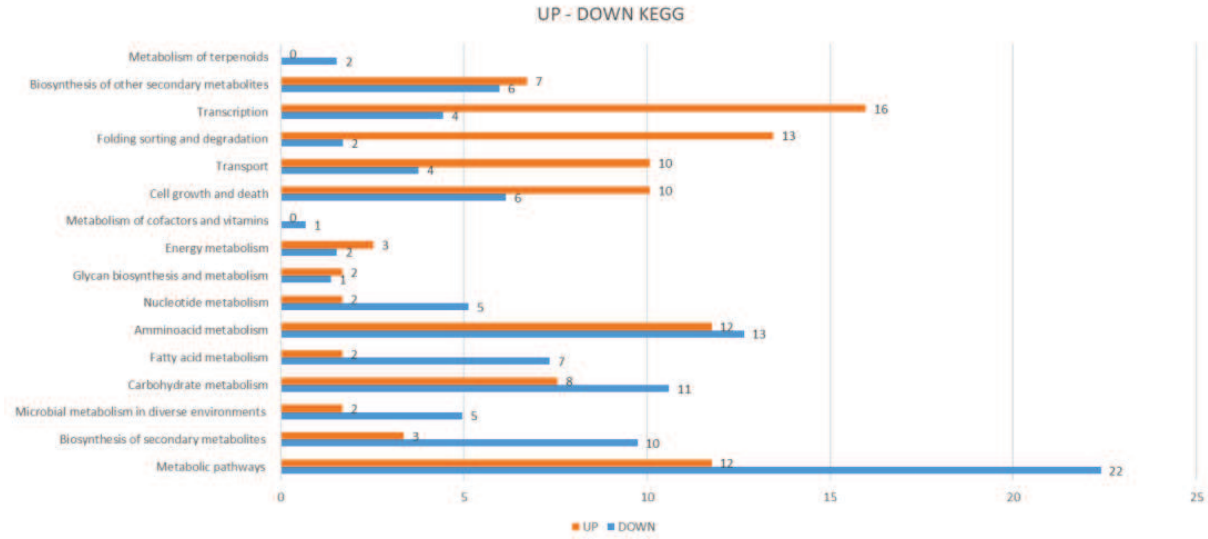


Figure 5. KEGG classification of DEPs proteins expressed in post-harvest fruiting bodies grown on oak log vs the sawdust substrate grown

The value of the average Pearson correlation between FS and FL samples of 0.62±0.012 (see Figure S1) indicates that the experimental groups were clearly separated, suggesting that the medium of cultivation deeply affect the post-harvest fruiting body proteome profile. In fact, quantitative analysis has revealed 1112 differentially expressed proteins (DEPs) that change in abundance comparing FL versus FS samples (Table S5, ANOVA multiple sample test, FDR < 0.01): 303 proteins were more abundant (34 present exclusively in FL) and 809 were less abundant in log-cultivated fruiting bodies as compared with substrate-cultivation (200 present exclusively in FS). For a functional classification, DEPs were mapped to KEGG pathways (Figure 5). Metabolic pathways including the metabolism of carbohydrate, fatty acid, and amino acid were the most represented by proteins up-regulated in substrate-cultivated mushrooms. The proteins up-regulated in oak log fruiting bodies belong mostly to “transcription”, “folding sorting and degradation”, “transport” and “cell growth and death” categories.

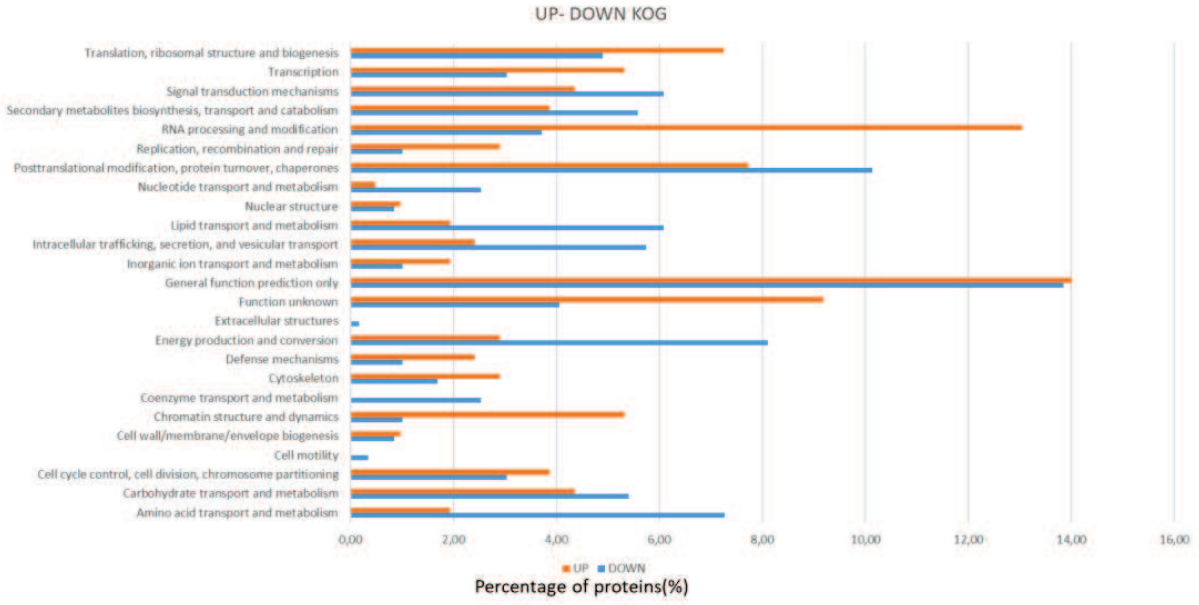


Figure 6. KOG classification of DEPs proteins expressed in post-harvest fruiting bodies grown on oak log vs the sawdust substrate grown

Similar results were obtained using the annotation based on the KOG database (Fig 6). Up-regulated proteins in substrate grown fruiting bodies belong to energy production and aminoacid, lipid, carbohydrate and nucleotide transport, and metabolism. Also, the proteins involved in the production of secondary metabolites showed higher levels in substrate-grown mushroom. The proteins increased in oak log-grown mushrooms belong mainly to transcriptional and translational processes, cell cycle, RNA processing, Chromatin structure, and dynamics and cytoskeleton. The category "defense mechanisms" was also more represented in FL samples.

CAZyme

Using the db CAN meta server tools we identified, in the proteome dataset of post-harvested *L. edodes* fruiting bodies, a total of 102 proteins (3.5%) candidate carbohydrate-active enzyme genes (CAZymes), including 60 glycoside hydrolases, 26 glycosyl transferases, 1 carbohydrate esterase, 1 polysaccharide lyases and 14 Auxiliary redox enzymes (Table S6). Among these 22 (2.7%) proteins belong to the DEPS down-regulated in log grown mushroom and 13 (4,3%) belong to were DEPs up-regulated in

log. (22/810, 4.9%). The proteome of *L.edodes* revealed that 22 proteins (Table S7) were involved in lignocellulose decomposition. We identified 3 putative endoglucanase, 1 cellobiohydrolase, and 1 pectinase, 4 putative β-glucosidase, 4 peroxidases of which 3 laccases, and 10 auxiliary enzymes. Six of these lignin decomposition proteins are DEPS: 4 are more abundant in sawdust substrate mushrooms and 2 are up-regulated in log-grown fruiting bodies.

Antibacterial activity of a serine protease inhibitor accumulated in mushroom cultivated on oak log

We have previously pointed out that the aqueous extract from dried fruiting bodies of shiitake showed an antibacterial activity which was completely lost by mild chemical treatments (heating above 50°C the crude extracts or lowering the pH to a value of 4-3.5) [80]. These results led us to the hypothesis that the antibacterial activity was due the fraction of proteins solubilized during water extraction. For this reason, proteins from aqueous extracts were separated by ammonium sulphate precipitation and further separated by size exclusion cut-off membranes into three different fractions namely HMW (high molecular weight fraction; >30 KDa), MMW (medium molecular weight fraction, 5 - 30 kDa) and LMW (low molecular weight fraction, <5KDa).

The antibacterial activity of the different fractions against *P. aeruginosa* PAO1 was examined by Kirby-Bauer assay and only the MMW fraction showed an antibacterial activity; the higher activity was found in an extract from oak grown mushroom and it was similar to that measured in the crude extract (Table 1). This antibacterial activity was lost after heating at 95°C or treatment with Trypsin confirming that it was due to a protein**.**

Shiitake sample	Crude extract inhibition zone (mm)	Crude extract MIC (µg/ml)	Inhibition (mm) cut-off fraction (<5 Kda)	Inhibition (mm) cut-off fraction (5 to 30 Kda)	Inhibition (mm) cut-off fraction (>30 Kda)	Activity reduction after trypsin treatment on MMW fraction	Band 2 MIC (µg/ml)
FS	2.5 ± 0.52	12.5	0	0.7 ± 0.29	0	99.9 ± 0.13 %	20
FL	7.0 ± 0.43	2.5	0	7.8 ± 0.31	0	88.2 ± 0.35 %	2,5

Table.1: antimicrobial activity of L.edodes crude extracts and cut-off fractions on P. aeruginosa.

The MMW fraction was separated using a native electrophoresis and gel bands were excised, and the antimicrobial activities of the proteins eluted from bands were measured.

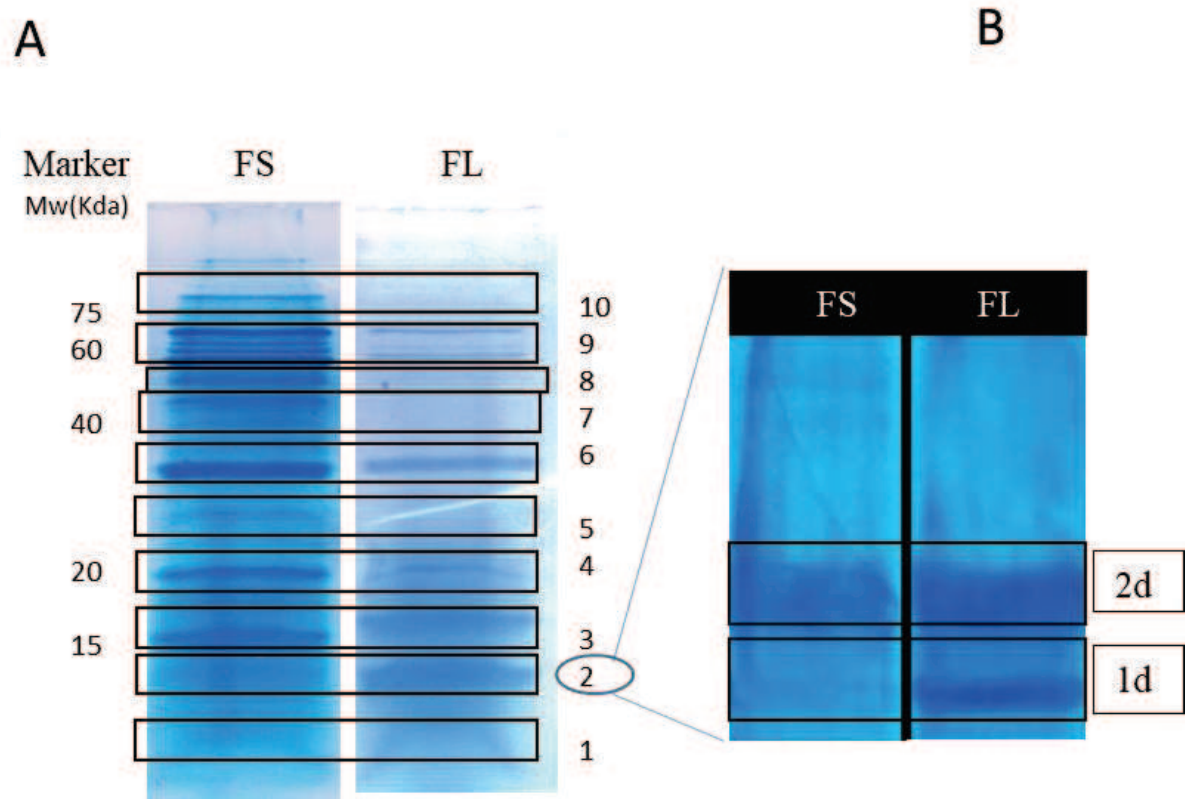


Figure 7: (A) Native protein separation on polyacrylamide; gel bands studied for antibacterial activity are highlighted. (B) Band pattern emerged from SDS-PAGE analysis of native-PAGE extract of band 2 region. A - substrate; B - oak. Most representative and further studied band are highlighted

Only the eluates of gel band number 2 showed a microbial growth inhibition effect that was eight times higher for FL versus FS extracts (Table 1). These two active eluates were further separated by SDS-PAGE. As showed in figure 7B, the oak sample revealed one more band in comparison to the substrate sample. LC-MS/MS analysis of this unique band indicated that the 15KDa band corresponds to a serine protease inhibitor (P81639; LE01Gene05309.t1) Our results are also supported by the proteomic analysis: this protein was the most upregulated protein in oak fruiting bodies with respect to the substrate. When the sequence of this protein was aligned to CDD (conserved domain database) entries, we found a Ricin-type beta-trefoil domain (Superfamily cl23784, e-value = 1.61e-46) typical in lectins. Lectins have been reported to have a direct antimicrobial activity [107]. The serine protease inhibitor was also compared to antimicrobial peptides

database DRAMP (data repository of antimicrobial peptides) by means of BLAST algorithm using the BLOSUM62 score matrix. This allowed the identification of a similar protein entry (identity 33%, positives 50%), DRAMP04479, belonging to the Defensin protein family that was previously reported in the literature as composed of evolutionary ancient antimicrobial peptide [108]. Overall these data suggest that the observed antibacterial activity of the aqueous extract from shiitake fruiting bodies cultivated on oak log may be associated, at least partially, to the increase of this serine protease inhibitor.

Discussion

This work is the first comprehensive and quantitative profiling of the proteome of post-harvest fruiting bodies of *L. edodes* mushroom. Proteins from fruiting bodies harvested from sawdust substrate and oak log were subject to a LCMS/MS analysis. A list of 2915 proteins, representing all the most important functional categories was identified to provide a valuable resource for further study of this mushroom. Interestingly, a large portion of the protein dataset belongs to the organelle cellular component category (Fig. 2). We focused our attention on proteins that differentially expressed in the two cultivation methods. By functional analysis, we found that most of the proteins that are up-regulated in substrate grown samples were assigned to metabolic categories such as energy, lipid, amino acid and carbohydrate-related metabolism (Fig. 5-6). Secondary metabolism was also increased. During the mushroom ripening process, the metabolism is concentrated in the fruiting body, so our results suggest a faster metabolism in mushrooms grown on substrate that could be due to a forced stimulation of the fructification. In fact, to reach high yields in mushroom cultivation, sawdust substrates are made using a limited amount of nutrients in order to induce a stress condition that stimulates fructification [109, 110]. The higher stress condition of mushrooms grown on the artificial substrate was indicated also by the increased production of proteins for the biosynthesis of antioxidant molecules such as terpenoids and vitamins.

On the contrary, hardwood log samples showed a net up-regulation of proteins related to cell growth, cellular transcription, RNA processing and modification, protein folding and

sorting, chromatin structure and dynamics (Fig. 5-6). These processes are important in the development of the fruiting bodies. In hardwood logs the myceliar growth and the fruiting process are both active in the harvest season: the log is not yet fully colonized and the fruiting bodies are not fully developed.

Post-harvest modification of fruiting bodies

In cultivated mushroom, the post-harvest quality loss is an important problem from the commercial viewpoint. Post-harvest mushroom may retrieve nutrients from the fruiting body itself. This triggers the senescence process with browning and softening of fruiting bodies. Gill browsing in *L.edodes* is mainly due to melanins and oxidation of tyrosin seems to be an important step [111]. It has been suggested that Tyrosinase activity (LeTyr) is involved in melanin synthesis in different tissues [112]. It has been reported that LeTyr increased after fruiting body post-harvest [113]. Moreover, the Laccase Lcc4 seems to be involved in melanin synthesis after harvesting [114, 115]. We found that two tyrosinase (LE01Gene00669.t2, LE01Gene04565.t1) and Lcc4 strongly increased in fruiting bodies from the substrate with respect to oak log. These data are in accord with the more evident gill browning effect on fruiting bodies cultivated on artificial substrates (Fig.1). Fruiting body softening occurs due to cell wall degradation. Several families of glucanases, GH55, GH5, GH16, GH30 and chitin related genes (GH18, GH20) are involved in the degradation of cell wall components and resulted up-regulated at transcriptomic level post-harvesting [86]. In substrate grown mushrooms we found the increase of three glucanases and two chitin related proteins indicating senescence in progress. In fruiting bodies grown on log, we found the increase of three chitinases and the exo-β-1,3 glucanase EXG1. The latter protein is involved in stipe elongation [116] and chitinases are important proteins for the morphogenesis of fungi. Moreover, two autophagy proteins and two conidiation specific proteins were more abundant in these fruiting bodies. All these data indicate that a ripening process and spore formation were active 2 days after harvest of the fruiting bodies grown on oak log. Finally, during the postharvest storage lentinan appears to be degraded by β-1,3 glucanases [117]. Controlling the amount of these enzymes is therefore very important to keep the lentinan quantity in the fruiting body after harvest. Some glucanases belonging to GH5 family have been reported to be

involved in lentinan degradation after harvesting [86, 118]. Some of them (such as LE01Gene01071.t1, LE01Gene12502.t2 LE01Gene04612.t1) were more abundant or exclusively present in sawdust grown fruiting bodies. This suggests that fruiting bodies derived from substrate cultivation were less suitable for a functional food product.

Proteins related to the unique aroma of *Lentinula edodes*

Lenthionine is the organosulfur compound responsible for the unique aroma of *L.edodes* [119]. Two of the enzymes involved in its biosynthesis are the gamma-glutamyl transpeptidase (GGT) and the C-S lyase (CSL). In the genome of *L. edodes* 7 *ggt* and 5 C*sl* genes have been found. In the proteome of shiitake fruiting bodies, we identified three proteins with high similarity to GGT 1, 2 and 3 and three proteins annotated as C-SL. GGT1 was more abundant in fruiting bodies grown on sawdust substrate with respect to those grown on oak log suggesting more aroma synthesis in fruiting bodies grown on artificial substrate.

Antimicrobial proteins

As observed in KOG database analysis, defense mechanisms are more expressed in oak log mushrooms. Among them, we found that a protein corresponding to the solanapyrone synthase (LE01Gene06800.t1) increased in log-cultivated fruiting bodies. Diverse fungal species produce the antibiotic secondary metabolite Solanopyrone A and it has been suggested that it plays an important antifungal activity against saprobic competitors [120]. We demonstrated that the higher antibacterial activity of aqueous extract from oak log fruiting bodies (Parola et al.) was associated with the presence of a serine protease inhibitor. The proteomic analysis confirmed that this protein was strongly accumulated in FL samples as compared with FS. This protein was purified for the first time by Odany et al (1999). Our data strongly support the antibacterial defensive role for this serine protease inhibitor. Serine protease inhibitors from plants or crustaceans are known for their bactericidal and bacteriostatic activities against different nosocomial pathogen microorganisms [121-123].

In conclusion, our results showed a higher metabolic activity in substrate grown fruiting bodies which allows a faster development and a higher content of secondary metabolites with respect to oak log mushrooms. On the other hand, 2 days post-harvest, the senescence process has already begun in substrate fruiting bodies while in oak log mushrooms morphological development is still in progress. Finally, fruiting bodies grown on log can be producers of molecules with important antimicrobial functions.

Conclusion

This PhD project focused on two important medicinal mushrooms, namely *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster). Many recent studies have demonstrated their relevance for human health. The company IoBoscoVivo srl has supported this research project aiming to produce new functional food products for the high-end Italian market. The central point of the thesis is a deep comparison of fruiting bodies obtained from two different cultivation methods. Mushroom cultivation is actually highly influenced by the cost of the raw materials used as substrates and by productive processes. On the other hand, to achieve a functional food product is necessary a high quality of the harvested fruiting bodies. A comparison of fruiting bodies taking into account their macroscopical characteristics, healthy activities and nutrient content is mandatory to achieve this final aim. Both the cultivation on sawdust substrates and on logs produce fruiting bodies suitable for the Italian market. We selected some clones of *L.edodes* and *P. ostreatus* ables to give a good yield on different hardwood logs. The average size of a log-cultivated shiitake mushroom was lower than the one of a sawdust substrate mushrooms, but its flesh was stronger and harder. The antioxidant, antitumoral and antimicrobial activities were also strongly influenced by the cultivation method. In particular, log cultivated mushrooms showed the best performance and exhibited interesting radical scavenging (antioxidant) properties; additionally, shiitake was also able to produce metabolites such as a serine protease inhibitor with antimicrobial activity. Moreover, a high cytotoxic activity on tumoral cells without side effects can lead to the use of these mushrooms as main ingredients for functional food formulations. Senescence of fruiting bodies after harvesting is crucial for fresh mushrooms market. For this reason, a proteomics analysis on shiitake has been performed on post-harvest fruiting bodies. Understanding the mechanisms and the molecules involved in senescence process is important to find the best cultivation method that can guarantee the longest shelf life of fruiting bodies while keeping their characteristics unaltered as much as possible. Log-grown mushrooms exhibited a better resistance to the senescence process; proteomics analysis allowed us to identify the protein involved in the antimicrobial activity. Overall, these findings add understanding to the mushroom strains used by the company

IoBoscoVivo srl as flagships in their organic product line. Our results support the importance of these products as daily components of diets and as basic ingredients for the human health and nutrition.

Supplementary materials can be found at the following link:

https://www.dropbox.com/s/rhsx0q0ux3d5yk5/Full%20table_lentinula%20edodes.xlsx?dl=0

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