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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 amount of medicinal compounds, and are also an optimal source of fibres, proteins, vitamins (like groups B and D), and other micronutrients including potassium, magnesium, etc. Consequently, mushrooms are commonly considered to be functional foods. Many works report the high biological potentials of medicinal mushrooms involving their antibacterial, hypoglycaemic, anticholesterolemic, radical scavenging, and anti-inflammatory effects.

Context and purpose of this study: First off, this work aimed to find strains of *Lentinula edodes* and *Pleurotus ostreatus* from a bank of edible mushrooms bought from international strain banks (Table I) that could possess health benefit related properties, such as a radical scavenging activity (antioxidant effect), antibacterial effects against common pathogenic bacteria, and being able to produce interesting nutrients and secondary metabolites. As the fungal bank comprises of 20 strains of *L. edodes* and 20 strains of *P. ostreatus,* a first screening was made by the selection of 13 strains for each mushroom able to grow in multiple wood types or that were particularly productive and had proved good growth reproducibility over the last 5 years. This work also studied the correlation between culture conditions and mushroom quality in terms of the previously reported properties. Comparison among the selected strains was operated by the assessment of

antioxidant and antimicrobial activities after different sample treatments. Furthermore, an initial optimization of the analytic techniques was produced for the direct estimation of important secondary metabolites and nutrients by means of HPLC-MS/MS technique. Further research will encompass an evaluation of transformation processes (drying, freezing, rehydration, cooking, etc.) impact on radical scavenging, antibacterial activity, and possible degradation/loss of nutraceutically important substances such as vitamin D2, ergothioneine, eritadenine, lovastatin, lentinan, and lenthionine.

Results: 13 strains of each mushroom species have been cultivated on different wood logs. Seven strains of shiitake and six strains of oyster mushroom were able to produce sporocarps. Antioxidant levels in water extracts from dried mushrooms produced significatively different results on the basis of strains and of wood. Both mushrooms demonstrated higher radical scavenging activity in log cultivation than substrates cultivation, which was subsequently used as reference. Furthermore, all strains of *P. 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.

Conclusion: 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.

Keywords: Shiitake, Oyster, *Pleurotus ostreatus, Lentinula edodes, Pseudomonas aeruginosa, Staphylococcus aureus*, Log grown mushroom, antioxidant, radical scavenging, total phenolic content, DPPH, ABTS, Folin-Ciocalteu, antibacterial, fruitbodies, 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 compounds, in addition to their

taste and organoleptic properties. Nutritional value was found in many works due to their high content of proteins, fibres, vitamins, and mineral salts, in addition to their low-fat level [2-4], and high content of micronutrients [5-6].

Lentinula edodes (shiitake) and *Pleurotus ostreatus* (oyster) are among the most cultivated mushrooms in the world [7]. These can be grown using a wide range of methods, conditions, and substrates. Moreover, the choice of the cultivation technique was previously found both to affect the fruiting yield and the production of secondary metabolites.

Oyster and shiitake are characterized by their short growing time compared to other edible species, and feasible production during all the year. Accordingly, their cultivation and experimentation, and the knowledge of their nutritional and medical value has increased over the years [8], [9], [10], [11].

Shiitake and oyster fruit bodies have actually been demonstrated to hold effective antioxidant activity due to a high phenolic compounds content [12], [13], [14], [15]. Additionally, shiitake extracts demonstrated antibacterial activity against *Staphylococus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* in previous research [16], [17], [18], [19], [20], [21].

Many studies regarding the medicinal properties of these mushrooms have been conducted, which have also allowed the recovery of interesting secondary metabolites. In the shiitake mushroom, one of the most relevant is lentinan, a β-glucan compound demonstarting several activities that comprise the antitumor effect due to induced stimulation of the host immune system [6]. Other interesting metabolites are ergothioneine, showing antioxidant activity and eritadenine, which holds an hypocholesterolemic effect [22], [23], [24].

Moreover, *Pleurotus ostreatus* also contains a large number of medicinal compounds. In particular, lovastatin is a secondary metabolite which is capable of lowering blood cholesterol levels and has been therefore suggested to prevent cardiovascular diseases [25], [26].

In this work, 13 strains of *L. edodes* and *P. ostreatus* were selected on the basis of their good and reproducible growth yield over the last five years. All the mushrooms were evaluated for antimicrobial activity against two bacterial strains, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Radical scavenging activity was studied by means of ABTS and DPPH assay, and total phenolic content was also measured by means of a Folin-Ciocalteu assay.

Currently, there are not enough studies regarding temperature 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)

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

***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	KCS0147	Europe
Mycelia	M2191	KCS0148	Europe
Mycelia	M2153	KCS0149	Europe
Aloha colture bank	JB	KCS0150	USA
Società agricola IoBoscoVivo	KCS0160	KCS0160	Italy
Società agricola IoBoscoVivo	KCS0050	KCS0050	Italy
Società agricola IoBoscoVivo	KCS0152	KCS0152	Italy

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 KCS0160 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

The grain spawn method was used for mycelia cultivations [11], [29], [30]. 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 holes in each wood species by means of an inoculator gun. Afterwards, holes were 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 in order to obtain the production of fruitbodies [29]. Within 12 months, every harvest of fruitbodies was performed.

Sample thermal treatments

Each mushroom strain was dried at 37°C for 2 days, subsequently 5g aliquots of each substrate grown strain (KCS0140 and KCS0160) were subjected to thermal treatments either at 4°C or 37°C or 50°C or 80°C for 2h in dark conditions. After treatment, materials were powdered and immediately subjected to extraction.

Sample water extraction

The extraction method reported by N.M. Tonucci *et al.* (2015) was modified. Briefly, 5 g of all fruitbodies that underwent thermal treatment were powdered and extracted for 72 h at 4°C in 100 ml of water [31]. 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 (coded **W1**-*strain-log*) were conserved at -20°C.

This procedure was repeated more than 5 times during the assessments. Extraction yields were reported as the average yield values.

Fractionation of KCS0140 strain crude water extract

Solvent counter-extraction: The crude water extract W1-kcs0140-beech was subjected 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 a 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).

Fruitbodies production from log cultivation

Shiitake strains KCS0128, KCS0138, KCS0139, KCS0141, KCS0142, and KCS0144, showed the production of fruitbodies 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 fruitbodies after six-month from the inoculum. Concerning *Pleurotus ostreatus*, KCS0160 strain was able to grow only on substrate. All the oyster strains produced fruitbodies on poplar logs. Strains KCS0050, KCS0146, and KCS0152 showed fruitbodies on robinia logs. Strains KCS0147, KCS0148, KCS0150, and KCS0152 showed fruitbodies production on willow logs. Only the strain KCS0150 showed fruitbodies 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* KCS0160 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.

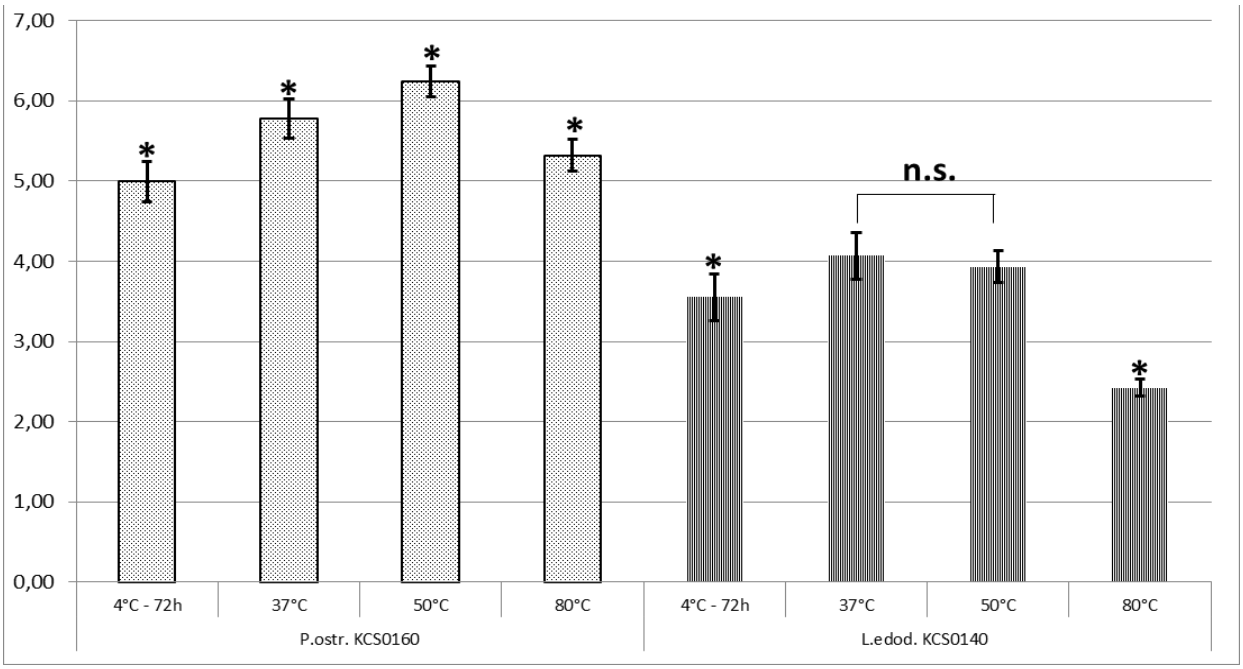
As it concerns shiitake, this strain showed the highest activity both at 37°C and 50°C, a significatively ($p < 0.05$) lower activity was observed either at 4°C and 80°C (Figure 1).

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* KCS0160 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

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 (Table 1, Figure 2). KCS0152 was more effective on poplar, demonstrating a lower IC_{50} when compared to the cultivation on

robinia (Table 1, Figure 2). KCS0148 instead showed the same IC_{50} as the previous strain whether grown on poplar or willow logs. A higher value in respect to the previous mentioned strains was observed for KCS0050 and also this strain did not yield significant differences either on poplar or robinia. KCS0150 yielded a slightly lower IC_{50} on robinia comparing to the cultivation on poplar and willow.

In regards to *L. edodes* ABTS radical scavenging activity, substrate grown KCS0140 had the lowest efficacy. The same strain grown on oak wood revealed the second highest activity, a lower efficacy was detected when it was cultivated on beech. Strain KCS0144 allowed the observation of the highest activity among shiitake strains. KCS0139 demonstrated an activity less than 5 % lower than oak grown KCS0140. All the other strains (KCS0128, KCS0138, KCS0141, and KCS0142) resulted in a similar and lower activity as compared with the previously mentioned strains (Table 1, Figure 3).

As DPPH assay was concerned, the most efficient were KCS0140 and KCS0144 both grown on oak logs that demonstrated the lowest IC_{50}. Substrate grown KCS0140 showed the lowest activity, resulting in the highest IC_{50}. When KCS0140 grown on beech and oak were considered, significatively different results were observed, with the former showing an IC_{50} about 60% higher than the latter (Table 1, Figure 3).

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.

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 KCS0160	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

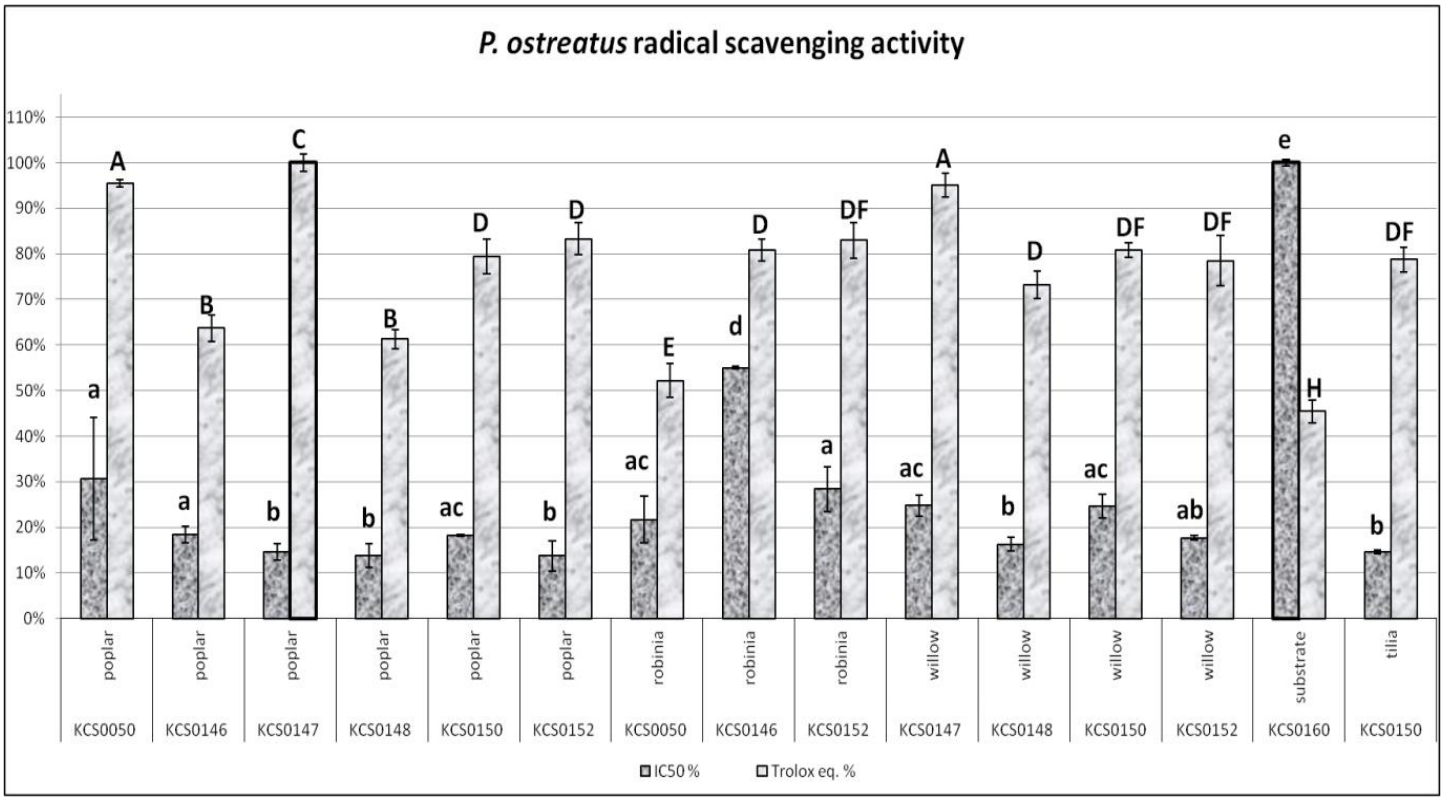


Figure 2. Comparison between *P. ostreatus* strains antioxidant levels either substrate or log grown. Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant differences ($p < 0.05$) between strains and log species are indicated with different letters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

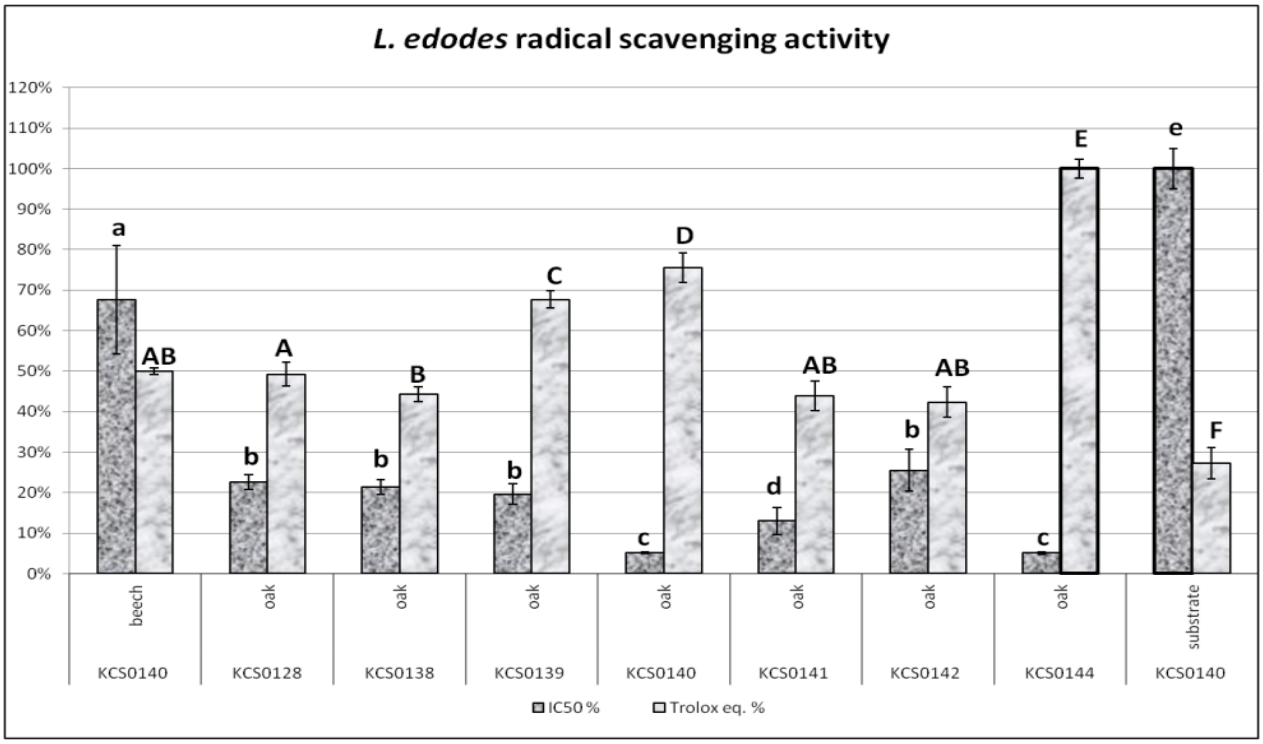


Figure 3. Comparison between *L. edodes* strains antioxidant levels either substrate or log grown. Activities are reported as normalized percentages to the highest one in each series (highlighted). Significant differences ($p < 0.05$) between strains and log species are indicated with different letters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

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).

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
KCS0160	sawdust	1.025 ± 0.010
KCS0150	tilia	1.561 ± 0.025

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

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.

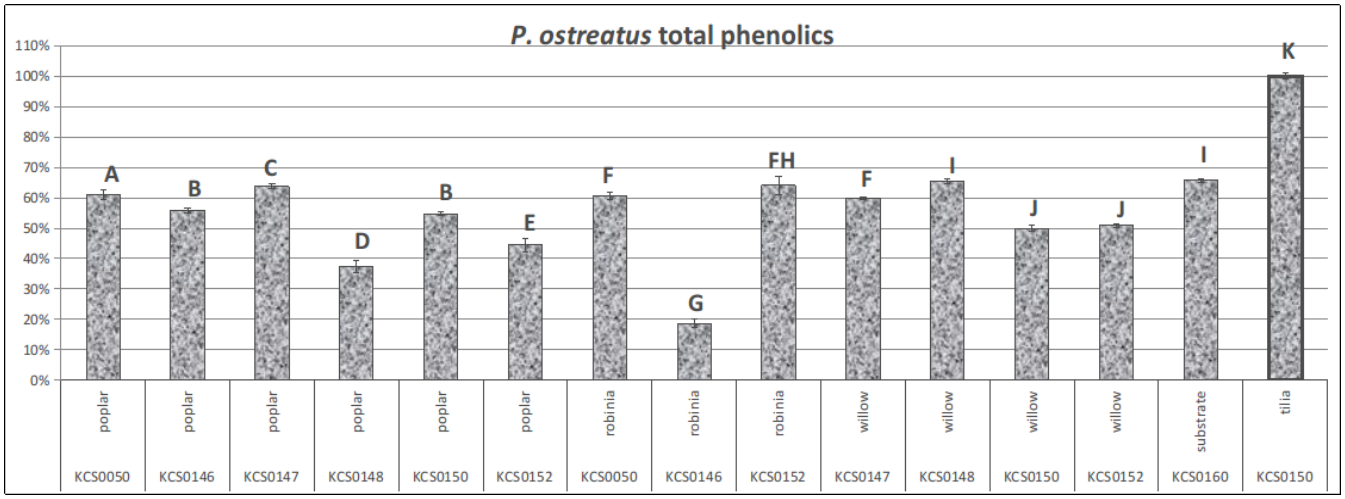
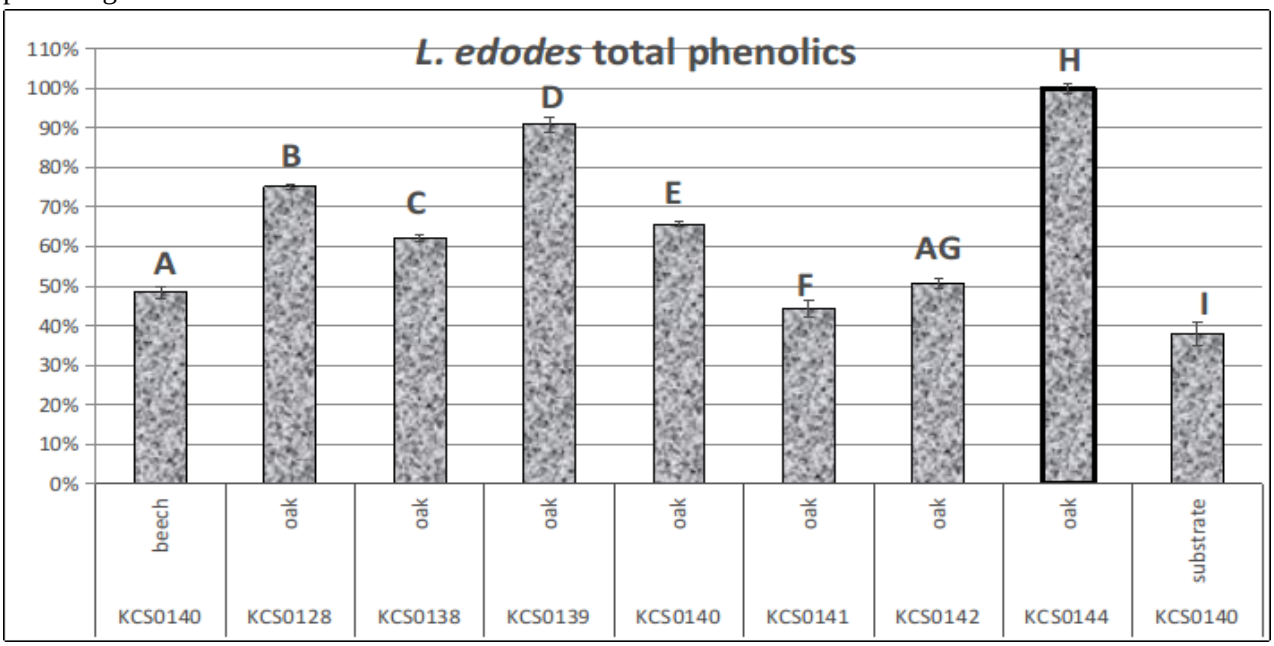


Figure 5. Phenolic content in *L. edodes* strains grown on different log types and substrate. Values reported are normalized to the highest content observed (KCS0144 on oak) and indicated as the respective percentages.



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.

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.

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

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 an 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.

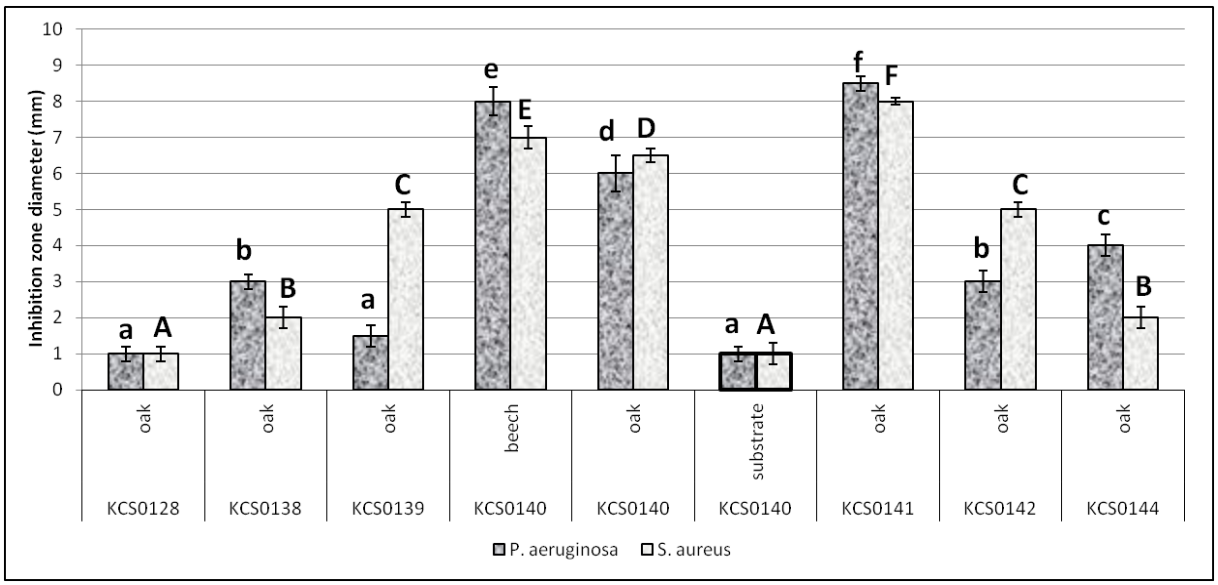


Figure 6. Inhibition zones diameter of water extracts from the shiitake strains grown on logs or substrate. The same strain KCS0140 cultivated on substrate is highlighted. Significance ($p < 0.05$) reported with different letters.

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.

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*.

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

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).

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.

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

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).

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

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

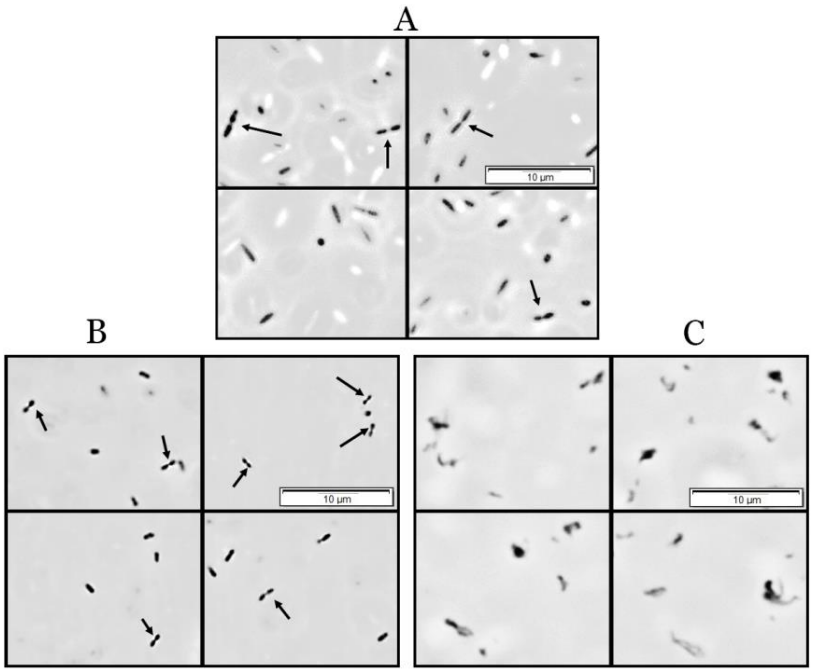


Figure 7. (A) fresh inoculum of *P. aeruginosa* 30 min after dilution of an overnight LB inoculum to 10^6 cfu/ml; arrow indicates an example of cell under division. (B) bacteria after 2 h of incubation with 25 mg/ml of treatment; arrows indicate cell presumably under division. (C) treated inoculum after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

CONCLUSION

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.

Authors' Contribution: All authors contributed to this study.

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

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