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GastronOmics: Edibility and safety of mycelium of the oyster mushroom *Pleurotus ostreatus*

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ABSTRACT

Food production is one of the most environmentally damaging human activities. In the face of climate change, it is essential to rethink our dietary habits and explore potential alternative foods catering both towards human and planetary needs. Fungal mycelium might be an attractive alternative protein source due to its rapid growth on sustainable substrates as well as promising nutritional and organoleptic properties. The natural biodiversity of filamentous fungi is vast and represents an untapped reservoir for food innovation. However, fungi are known to produce bioactive compounds that may affect human health, both positively and negatively. To narrow the search for safe and culinarily attractive fungal species, mycelia of edible fruiting-body forming fungi provide a promising starting point. Here, we explore whether the culinary attractiveness and safety of the commonly eaten mushroom, *Pleurotus ostreatus,* can also be translated to its mycelium.

Whole-genome sequencing and pan-genome analysis revealed a high degree of genetic variability within the genus *Pleurotus*, suggesting that gastronomic traits as well as food safety may differ between strains. A representative strain, *P. ostreatus* M2191, was further analyzed for the food safety, nutritional properties and culinary applicability of its mycelium. No regulated mycotoxins were detected in either the fruiting body nor the mycelium. Yet, *P. ostreatus* is known to produce four peptide toxins, Ostreatin, Ostreolysin and Pleurotoysin A/B. These were found to be lower in the mycelium compared to fruiting bodies, which are already considered safe for consumption. Instead, a number of secondary metabolites with potential health benefits were detected in the fungal mycelium. *In silico* analysis of the proteome suggested low allergenicity. In addition, the fruiting body and the mycelium showed similar nutritional value, which was dependent on the growth substrate.

To highlight the culinary potential of mycelium, we created a dish served at the two-star restaurant the Alchemist in Copenhagen, Denmark. Sensory analysis of the mycelium dish by an untrained consumer panel indicated consumer liking and openness to fungal mycelia.

Based on sustainability, safety, culinary potential, and consumer acceptance, our findings suggest that *P. ostreatus* mycelium has great potential for use as a novel food source.

1. Introduction

The food production system is one of the most important drivers of global environmental damage, resource depletion, eutrophication, and biodiversity loss (Willett et al., 2019). Future projections forecast a growing world population and an increasing demand for food, coupled with a reduction in fertile farmland due to climate change (Dietz and Lanz, 2020). Accordingly, we need to identify new ways of sustainably producing food.

Filamentous fungi are an attractive choice for the production of

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sustainable foods for the future. Fungi play a central role in decomposing organic and inorganic materials in natural ecosystems, and are capable of using complex carbohydrates found in plant materials (Esser et al., 1994). Consequently, filamentous fungi can be grown on nearly all organic waste streams generated in our current food production system. Leveraging the potential of fungi to turn waste into food is an important step towards increasing the sustainability and efficiency of our food production system.

In addition to environmental benefits, the abundant secretion of enzymes and metabolites by fungi allows the creation of foods with new flavors and textures. Secretion of enzymes like amylases or proteases aid in the conversion of starch and protein into simple sugars, peptides or free amino acids, ultimately translating into sweet, kokumi and umami taste perceivable by humans (Kusumoto and Rai, 2017). In addition to enzymes, fungi also produce many primary and secondary metabolites, for example organic acids that can be perceived as sour (Sahasrabudhe and Sankpal, 2001) or volatile compounds with aromatic properties (Dickschat, 2017). Not only flavor but also texture can be created using fungal mycelia. Mycelia consist of filamentous hyphae that can be used to create fibers resembling those of meat or seafood (Sadler, 1990). Additionally, mycelia can bind loose ingredients together to form a firm texture or provide a food scaffold, for example in tempeh or certain cheeses.

Humans already have a long history of consuming food created from or with fungi. Yeasts and molds like *Saccharomyces cerevisiae* or *Aspergillus oryzae* have been domesticated by humans for more than 12,000 years for food and beverage production (Gallone et al., 2016; Gibbons et al., 2012) and even the biomass is consumed as a nutritional supplement, flavor enhancer, or meat alternatives (Rousta et al., 2021). The fruiting bodies of fungi are also consumed, and studies of ancient human microbiomes suggest there may have been a higher proportion of mushrooms in early human diets compared to today (Wibowo et al., 2021). However, the mycelium of fruiting-body forming fungi has traditionally not been cultivated for human consumption. Only a few mold species seem to have safely been used for human food production (Nain et al., 2020), whereas thousands of mushroom species are consumed safely by humans across the globe (Li et al., 2021). Tapping into the biodiversity of the mycelia of these mushroom-forming species and exploring their culinary potential will open new avenues for more sustainable and delicious food production.

There is limited research on mycelium as a food source compared to other food sources, with recent studies showing promising results regarding its safety, nutritional value, and potential as a sustainable protein alternative. To widely adopt fungal mycelium into our diets several challenges should be addressed: (i) Safety. All fungi can produce secondary metabolites with bioactive capabilities. Some compounds have health promoting effects, while others might be toxic. Their production depends on specific growth conditions (Tudzynski, 2014) and substrates, and it is therefore important to determine if fungal mycelia produced for food is equally safe as traditionally- or naturally-grown fruiting bodies. (ii) The culinary potential of fungal mycelium needs to be explored with the aim of producing delicious and sustainable products. (iii) The consumer preferences and their willingness to adopt fungal mycelium in their diets.

In this interdisciplinary study we present a unique approach to food development and analysis. We combined gastronomy with microbiology, genetics, analytical chemistry, and sensory analysis to develop a dish based on the mycelium of the oyster mushroom *Pleurotus ostreatus*, and evaluated its safety and consumer acceptance. This study provides insight into the potential of using edible filamentous fungi as a more sustainable food source and highlights the importance of considering multiple factors, including microbiological and sensory considerations, in the development of new food products.

2. Materials and methods

2.1. Cultivation media

Yeast malt broth (YMB) and yeast malt agar (YMA) were prepared by mixing 11 g/L D-glucose monohydrate, 3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptidic digest of soy, 1.31 g/L $K_2HPO_4{\cdot}3H_2O$, 1 g/L KH_2PO_4, 3 g/L $NaNO_3$, 0.0001% salicylic acid, 0.25 mM $CaCl_2.2H_2O$, 0.077 mM $ZnSO_4.7H_2O$, 2.24 mM $MgSO_4.7H_2O$ (VWR), and the pH was set at 6.2 using a 1 M HCl solution. YMA medium was supplemented with 1.5% of agar. A trace element solution (containing 14.1 g/L Fe $(NH_4)_2(SO_4).6H_2O$, 731.2 g/L $CuSO_4$, 81 g/L $CoCl_2.6H_2O$, 51 g/L $Na_2MoO_4.2H_2O$, 81 g/L $NiCl_2.6H_2O$ and 38 g/L $SnCl_2.2H_2O$) was added at 1 mL/L at room temperature. 0.2% Tween80 was added to the YMB at room temperature. PDA (70,139) was prepared at 39 g/L according to the manufacturer's instructions. Yeast Nitrogen Agar (YNA) was prepared using 1.7 g/L Yeast Nitrogen Base (Y1251) 10 g/L D-glucose, 5 g/L ammonium sulphate, and 1.5% agar at pH 6.2 using a 1 M HCl solution. All chemicals were purchased from Sigma-Aldrich, unless otherwise stated.

Based on the media yeast malt broth (YMB) routinely used for the cultivation of filamentous fungi in laboratories (Atlas, 2005), an edible variation suitable for human consumption was developed. The newly developed medium is called yeast malt mushroom broth iota medium (YMBI). It was created from a base stock consisting of 150 g/L oyster mushrooms (Beyond Coffee ApS, Copenhagen), 45 g dried ceps (Condi) in 1 L deionized water. The ingredients were mixed in a vacuum bag, followed by steaming for 4 h at 80 °C. The base stock was strained through a fish net strainer (Knud Hansen Net, mesh size 13 rows and 30 knots per cm^2). The YMBI was prepared by dissolving 120 g/L malt extract (Aurion), 10 g/L iota (Texturas ElBulli), 4 g/L agar agar (Texturas ElBulli), 10 g/L dry yeast flakes (Natur Drogeriet), 10 g/L NaCl (Maldon) and 8 g/L raw brown sugar (Dan Sukker) in the base stock. The YMBI was boiled for 3 min, cooled to 3 °C, transferred to vacuum bags, and frozen. Before use, the frozen YMBI was thawed overnight at 4 °C, heated to 60 °C and homogenized by stirring. Subsequently, 15 mL YMBI was dispensed inside glass petri dishes (Ø100 mm) and wrapped inside sterilization paper. The petri dishes were autoclaved at 125 °C for 20 min.

2.2. Cultivation of Pleurotus ostreatus on YMBI

Winter oyster mushroom *Pleurotus ostreatus* (*P. ostreatus*) was obtained from Mycelia.be (strain M2191, Mycelia NV, BE 0427321424) and was grown on YMB medium at 26 °C for 14 days unless stated otherwise. Mycelium plugs (Ø5 mm) were transferred to inoculate YMBI plates. The inoculated petri dishes were wrapped with parafilm and incubated at 26 °C for 2–4 weeks until fully colonized. The mycelium (Ø5 mm) was used to inoculate further YMBI plates followed by an incubation at 26 °C for 2–4 weeks. Plates with regular mycelium growth were sealed with parafilm and stored upside down to prevent condensation at 4 °C for up to 1 week before the sensory analysis. The petri dishes were allowed to adjust to room temperature for 1 h before being served. Parafilm was removed just before serving.

2.3. Cultivation of P. ostreatus fruiting bodies

For spawn production, rye was washed and soaked overnight in water and autoclaved at 121 °C for 15 min in 1.5 L glass jars (Quattro, H. W. Larsen, Denmark) with ventilation. The mycelium (Ø5 mm plugs) of *P. ostreatus* (M2191, Mycelia NV, BE 0427321424) grown on YMBI was used to inoculate the rye, followed by incubation at 25 °C for 2–4 weeks until fully colonized.

Since YMBI was not suitable for fruiting body production, we used waste coffee grounds, mixed with cardboard (20 × 20mm) at a 1:1 ratio (w/w) for fruiting body production. 150 g of the mix was added to a

wide-mouth clue cap jar of 250 mL capacity (Duran, Germany, 212863658) and autoclaved at 121 °C for 15 min. Inoculation was done with 10% of rye spawn followed by incubation at 25 °C in the dark for 2–4 weeks. Fully colonized samples were cold-shocked overnight at 4 °C and then incubated at RT with 80–85% humidity to initiate fruiting.

2.4. High molecular mass DNA extraction

The following method was developed for high-molecular mass DNA extraction based on a user-adapted protocol for the genomic tip kit (Qiagen). *P. ostreatus* (M2191, Mycelia NV, BE 0427321424) was grown on cellophane on YMA plates containing 50 μg/ml ampicillin and incubated at 25 °C for up to 14 days. The mycelium was harvested from the cellophane and freeze-dried. The dried biomass of two plates corresponded to 69 mg 60 mg of mycelium was further utilized by freezing at −20 °C and subsequent pulverization with mortar and pestle, that were pre-cooled to −80 °C and placed in liquid nitrogen. 6 mL of G2 lysis buffer (genomic tip kit, Qiagen) were added to the pulverized mycelium and the mixture was aliquoted into three 2 mL tubes placed on ice. 10 μl Zymolase T20 (VWR) was added to the tubes, which were carefully inverted for mixing. Samples were incubated at 37 °C at 750 rpm and occasional inversion of the tubes for 2 h. Thereafter, 4 μL of RNAse (Qiagen) were added. The tubes were briefly vortexed and incubated for 30 min under previous conditions. 30 μL of proteinase K (Sigma-Aldrich) were added, mixed and incubated for 2 h at 50 °C and 750 rpm. The samples were centrifuged for 20 min at 4 °C and 15,000 g. During the centrifugation, GT columns (Qiagen) were equilibrated with QBT buffer (Qiagen). The supernatant from the tubes was transferred to a genomic tip column. The column was washed three times with 2 mL QC buffer (Qiagen). The DNA was eluted with 800 μL of QF buffer (Qiagen). 560 μL isopropanol were added at room temperature to each sample, which were subsequently centrifuged at 10,000 g and 4 °C for 20 min. The supernatant was discarded and 500 μl of 70% ethanol were added. After repeating the centrifugation step for 15 min, the supernatant was removed and pellets were dried at room temperature. The pellets were dissolved overnight in 50 μL TE buffer (pH 8) at 4 °C. DNA quality was assessed by loading 5 μL on a 1% agarose gel, by measuring absorption ratios via Nanodrop and DNA concentration via Qubit according to manufacturer's instructions. 75 ng of sample were loaded on a fragment analyser (Agilent) using the HMW DNA kit DNF-467 (Agilent). The final DNA concentration was 19.3 ng/μL and the average fragment size was 58 kb.

2.5. Whole-genome sequencing and assembly

The extracted DNA was submitted to Illumina NextSeq and Oxford Nanopore gridION. NextSeq library preparation and barcoding was performed using plexWell 24 plus library preparation kit according to the manufacturer's protocol except 11 cycles were used for library amplification. The library was sequenced using NextSeq 300 cycles midoutput kit with 8 isolates per flow cell. GridION library preparation was performed using rapid barcoding 96 according to the manufacturer's instruction and sequenced using R9.4.1 flow cell. Basecalling was performed using Guppy 5.0.11 (nanopore) with high accuracy base calling model and reads were exported as FastQ files.

The sequencing runs yield 8.7 gigabases of sequence reads, 5.5 GB of which were Illumina paired-end reads with 150 bases; and 3.2 gigabases were nanopore long reads with an average read length of 3 kilobases. These reads were used for hybrid assembly with MaSuRCA version 4.0.9 (Zimin et al., 2013). The assembly includes 64.7 megabases of sequence in 414 contigs with an N50 value of 365,745 bases and G + C content of 51%. The length of this assembly is 38% larger than the average length of available *Pleurotus* genomes (47 Mb; Table 1) implying extensive sequence repetition.

To annotate the genome, we first predicted the protein coding sequences in the assembly with Augustus release 3.5.0 (Stanke et al., 2006). To do so, we used a training dataset created with the *Pleurotus cornucopiae* annotations (GenBank accession number: WQMT02000000). The gene-calling process led to the prediction of 19,496 proteins of which 21% are redundant sequences, (i.e. paralogs) with 90% sequence identity.

2.6. Pan-genome analysis and taxonomic classification of P. ostreatus M2191

We used phylogenomics to confirm the taxonomic assignment of the M2191 strain. First, we used the BPGA pipeline (Chaudhari et al., 2016) to calculate the core genome of a set of basidiomycetes species (Fig. 1) including 38 *Pleurotus* sp. (Table 1) and other members of the order *Agaricales*, a *Lentinula* and *Agaricus* strains as outgroups. This analysis resulted in a set of 428 conserved proteins used to calculate a species tree. Each set of orthologous proteins was aligned with Muscle v3.8.1551 (Edgar, 2004) and trimmed with GBlocks (Castresana, 2000). After these steps, 354 individual alignments were useful for phylogenetic analysis while the remaining sets of orthologs yield alignments with large, gapped regions that were trimmed. The 354 alignments were then concatenated to generate a supermatrix with 73,720 columns that we used for phylogenetic analysis. An evolutionary model was calculated for each one of the 354 partitions. These models were used to calculate a phylogenetic tree with IQtree (Nguyen et al., 2014) for which branch support was calculated using 100,000 ultrafast bootstrap replicates. The entire process was performed automatically using code available at https://github.com/WeMakeMolecules/Core-to-Tree.

Once the taxonomic reconstruction was obtained and the M2191 assignment was confirmed. We used BPGA to calculate the core and pan genome for the genus *Pleurotus* using the 38 genomes available (Table 1 and Fig. 1). The functional annotations were obtained using the COG system through eggNOG-mapper 2.1.12 (Cantalapiedra et al., 2021).

The core of the genus is formed by 754 protein families, representing only 6.1% of the average proteome of the genus (12,297 proteins). The Heaps law model parameter α was estimated to 0.44 using the micropan R package (Snipen and Liland, 2015), indicating an open pan-genome, the threshold being 1.0 (Tettelin et al., 2008).

Functional annotation of the core genome using the Clusters of Orthologous Genes (COG) system allowed the annotation of 99, 70 and 48% of the core, accessory and unique genome, respectively (Fig. 1F and S1C). To confirm the taxonomic annotations we calculated the average nucleotide identity (ANI) of all the *Pleurotus* strains in our tree using PyANI (Pritchard et al., 2015).

2.7. Identification of orthologs to toxin-producing genes

For identification of toxin-encoding protein orthologs among the *Pleurotus* spp. Genomes in our study, we used the amino acid sequences of pleurotolysin A (uniprot accession Q8X1M9) and ostreatin (uniprot accession A0A067NAU1) as queries for searches with the *Core Analysis of Syntenic Gene Clusters*, pipeline (CORASON) (Navarro-Muñoz et al., 2020) using a modified script (https://github.com/WeMakeMolecules/fungison). The search was performed with a bit score cutoff of 200 and an e-value cutoff of 1e-7. To distinguish between orthologs and paralogs, we used the CORASON output to evaluate their synteny and evolutionary relationships (Fig. S3). Notably, Ostreolysin, Pleurotolysin A and Pleurotolysin B are located in the same genomic neighborhood.

2.8. Identification of biosynthetic gene clusters

To identify biosynthetic gene clusters encoded in the genomes included in our study, we used antiSMASH v7.0. Which ran using the genome assemblies and the gene calling files generated with Augustus release 3.5.0 obtained as described above. The graphic in Fig. S2 and Table 3 were created with BGC counts obtained from the outputs generated by antiSMASH using the antismash_region_parser.pl script

Table 1
Genome metrics of available *Pleurotus* genomes
The 38 *Pleurotus* genomes were used to confirm the taxonomic assignment of the *Pleurotus ostreatus* M2191 strain. The assembly includes 64.7 megabases of sequence split in 414 contigs with an N50 value of 365,745 bases and G + C content of 0.5151%. *Agaricus bisporus* and *Lentinula edodes* were used as outgroups for the phylogenetic tree. See accompanying Fig. S1C.

Species	Strain	GenBank	Length (Mb)	Contigs	GC content	N50	CDSs	Core genes	Accessory genes	Unique genes	Exclusively absent genes
Pleurotus citrinopileatus	HfpriPC05-1-Y1	GCA 17312325.1	38.6	88	0.49	2853238	7024	754	6678	262	0
Pleurotus citrinopileatus	MG63	GCA 3314595.1	36.8	10,689	0.5	9672	7263	754	6907	539	2
Pleurotus cornucopiae	CCMSSC00406	GCA 19677325.2	32.4	11	0.51	3564128	6839	754	6504	4	0
Pleurotus djamor	MPG 5	GCA 29747585.1	62.5	2603	0.5	52,533	8389	754	8030	785	16
Pleurotus eryngii	183	GCA 1717165.1	43.8	166	0.49	508,674	6915	754	6582	161	125
Pleurotus eryngii	ATCC 90797	GCA 15484515.1	44.6	609	0.5	241,626	7598	754	7261	58	0
Pleurotus eryngii	JKXB130DA	GCA 3243765.1	49.9	153	0.49	562,808	6876	754	6540	671	75
Pleurotus eryngii	MG61	GCA 3314755.1	174.6	104,407	0.47	4216	10,510	754	9936	5763	0
Pleurotus eryngii	var. elaeoselini CCMJ2131	GCA 29874175.1	51.1	42	0.5	2983285	7463	754	7128	262	0
Pleurotus eryngii	var. energii E10	GCA 29467805.1	53.5	47	0.49	3172794	7702	754	7372	250	6
Pleurotus eryngii	var. ferulae CCMJ970	GCA 29467795.1	48.0	51	0.5	2342731	7361	754	7037	235	1
Pleurotus floridanus	CCMSSC00406	GCA 19395345.1	35.1	425	0.51	331,558	7250	754	6915	8	0
Pleurotus giganteus	PG46	GCA 32158425.1	40.1	16	0.5	2887167	5251	754	4911	2309	8
Pleurotus ostreatoroseus	DPUA 1720	GCA 5298045.1	38.6	619	0.53	180,995	2627	754	2160	5865	512
Pleurotus ostreatus	595	GCA 24195665.1	79.7	329	0.51	365,186	8383	754	8008	1198	4
Pleurotus ostreatus	CCMSSC00389	GCA 1956935.2	35.1	136	0.51	3067903	7220	754	6888	22	1
Pleurotus ostreatus	CCMSSC03989 v2	GCA 3313235.2	34.4	89	0.51	2851238	6785	754	6445	120	6
Pleurotus ostreatus	gfPleOstr1.1	GCA 947034855.1	40.6	16	0.51	4316464	7286	754	6952	67	1
Pleurotus ostreatus	gfPleOstr1.1 alt haplotype	GCA 947034875.1	40.8	53	0.51	1811266	7278	754	6940	67	0
Pleurotus ostreatus	**M2191**		**64.7**	**414**	**0.51**	**365,745**	**19,496**	**754**	**8085**	**278**	**0**
Pleurotus ostreatus	PC15	GCA 697685.1	34.3	12	0.51	3270165	6880	754	6545	55	0
Pleurotus ostreatus	PC9	GCA 14466165.1	35.0	17	0.51	3500734	6958	754	6620	9	0
Pleurotus ostreatus	PC9.15	GCA 29852705.2	35.4	14	0.51	3339398	6813	754	6477	6	3
Pleurotus platypus	MG11	GCA 3313735.1	40.0	6484	0.51	62,119	7609	754	7271	27	3
Pleurotus pulmonarius	CCMSSC 4423	GCA 32158505.1	42.3	111	0.51	2663750	7667	754	7325	27	0
Pleurotus pulmonarius	PM ss13	GCA 12979565.1	42.6	17	0.51	3242126	7590	754	7246	47	1
Pleurotus pulmonarius	PM ss2	GCA 12980525.1	39.2	23	0.51	3175356	7333	754	6989	87	8
Pleurotus pulmonarius	PM ss5	GCA 12980535.1	**39.9**	23	0.51	3374720	7596	754	7254	28	1
Pleurotus salmoneostramineus	N4	GCA 2933715.1	39.8	1597	0.5	107,283	7964	754	7620	257	2
Pleurotus salmoneostramineus	N60	GCA 2583695.1	39.2	1372	0.5	108,301	7908	754	7562	169	1
Pleurotus salmoneostramineus	NBRC 31859	GCA 1742905.1	65.9	26,934	0.5	4878	9099	754	8707	2922	6
Pleurotus sapidus	DSM 8266	GCA 26743855.1	46.9	63	0.51	1656594	7204	754	6870	547	3
Pleurotus tuber-regium	ACCC 50657-18	GCA 14058305.1	35.8	65	0.48	2556151	6156	754	5817	119	2
Pleurotus tuber-regium	JN18	GCA 19693315.1	39.4	123	0.47	1181580	6200	754	5867	198	8
Pleurotus tuber-regium	KL18	GCA 3314355.1	33.6	1283	0.48	223,672	6321	754	5989	107	2
Pleurotus tuoliensis	CCMSSC00489P1	GCA 2847145.1	41.1	1409	0.5	628,135	7345	754	7008	137	0
Pleurotus tuoliensis	JKBL130LB	GCA 3243755.1	48.2	106	0.5	1168774	7397	754	7065	479	10
Pleurotus tuoliensis	MG79	GCA 3313115.1	43.5	7458	0.5	22,588	7794	754	7458	111	0
		Average	**47.0**	**4423.0**	**0.5**	**1651567**	12,293	**754**	**6920**	**638**	**21**
		Total				**51,380**			**26,370**	**24,256**	**807**
Agaricus bisporus	AB58	GCA 8271545.1	30.1	18	0.47	2300414	2105	428	2105	3418	91
Lentinula edodes	135 A	GCA 2195965.1	36.7	2997	0.46	52,820	2242	428	2242	4872	93

Table 2
Secondary metabolite biosynthetic gene cluster discovery in Pleurotus ostreatus M2191.
Predicted genes were annotated using AntiSMASH fungal version 7.0.1. Secondary metabolite regions were identified using strictness 'relaxed'. Two ochratoxin A BCG's were detected.

Region	Type	from	to	Most similar known cluster	Biosynthetic class (es)	Similarity
Region 14.1	fungal-RiPP-like	408,700	456,394			
Region 16.1	fungal-RiPP-like	59,552	150,529			
Region 18.1	fungal-RiPP-like	1	77,492			
Region 22.1	NRPS-like	3950	63,737			
Region 22.2	NRPS-like	222,496	286,117			
Region 22.3	terpene	292,385	327,576	clavaric acid	Terpene	100%
Region 30.1	terpene	405,368	436,207			
Region 30.2	NRPS-like	550,995	615,267			
Region 48.1	terpene	21,297	52,475			
Region 49.1	NI-siderophore	528,664	549,459			
Region 49.2	terpene	567,308	598,486			
Region 51.1	T1PKS,terpene	326,323	421,652	(+)-δ-cadinol	Terpene	40%
Region 52.1	fungal-RiPP-like	118,526	209,500			
Region 52.2	NRPS-like,NRPS	506,551	620,223			
Region 52.3	NRPS-like	812,641	876,050			
Region 52.4	NRPS-like	877,789	941,482			
Region 55.1	terpene	28,582	82,233			
Region 60.1	NRPS-like	69,131	132,112			
Region 69.1	terpene	2727	32,891	(+)-δ-cadinol	Terpene	100%
Region 69.2	terpene	92,976	115,912			
Region 72.1	NRPS-like	144,865	209,136			
Region 72.2	terpene	356,993	387,913			
Region 82.1	fungal-RiPP-like	1	63,411			
Region 84.1	terpene	41,233	72,567			
Region 84.2	NRPS-like	322,517	368,093	ochratoxin A	NRP + Polyketide	100%
Region 90.1	fungal-RiPP-like	2489	94,419			
Region 102.1	NRPS-like,terpene	1	59,517			
Region 105.1	fungal-RiPP-like	1	66,457			
Region 110.1	terpene	56,492	87,643			
Region 111.1	NRPS	459,503	526,020			
Region 111.2	NRPS-like	672,511	710,577			
Region 112.1	terpene	64,186	95,310			
Region 125.1	fungal-RiPP-like	88,880	180,810			
Region 131.1	fungal-RiPP-like	1	77,974			
Region 133.1	terpene	18,711	49,969			
Region 142.1	NRPS-like	41,203	105,394			
Region 145.1	NI-siderophore	26,759	43,039			
Region 147.1	terpene	1	27,112			
Region 147.2	NI-siderophore	60,832	81,627			
Region 154.1	T1PKS,terpene	618,305	704,862	(+)-δ-cadinol	Terpene	40%
Region 157.1	terpene	209,141	241,731			
Region 176.1	terpene	132,396	165,842			
Region 179.1	terpene	1,223,426	1,249,666			
Region 179.2	terpene	1,333,810	1,365,107	(+)-δ-cadinol	Terpene	100%
Region 184.1	fungal-RiPP-like	3897	95,731			
Region 193.1	fungal-RiPP-like	148,253	202,769			
Region 206.1	NRPS-like	18,064	81,810			
Region 214.1	terpene	58,718	89,988			
Region 216.1	terpene	272,692	303,962			
Region 225.1	NRPS-like	58,489	122,662	ochratoxin A	NRP + Polyketide	100%
Region 225.2	terpene	350,950	382,290			
Region 240.1	terpene	34,113	83,013	clavaric acid	Terpene	100%
Region 248.1	fungal-RiPP-like	180,119	271,683			
Region 251.1	NRPS-like	241,997	304,978			
Region 252.1	fungal-RiPP-like	159,688	251,106			
Region 256.1	terpene	232,581	265,217			
Region 257.1	terpene	271,672	302,866	(+)-δ-cadinol	Terpene	100%
Region 264.1	fungal-RiPP-like	261,114	352,121			
Region 282.1	NRPS-like	109,368	173,668			
Region 282.2	fungal-RiPP-like	216,095	263,794			
Region 287.1	terpene	361,283	383,800	(+)-δ-cadinol	Terpene	100%
Region 291.1	NRPS-like,terpene	72,797	129,741			
Region 300.1	fungal-RiPP-like	1	61,221			
Region 301.1	terpene	573,335	633,832			
Region 304.1	fungal-RiPP-like	234,144	290,941			
Region 319.1	fungal-RiPP-like	136,214	227,002			
Region 328.1	fungal-RiPP-like	21,759	114,294			
Region 344.1	terpene,fungal-RiPP-like	1	96,569	(+)-δ-cadinol	Terpene	100%
Region 356.1	terpene	25,596	46,885			
Region 356.2	terpene	106,313	137,578	(+)-δ-cadinol	Terpene	100%
Region 363.1	fungal-RiPP-like	71,987	162,782			
Region 367.1	terpene	110,100	141,969			

(*continued on next page*)

Table 2 (*continued*)

Region	Type	from	to	Most similar known cluster	Biosynthetic class (es)	Similarity
Region 375.1	NRPS-like	16,276	125,072			
Region 375.2	NRPS,NRPS-like	375,001	479,293			
Region 382.1	fungal-RiPP-like	1259	95,073			

(https://github.com/WeMakeMolecules/Megasynthase_string_miner/blob/main/antismash_region_parser.pl).

2.9. Quantitative reverse-transcriptase PCR of toxin-producing genes in mycelium and fruiting body

RNA isolation: Total RNA was isolated from ~200 mg of *P. ostreatus* M2191 mycelium and fruiting bodies using the Direct-zol™ RNA Mini-Prep Plus Kit (Zymo Research, USA) according to the manufacturer's instructions. Cells were lysed with 0.5 mm glass beads (Z250465, Sigma-Aldrich) on a Mini-Beadbeater-16 (607, BioSpec). RNA quantification was done using a NanoDrop ND-2000 UV–Vis spectro-photometer (NanoDrop Technologies Inc., Wilmington,DE, USA).

cDNA synthesis: 75 ng RNA was used as input for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Scientific) as per the manufacturer's instructions in a final reaction volume of 10 μL. A sample without reverse transcriptase was prepared as a negative control.

Primer design: The primer designing tool PrimerQuest (IDT) was used to design primers for Ostreolysin, Pleurotolysin A, Pleurotolysin B and Ostreatin based on the coding sequence from *P. ostreatus* M2191 genome (contigs 170.g14054.t1, 170.g14054.t1, 170.g14062.t1, 170.g14062.t1, 311.g6706.t1, 311.g6706.t1, 311.g6706.t1, 311.g6705.t1, 170.g14053.t1, 91.g12438.t1, 91.g12438.t1, 91.g12438.t1). The primers were checked for self-annealing, mismatches and hairpin formation using OligoCalc (Kibbe, 2007). Primer sequences can be found in Table S2. We used secretion-associated methylthioadenosine phosphorylase (*phos*) as housekeeping gene according to the literature (Castanera et al., 2015; Fernández-Fueyo et al., 2014). MIQE guidelines were followed at every step, wherever possible (Bustin et al., 2009).

RT-qPCR assay: 2 × SensiFAST SYBR® Lo-ROX mix (BioLine) was used for all qPCR reactions in a 10 μL reaction mix. Briefly, reactions consist of 5 μL of SensiFAST SYBR® Lo-ROX mix, 400 nM of each primer (Supplementary Table 1) and 2 μL of 1:100 diluted template cDNA. All qPCR reactions followed the same thermocycler program that consisted of an initial 3 min step at 95 °C, followed by 40 cycles of 95 °C for 10s, 60 °C for 15s, and 72 °C for 30s, followed by a melting curve protocol consisting of a single cycle of 95 °C for 5s, 60 °C for 1 min, 95 °C for 5s. qPCR runs were performed on the Roche LightCycler® 480 Real-time PCR System in LightCycle® 480 Multiwell Plate 384, clear plates. All samples were run in triplicate.

A non-template control (NTC) was run in each plate to confirm the absence of nucleic acid contamination. A melting curve analysis was performed to determine the specificity of the qPCR assay and to check for minimal primer-dimer formation. RT-qPCR products were subjected to agarose gel electrophoresis to assess specific product formation.

RT-qPCR data and statistical analysis: The mean sample Ct values for different transcripts were calculated for triplicate samples and relative expression ratio of gene expression was calculated using the ΔΔCt method using the geometric mean of the reference genes. Data is expressed as normalized relative expression values (2^ΔΔCt), relative to mycelium. Error bars indicate error propagated SD, p-values were calculated using an unpaired two-sided *t*-test. R version 4.3.1 was used for the visualization of the data based on relative expression.

2.10. Metabolite extraction from P. ostreatus mycelium and fruiting bodies

6 mm diameter plugs were sectioned from 4 biological replicates and a corresponding negative control sample (substrate only), *P. ostreatus* fruiting bodies were first cut and smashed in a mortar. In each vial, 0.6 mL of the solvent mixture MeOH:AcOEt:CH_2Cl_2 (1:2:3) with 0.1% formic acid was added. Vials were placed in an ultrasound bath for 60 min. Supernatant was transferred to a 2 mL microtube followed by evaporation of the solvent. The contents were resuspended in HPLC grade MeOH, centrifuged (15,000 rpm, 10 min), transferred to a 1.5 mL glass vial for LC-MS/MS analysis.

2.11. LC-MS/MS data acquisition

LC-MS/MS data acquisition of both mycelium and fruiting body extracts was performed using LC-MS/MS performed on an Agilent 1290 Infinity UHPLC coupled to an Agilent 6545 QTOF. Chromatographic separation was achieved using an Agilent InfinityLab Poroshell 120 Phenyl Hexyl column (2.1 × 150 mm, 1.9 μm) kept at 40 °C and a constant flow rate of 0.35 mL/min using a linear gradient. The mobile phase eluents were A: LC-MS grade water, and B: LC-MS grade acetonitrile, both containing 20 mM formic acid. Starting conditions were 90% A and 10% B, followed by and increase to 100% B over 10 min, and then held at 100% B for 2 min. The system was re-equilibrated prior to subsequent sample injections.

Ionization of eluting metabolites was performed in positive and negative electrospray ionization (ESI) mode with a voltage of 3500 V and 2500, respectively. Spectra were acquired at a rate of 10 Hz, with a *m/z* range of 50–1700. Fragmentation spectra were acquired in automated fragmentation DDA using multiple collision energies (10, 20, and 40 eV) with selection of three precursor ions per MS level scan.

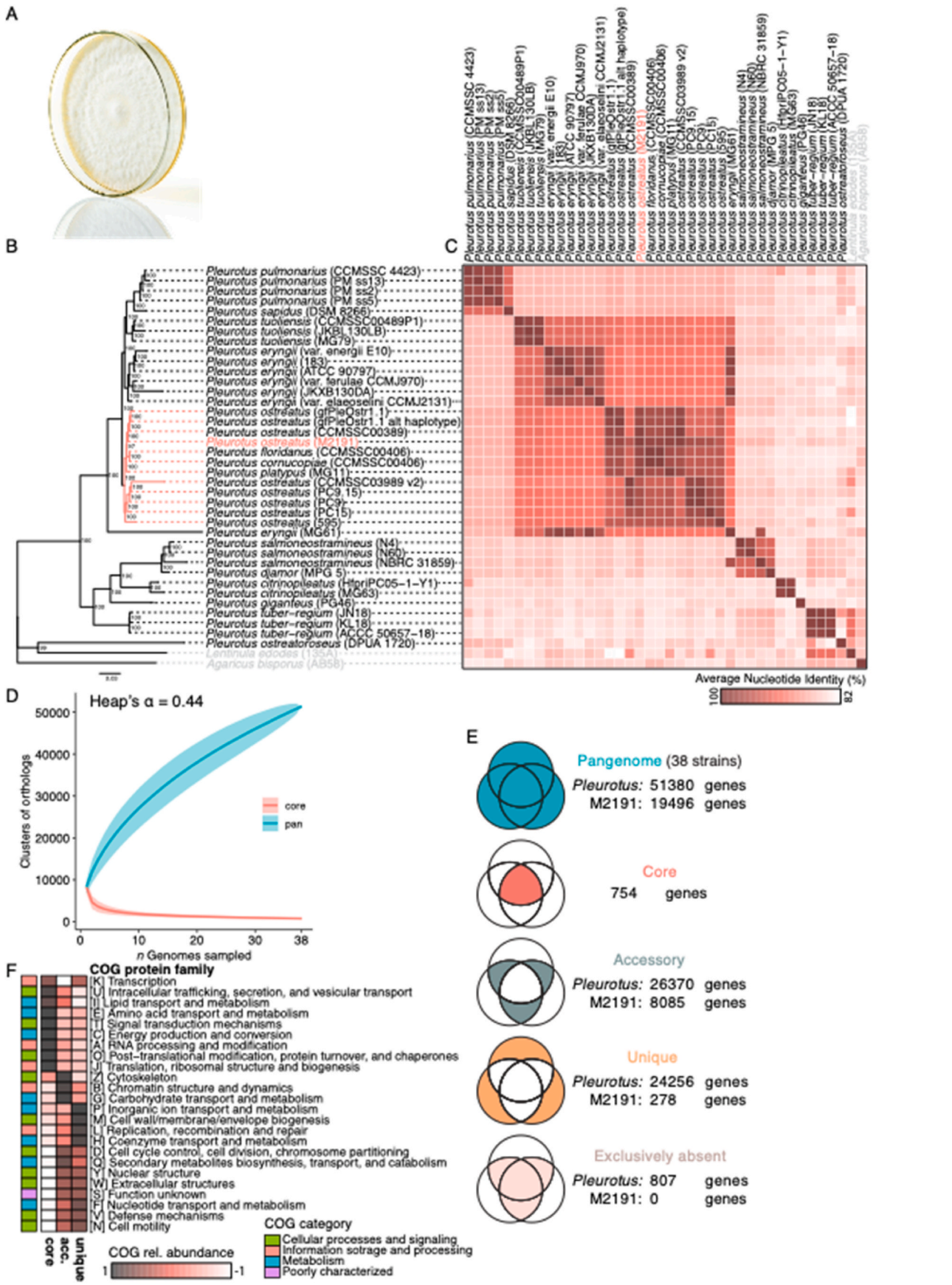
2.12. LC-MS/MS data analysis

Data analysis was performed using the MassHunter Qualitative Analysis Software B.07.00 (Agilent). To search for known mycotoxins and other natural products the *P. ostreatus* tandem mass spectrometry data was analyzed using the auto MS/MS feature followed by matching in the DTU Mycotoxin-Fungal Secondary Metabolite MS/HRMS library (Klitgaard et al., 2014; Nielsen and Smedsgaard, 2003). Matches were filtered by the following criteria: (i) the library match score for the target feature was >90; (ii) The target feature was not found in blank samples or its corresponding negative control; (ii) The experimental *m/z* had a mass error of less than 5 PPM relative to the theoretical *m/z* to the matched molecule; (iii) The fragmentation ions found in the spectrum for the target feature were consistent with the matched metabolite in the library. Note that this filtering allowed a high sensitivity for the detection of mycotoxins in the library. Downstream analysis and visualization were done using R version 4.3.1.

2.13. Substrate growth assay

Inoculum was prepared by growing mycelium on 20 mL YMB medium in a Ø100 mm Petri dish for 10 days at 25 °C. Mycelium was washed three times with sterile water, and transferred to a gentleMACS C tube (Miltenyi Biotec) with sterile water to the 10 mL mark. Mycelium was dissociated for 2 × 10s at 4000 rpm using the GentleMacs Octo Dissociator (Miltenyi Biotec) and filtered through a sterile gauze to avoid bigger clumps. The turbidity was adjusted to 62% T using a turbidimeter (Biolog). Inoculation fluid was prepared by adding 50 μl to 24 mL of sterile water.

4 replicate Biolog PM01 and PM02 plates were prepared following



(caption on next page)

Fig. 1. Taxonomic assignment of the *Pleurotus ostreatus* M2191 and pangenome analysis for *Pleurotus* spp.
A. *Pleurotus ostreatus* M2191 mycelial growth on a YMBI plate after 7 days incubation at 22 °C. **B.** Phylogenetic tree constructed with a set of 353 conserved proteins. **C.** Average nucleotide identity among the members of the genus *Pleurotus*. **D.** Pangenome plot of *Pleurotus* genomes used for pangenome construction. The plot shows the number of core and accessory gene families shared by a growing set of genomes. This process was repeated 20 times to obtain the error bars, they represent the standard deviation of the number of core/accessory gene families found for step. The graph also shows a curve analysis using a power law regression model (Heap's law, $N = kn^{-\alpha}$). The Heaps law α was estimated to 0.40 and $k = 3945$, indicating an open pan-genome. For $\alpha > 1$, the pan-genome is closed, and for $\alpha < 1$, the pan-genome is open (infinitely growing with n). **E.** Genes in the pangenome (32,337 genes) are divided into the core (comprising 1161 genes), accessory (comprising 2940 genes), and rare (comprising 19,555 genes) genomes. For details see accompanying Table 1 and Fig. S1C. **F.** Heatmap representation of functional annotation of the core, accessory and unique genome of *P. ostreatus* M2191 using the Clusters of Orthologous Genes (COG) system, scaled row-wise.

Table 3
Genome analysis for secondary metabolites across the genus *Pleurotus*
Comparative analysis of genomes deposited in the GenBank as *Pleurotus* strains using AntiSMASH version 6.0.1.64 *loci* encoding biosynthetic gene clusters for secondary metabolites were predicted, 23 of which contained domains related to Non-Ribosomal Peptide Synthases (NRPS), 2 Polyketide Synthases (PKS), and 36 to terpene biosynthesis. See also the corresponding Supplementary Fig. S2.

Species	Strain	fungal-RiPP	NRPS and NRPS-like	siderophore	T1PKS	terpene	total
Pleurotus citrinopileatus	HfpriPC05-1-Y1	6	15	1	5	18	45
Pleurotus citrinopileatus	MG63	7	9	1	5	21	43
Pleurotus cornucopiae	CCMSSC00406	10	12	1	1	16	40
Pleurotus djamor	MPG 5	13	21	1	10	34	79
Pleurotus eryngii	183	8	7	1	1	13	30
Pleurotus eryngii	ATCC 90797	8	7	1	1	13	30
Pleurotus eryngii	JKXB130DA	7	4	1	1	7	20
Pleurotus eryngii	MG61	20	8	2	2	16	48
Pleurotus eryngii	var. elaeoselini CCMJ2131	10	9	1	1	14	35
Pleurotus eryngii	var. energii E10	8	8	1	1	12	30
Pleurotus eryngii	var. ferulae CCMJ970	9	8	1	1	14	33
Pleurotus floridanus	CCMSSC00406	11	12	1	1	16	41
Pleurotus giganteus	PG46	7	12	1	3	17	40
Pleurotus ostreatoroseus	DPUA 1720	10	12	0	5	17	44
Pleurotus ostreatus	595	19	22	1	2	33	77
Pleurotus ostreatus	CCMSSC00389	11	14	1	1	18	45
Pleurotus ostreatus	CCMSSC03989 v2	10	8	1	1	11	31
Pleurotus ostreatus	gfPleOstr1.1	10	13	1	1	16	41
Pleurotus ostreatus	gfPleOstr1.1 alt haplotype	10	13	1	3	15	42
Pleurotus ostreatus	**M2191**	**0**	**23**	**3**	**2**	**36**	**64**
Pleurotus ostreatus	PC15	12	10	1	1	16	40
Pleurotus ostreatus	PC9	12	10	1	1	16	40
Pleurotus ostreatus	PC9.15	13	10	1	1	16	41
Pleurotus platypus	MG11	11	15	1	1	17	45
Pleurotus pulmonarius	CCMSSC 4423	8	14	1	2	22	47
Pleurotus pulmonarius	PM ss13	8	15	1	4	22	50
Pleurotus pulmonarius	PM ss2	8	12	1	1	19	41
Pleurotus pulmonarius	PM ss5	8	13	1	1	18	41
Pleurotus salmoneostramineus	N4	6	15	1	5	21	48
Pleurotus salmoneostramineus	N60	7	14	1	5	19	46
Pleurotus salmoneostramineus	NBRC 31859	9	11	3	6	36	65
Pleurotus sapidus	DSM 8266	9	16	1	2	21	49
Pleurotus tuber-regium	ACCC 50657-18	5	12	1	4	24	46
Pleurotus tuber-regium	JN18	7	15	1	4	21	48
Pleurotus tuber-regium	KL18	4	15	1	4	22	46
Pleurotus tuoliensis	CCMSSC00489P1	9	10	1	2	11	33
Pleurotus tuoliensis	JKBL130LB	7	10	1	2	11	31
Pleurotus tuoliensis	MG79	11	11	1	2	13	38
	Average	**9.2**	**12.2**	**1.1**	**2.5**	**18.5**	**43.5**
Agaricus bisporus	AB58	0	7	1	2	9	19
Lentinula edodes	135 A	3	13	2	5	9	32

the manufacturer's instructions. Each BiologTM plate contains a different carbon source including carbohydrates, carboxylic acids, amino acids, amines, polymers, phenolic acids, and a control. In short, 100 μL inoculation fluid was distributed over each well, and all plates were incubated for 12 days at 25 °C. Data collected using the OmniLog Analysis Software 1.7. Data (in OmniLog units) is normalized using parametric background subtraction and corrected for t = 0. Data is expressed as growth relative to glucose.

2.14. Growth curves

100 μL inoculum (described above) was inoculated on YMA plates. Radial growth was recorded daily, and expressed as area (mm^2). Growth in liquid medium is recorded as described above, OmniLog units are normalized relative to the largest mean in the data. Data is visualized using GraphPad Prism v10. μmax is expressed as either mm2/day or % growth per day. The average growth rate values of 4 replicates were reported for each sample. For the liquid medium, relative growth was calculated using the OmniLog units normalized to 100% for n = 2 biological replicates.

2.15. Measurement of relative protein content

Mycelia and fruiting bodies were cut using a 4 mm bore. Visible agar was removed, samples were washed three times with deionized water and filtered with a 0.5 μm pluriStrainer (pluriSelect Life Sciences) under

vacuum. Samples were dried in a vacuum oven at 40 °C for ~2 h until stable weight. DI water is added to a final concentration of 20 mg (dry weight)/mL, after which ~350 mg of 0.5 mm glass beads (Z250465, Sigma-Aldrich) were added. Samples are lysed using a bead-beater (Precellys 24, Bertin Technologies) at 3 × 6500 rpm for 45s. 20 μL of sample was equally distributed on a well of the 96-well silica plate and dried at 40 °C in an oven with vacuum applied. DI was taken alongside, and one well was left empty for background measurement. Samples were measured twice with the INVENIO-S FTIR spectrophotometer linked to the high-throughput HTS-XT extension (Bruker Optics) using the DLaTGS detector for transmission measurements.

Baseline correction (rubberband) and offset correction re-processing steps were performed with OPUS 8.5 (Bruker Optics). Values are displayed as AUC Amide II (1605-1485 cm-1)/AUC Full spectrum (3999-401 cm-1), normalized by the fruiting body. One-way ANOVA was performed as a statistical test.

2.16. Allergenicity analysis

AllerCatPro 2.0 was used to identify potential allergenic proteins (Nguyen et al., 2022). It uses data from multiple databases including Allergome, WHO/International Union of Immunological Societies (IUIS), Comprehensive Protein Allergen Resource (COMPARE), Food Allergy Research and Resource Program (FARRP), and UniProtKB, to analyze similarity between input proteins and the allergen datasets using their amino acid sequences and predicted 3D structures. Predicted amino acid sequences in FASTA format were used as input and default parameters were used: protein sequence is >35% identical over an 80 aa sliding window, or >93% identity with aligned surface residues for a strong evidence hit. Data was filtered for hits with strong and weak evidence, and for food-related allergens.

2.17. Sensory analysis: consumer study

A panel of untrained panelists (hereafter consumers) was recruited from Kgs. Lyngby, Denmark, using a mailing list of individuals who have expressed interest in participating in such panels. A total of 72 consumers, all healthy individuals willing to try mycelium, participated in the study. The study was gender balanced and all participants were aged above 18 and within the target consumer age group of flexitarians (Hellstern et al., 2024). The protection and processing of personal data followed the Declaration of Helsinki and the 2016/679 EU Regulation. A written consent indicating voluntary participation in Novel food sensory analysis was obtained from each participant before the study. The study was conducted at the Biosustain DTU Center and organized into six sessions, each comprising 12 to 15 participants. Each session lasted approximately 20 min. Consumers rated the sample before and after tasting, to study the differences between the perception just seeing the product and after tasting. Each consumer rated liking using a 9-point hedonic scale (1 = extremely dislike, 9 = extremely like) as well as CATA (Check-All-That-Apply), before and after tasting. A total of 30 attributes for CATA were chosen from literature related to *P. ostreatus* flavor (Misharina et al., 2009; Tagkouli et al., 2021). Further data was obtained by additional questions, like "*Have you eaten this kind of mycelium before*" using yes or no, "*How often do you eat mushroom or derivative products*" using a 5-points scale (1 = rarely, 5 = daily) and "*How much would you be willing to eat it*" using 7-points scale (1 = not interested at all, 7 = extremely interested). The study was explained in detail to the participants including each CATA attribute prior to the tasting. The study was conducted in a room with room temperature and relative humidity (20 ± 2 °C; 75 ± 5% RH); the illumination was a combination of natural and non-natural light. The sample was served in a Petri dish at room temperature (20 ± 2 °C). A students' t-test was conducted using "liking" between before and after tasting, as well as for 'have you eaten mycelium before' (Supplementary Fig. S4). For the radar chart, data was rescaled based on the maximum of 72 consumers (=100%). To analyze the differences among attributes a Wilcoxon rank test was used. Statistical analyses were conducted using XLSTAT Version 2021.6.03 (Addinsoft) and R version 4.3.1.

3. Results

3.1. Taxonomic assignment of P. ostreatus M2191 and pangenome analysis for Pleurotus spp. On the genus level reveals high genetic variability

In this study we investigated the gastronomic potential of the mycelium of the oyster mushroom *P. ostreatus* strain M2191 (Fig. 1A). We chose the M2191 strain from Mycelia, a leading Belgian mycelium production company, known for producing winter oyster mushrooms with a dense, meaty texture and large size (Aditya et al., 2024; Mycelia.be, 2024). *Pleurotus* spp. Have high intra-species genetic variability (Bunyard et al., 1996), which can lead to misidentifications of strains, leading to significant implications for their gastronomic properties and cultivation. To confirm the taxonomic classification of the strain and understand the taxonomic relations within this clade, we sequenced the genome of strain M2191 (Table 1). As a first step, the taxonomic assignment of M2191 was analyzed by constructing a phylogenetic tree for the *Pleurotus* genus. To construct this tree, we analyzed 354 sets of orthologous proteins from 37 previously sequenced *Pleurotus* genomes to compare to strain M2191. The genomes ranged in size from 32.4 to 117.6 Mb (Table 1), indicating a large variation in genome sizes in these fungi, potentially related to ploidy. We confirmed the classification of the strain M2191 as *P. ostreatus* with strong branch support, as can be seen from the species tree (Fig. 1B) and from the average nucleotide identity (ANI) of all the *Pleurotus* strains in the tree (Fig. 1C). Strain M2191 shares 97% average nucleotide identity with strain PC9, considered the model for the species. Our analysis incidentally showed that three strains previously classified as *P. platypus, P. floridanus* and *P. cornucopiae* were misclassified and are actually *P. ostreatus* (95% sequence identity with strain PC9).

After we established the taxonomy of strain M2191, we perfomed a pan-genome analysis of the *Pleurotus* genus (Fig. 1D) to quantify its genetic diversity, and to provide information about whether M2191's safety, substrate use and culinary potential align with strains of the same genus. The pan-genome was vast, comprising a total of 51,380 genes. 754 gene clusters were shared by the core genome of all the strains, comprising only 6.1% of the average proteome of the genus (12,297 proteins; Fig. 1E).

The core genome, as expected, contained genes associated with functions related to central metabolism and housekeeping functions (Fig. 1F and S1D). The accessory genomes, consisting of genes present in two or more genomes, but not in all the genomes, included 26,370 gene clusters (51% of the pangenome), of which 28% could be annotated using KEGG. A total of 24,256 gene clusters were unique (found in only one genome) and most of these gene functions are uncharacterized (22% was annotated using KEGG). The number of strain-specific genes in each genome varied from 4 to 5756 (Fig. 1E and S1A).

The most distant strain in the genus is *P. ostreatoroseus*, an edible Brazilian mushroom, which clusters closest to the outgroups rather than other *Pleurotus* species and had the largest number of unique genes. Interestingly, *P. ostreatus* strain M2191 also had more accessory and unique genes compared to other strains from the same species (16 and 37% larger, respectively; Table 1). The accessory and unique genomes of the *Pleurotus* genus have a relatively high number of genes associated with secondary metabolism, such as the production of vitamins, compared to the core. The accessory genome also contains genes associated with carbohydrate- and lipid metabolism, suggesting that the accessory genome provides adaptive advantages in specific substrate environments (Fig. 1F and S1D).

The *Pleurotus* pan-genome showed characteristics of an "open" pan-genome, as the number of new genes did not converge to zero upon

addition of new strains (at $n = 38$, an average of 635 new genes were contributed to the gene pool; Fig. S1B). Pan-genome openness can be estimated using a power law regression model as suggested by Tettelin et al. using a Heaps law model (Tettelin et al., 2008). A pangenome is said to be open (infinitely growing with n) if the Heap's law $\alpha < 1$. The Heaps law model parameter α was estimated to be 0.44 for the *Pleurotus* genus, indicating an open pan-genome and vast diversity in its gene pool (Fig. 1D) (Snipen and Liland, 2015).

The pangenome analysis indicates that the genus is genetically diverse, implying that the number of gene families will continue to expand as more genomes are sequenced. This diversity means strains may vary greatly in their gastronomical characteristics, safety profiles,

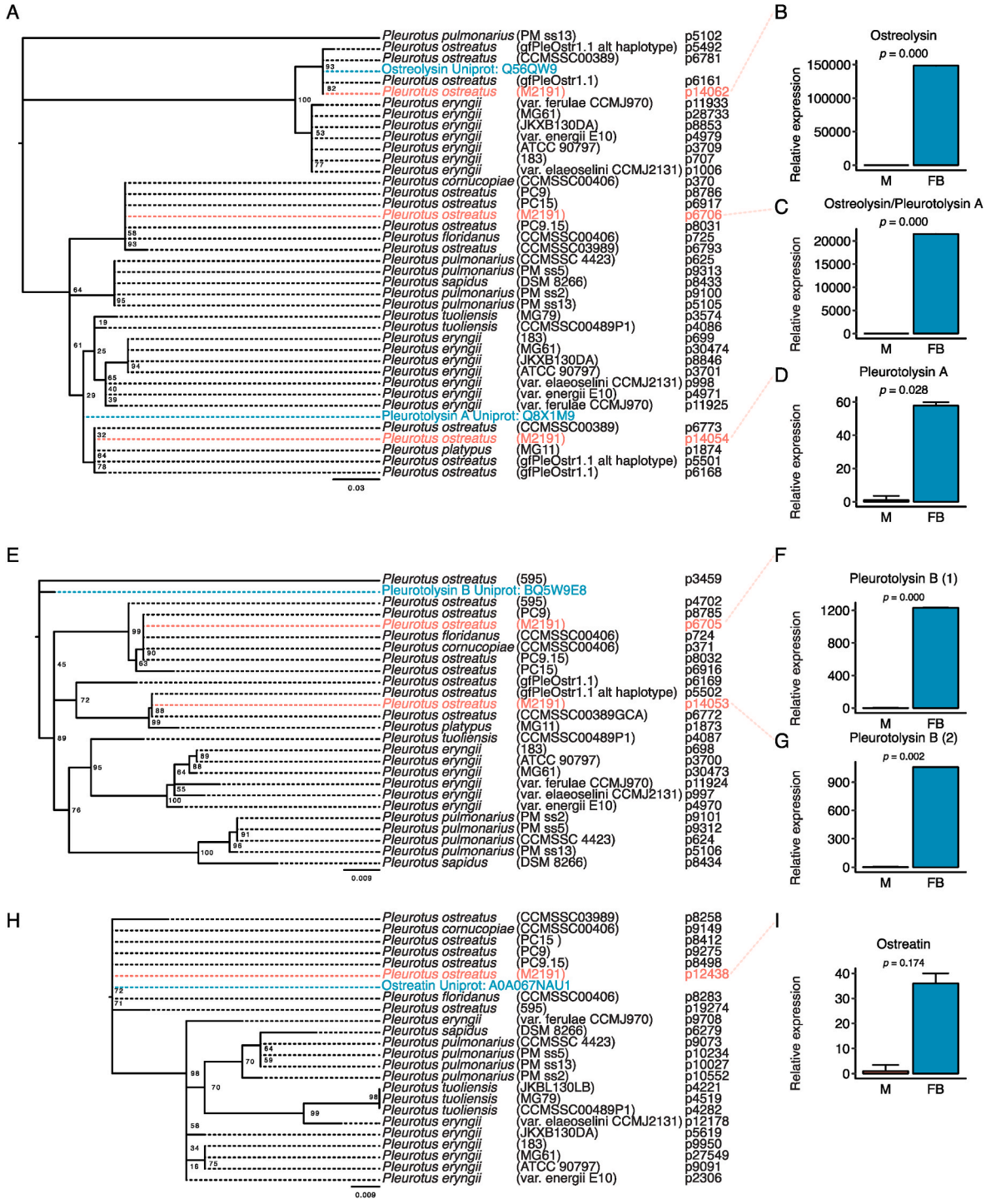


Fig. 2. Peptide toxin discovery in the *Pleurotus* genus

A. Phylogenetic relationship of related Ostreolysin (UniProt Q56QW9) and Pleurotolysin A (UniProt Q8X1M9) orthologs. **E.** Phylogenetic relationship of Pleurotolysin B (UniProt Q5W9E8) orthologs. **H.** Phylogenetic relationship of and Ostreatin (UniProt A0A067NAU1) orthologs. Known orthologs used as query are shown in blue and orthologs found in strain M2191 fun 3 are shown in red. Numbers indicate branch support. **B-D, F, G, I.** RT-qPCR analysis showing the gene expression of peptidic toxins in *P. ostreatus* M2191 mycelium (M) and fruiting body (FB). The mean fold change plus SD is displayed from $n = 3$ biological replicates, as well as the p-value for an unpaired two-sided *t*-test.

substrate use and potential applications. This diversity offers opportunities to develop a wide range of mycelium-based protein products with unique gastronomical properties. However, research on a single strain may not apply to the whole genus, and careful consideration will be required when applying findings to other strains. Furthermore, the presence of many uncharacterized genes within the genus indicates the need for further research to fully understand the functional diversity and potential of the genus.

3.2. Peptide toxin discovery in the pleurotus genus

The fruiting bodies of *P. ostreatus* are known to produce the peptide toxins Ostreatin, Ostreolysin and Pleurotolysin A/B (Landi et al., 2020; Vidic et al., 2005). Ostreatin is a ribotoxin-like protein that specifically targets ribosomes, compromising protein synthesis (Landi et al., 2020). Ostreolysin and Pleurotolysin are part of a cytolytic transmembrane pore-forming protein complex belonging to the aegerolysin protein family (Sakurai et al., 2004; Sepčić et al., 2004). These peptide-based toxins play a role in fungal defense against predatory nematodes or insects and in protecting against pathogenic microorganisms. Furthermore, their specific expression in peripheral parts of fruiting bodies and lamellae suggests a link between expression and fungal differentiation (Vidic et al., 2005).

As these peptides are also bioactive against humans (Butala et al., 2017; Tomita et al., 2004; Vrecl et al., 2015, Zužek et al., 2006), it is important to evaluate their presence in *P. ostreatus* to establish the safety of its use as a food. To this end, we analyzed the genomes of 38 *Pleurotus* strains for the presence of any orthologs of Ostreolysin, Pleurotolysin and Ostreatin. Despite the high overall genetic variability observed within the genus, the genes are conserved across *Pleurotus* species, suggesting an important function of the toxins in the ecological niche of the fungi. Three orthologs in total were identified in M2191 matching the related toxins Ostreolysin and Pleurotolysin A (shown Fig. 2A). Additionally, two genes were identified for Pleurotolysin B, and one gene for Ostreatin (Fig. 2E and H, respectively).

We next assessed gene expression levels of these six identified genes in the mycelium and fruiting bodies of M2191, using RT-qPCR. Expression levels of all toxin genes were substantially lower in the mycelium than in the fruiting bodies (ranging from 35 to 150,000 times lower; Fig. 1E), Since oyster mushrooms are widely consumed, these levels are considered safe for consumption, suggesting that oyster mushroom mycelium may be a safe food source. These data are also in line with reports of the absence of these toxins in vegetative mycelium (Berne et al., 2007; Vidic et al., 2005). Notably, Ostreolysin and Pleurotolysin exhibit biological activity solely in tandem, so the deficiency of either factor further would reduce risk. Moreover, it is generally recommended to avoid consumption of raw or undercooked fruiting bodies. Heating the peptide mycotoxins denatures them and renders them safe to eat. Industrial thermal processing may also denature heat-labile mycotoxins, possibly in combination with extrusion to simultaneously enhance textural properties (Berger et al., 2022). Overall, our analysis indicates that *P. ostreatus* mycelium expresses lower levels of known peptide toxins compared to the fruiting bodies.

3.3. The mycelium of P. ostreatus M2191 does not produce known mycotoxins in detectable amounts

Besides peptide toxins, fungi are known to produce a wide range of secondary metabolites. Some fungi produce harmful secondary metabolites known as mycotoxins and their production is strongly dependent on environmental conditions such as substrates. While *P. ostreatus* is not typically associated with the production of regulated mycotoxins, our investigation aimed to confirm the safety of strain M2191 grown on different substrates.

The biosynthetic genes for mycotoxins are often clustered together in the genome. We used antiSMASH to analyze the M2191 genome for biosynthetic gene clusters (BGCs) associated with the production of mycotoxins. We annotated 60% of the predicted proteome, identifying 75 predicted *loci* encoding biosynthetic gene clusters for secondary metabolites. Forty-three of the loci which contained domains related to Non-Ribosomal Peptide Synthases (NRPS), 2 had domains related to Polyketide Synthases (PKS), and 36 had domains related to terpene biosynthesis (Table 2). For comparative analyses, the same annotation scheme was applied to other genomes deposited in the GenBank as *Pleurotus* strains. This analysis suggested there are more BCG's in strain M2191 compared to the average of the *Pleurotus* genus (43.5) and compared to *Agaricus bisporus* and *Lentinula edodes* (19 and 32, respectively; Table 3 and Fig. S2).

Surprisingly, we detected two clusters that are associated with Ochratoxin A (OTA), a toxin typically produced by *Aspergillus* and *Penicillium* species, but our analysis suggests that M2191 is not likely to produce OTA. The OTA gene cluster is highly conserved and well described for ochatoxigenic species (Fig. 3A, top) (Wang et al., 2018). Comparison of the AntiSMASH results to the known Ochratoxin A gene cluster from *Aspergillus steynii* (Fig. 3A) suggests that the hits are based on a minimally annotated BCG (MIBiG BGC0002608). Upon further investigation of the region of interest, we observed that it contains only one tailoring enzyme, and none of the core genes of the known BGC. Additionally, the orientation and order of genes does not match, and the cluster nor the genome do not seem to contain otaC P450, an essential gene in the OTA gene cluster. In conclusion, it is not likely that strain M2191 can produce OTA.

To further analyze the production of mycotoxins we performed untargeted metabolomics on fruiting bodies and mycelium grown on two distinct substrates, as the mycotoxin profile of fungi is strongly dependent on growth substrate and conditions. We used a Mycotoxin-Fungal Secondary Metabolite MS/HRMS library to annotate metabolites (Klitgaard et al., 2014). Initially, we tested for the presence of OTA and other mycotoxins that are under regulation by the European Food Safety Authority (EFSA) (EU, 2023) or the U.S. Food and Drug Administration (FDA) (FDA, 2016). None of the regulated mycotoxins were detected in any of the tested conditions (Fig. 3B).

While mycotoxins are an important concern, fungi have also been associated with multiple potential health benefits (Rousta et al., 2023). Numerous compounds have been identified in mushroom or mycelium extracts that support gut health, blood pressure modulation, and antioxidant and anti-inflammatory activity (Golak-Siwulska et al., 2018). To annotate metabolites that have been linked to health-beneficial bioactivities in mycelium and fruiting bodies grown on different substrates, we used a Mycotoxin-Fungal Secondary Metabolite database containing 1592 fungal secondary metabolites (Fig. 3C) (Klitgaard et al., 2014). Many secondary metabolites annotated seem to be substrate-dependent, which was in line with our expectations. Interestingly, several metabolites were annotated preferentially in mycelium or in the fruiting bodies, regardless of the substrate. For example, grisofulvin, a molecule commonly used as an oral antifungal drug (Aris et al., 2022), was detected more often in the fruiting bodies than in the mycelium of strain M2191, potentially due to non-sterile conditions during fruiting body formation, whereas tryptophol, a tryptophan derivative, was more prevalent in the mycelium (Fig. 3C and Table S2).

We also annotated the purine nucleotide adenosine, as there are concerns about the high RNA content of fungal-derived foods, since RNA consumption is linked to elevated uric acid levels and gout (Jonas et al., 2001). Our data are not quantitative, but suggest that there are potential differences in the adenosine content between fruiting bodies and mycelium. These differences between mycelium and fruiting bodies highlights the need for further investigation into the variations in metabolites between fungal morphologies.

We also annotated pantothenic acid (vitamin B5), and ergosterol (provitamin D2), an essential micronutrient, in both mycelium and fruiting body. This is interesting since UV-B treatment of mushrooms has been shown to greatly increase vitamin D levels, up to the weekly

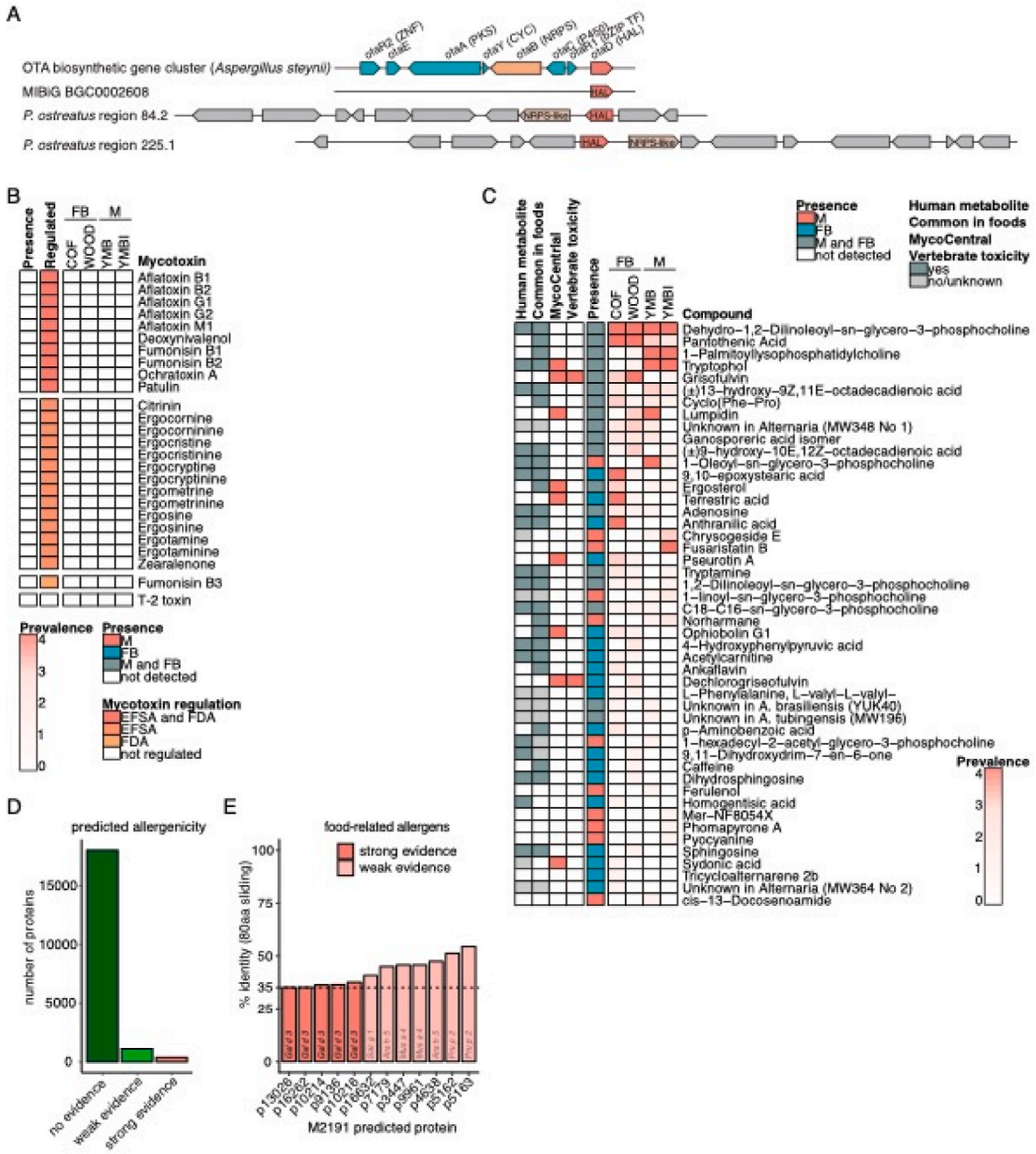


Fig. 3. The mycelium of *P. ostreatus* does not produce mycotoxins in detectable amounts.
A. Comparison of the AntiSMASH results (bottom) to the known OTA gene cluster from *Aspergillus steynii* (top) and the MIBiG BGC0002608 (OTA BGC hit from AntiSMASH). Shown is the schematic representation or the organisation of the well-conserved OTA cluster in *Aspergillus steynii*. Main features are the otaR2/ZNF, a zinc finger DNA binding protein; otaE, FAD-dependent oxidoreductase; otaA/PKS, polyketide synthase; otaY/CYC, PKS cyclase; otaB/NRPS, non-ribosomal peptide synthetase; otaC/P450, cytochrome P450 monooxygenase; otaR1/bZIP, basic leucine zipper transcription factor; otaD/HAL, halogenase. Bottom: biosynthetic gene cluster for Pleurotus ostreatus M2191 as predicted by antiSMASH. HA, Trp-halogenase; NRPS-like, a fragment of a non-ribosomal peptide synthetase gene. Genes with different predicted functions are colored, grey genes represent genes that are unknown or not predicted to be involved in the biosynthesis of OTA secondary metabolites. **B.** Heatmap of LC-MSMS results for regulated mycotoxins in the EU (EFSA) and the US (FDA). Fruiting bodies were cultivated on coffee (COF) and wood substrates; mycelia were cultivated on YMB and YMBI. **C.** Heatmap of other annotated compounds produced by *P. ostreatus.* The heatmap also indicates whether the compound is common in food or is a common human metabolite (as indicated in PubChem); whether it has published data on vertebrate toxicity; and whether it is included in the MycoCentral database for *predicted* toxicity. See accompanying Table S2 $N = 4$ biological replicates. **D.** Allergenicity of predicted *P. ostreatus* M2191 proteins using AllerCatPro 2.0 with an 80 aa sliding window. 2.0% of genes show strong evidence for allergenicity. **E.** Percentage identity of predicted M2191 proteins to the 12 food-related allergens. See accompanying Table 4.

demand for an adult per serving (Krings and Berger, 2014). If this could be done with the mycelium in the same way, it could lead to a new non-animal source of vitamin D, as has been shown on a small scale for *Pleurotus sapidus* (Ahlborn et al., 2019). Overall, the metabolomic analysis of strain M2191 indicates that the strain does not produce any known small molecule toxins, and revealed the presence of secondary metabolites that have been previously linked to health benefits.

3.4. Allergenic hazard of P. ostreatus

There is very little research on allergies to edible mushrooms, suggesting a low incidence of human mushroom allergies from ingestion (Berger et al., 2022; Finnigan et al., 2019). Nevertheless, the introduction of novel foods carries a risk of allergic reactions in individuals sensitive to specific proteins. We therefore set out to estimate the allergenic hazard of *P. ostreatus* mycelium as a novel food using AllerCatPro (Nguyen et al., 2022), a model developed for the characterization of the allergenic potential of proteins. Using an 80-aa sliding window, we tested all predicted proteins from strain M2191 for a sequence identity of >35% to known allergens according to the CODEX guidelines. This analysis led to the identification of 386 hits with high sequence homology or shared allergenicity epitopes, belonging to 92 unique allergens (Fig. 3D). The number of unique allergens was lower compared to other novel foods, such as *Fusarium* spp (used to produce the well-known mycoprotein Quorn). In this analysis, baker's yeast had 164 unique allergens, Fusarium spp. Had 316, and Candida albicans had 482 unique allergens (Abdelmoteleb et al., 2021).

The majority of predicted allergens identified elicit symptoms from human exposure by the airway, insect or skin contact. We therefore filtered our results for ingestion-related allergens (Table 4), leaving only genes with sequence homology to one unique allergen with a 'strong evidence' score, related to the egg white protein ovotransferrin (Gal d 3). However, considering that the identity score to Gal d 3 does not exceed 37.5% (Fig. 3E), it seems unlikely that *P. ostreatus* M2191 expresses this allergenicity epitope but further experiments on the allerginicity potential would be needed as confirmation. Of note, IgE-mediated food allergies are highly dependent on age, dose and frequency of exposure and therefore likely depends on the expression levels of the potential allergen, final protein levels, and frequency of consumption (Taylor, 2022). In addition, various studies indicate that IgE-mediated egg allergies typically resolve in childhood (Savage et al., 2016). Furthermore, the risk of an immune reaction further decreased by the notion that thermal treatment reduces the allergenic potential of Gal d3 (Urisu et al., 2015). Overall, these results indicate that our strain does not represent a significant risk of food allergy to the general population.

3.5. P. ostreatus M2191 mycelium as a sustainable protein source

Growth characteristics are an important factor in assessing mycelia for food production and biotechnology applications. Growth rates are highly dependent on strain, growth conditions, substrate and other environmental factors (Pilafidis et al., 2023; Zajul, 2017). We investigated the growth rates of strain M2191 mycelium in solid and submerged cultures, and compared them to the different phases of industrial mushroom cultivation (Dissasa, 2022; Melanouri et al., 2022). We found that mycelium growth in both cultures had a shorter timeline than fruiting body formation and even spawn running (full colonization of the substrate; Fig. 4A). This relatively fast growth suggests that mycelium cultivation could be an efficient way of accumulating mycoprotein as a sustainable food source.

The biological efficiency for oyster mushroom production is about 60% on average (Dissasa, 2022; Hoa et al., 2015; Muswati et al., 2021), yielding approximately 600 g of mushrooms per kilogram of dry substrate (total yield) in around 34 days (first harvest). In contrast, studies have shown that the biomass yield of *P. ostreatus* mycelium grown in

Table 4
Predicted allergenic proteins in *Pleurotus ostreatus* M2191
Proteins hits for food-related AllerCatPro 2.0 allergens, corresponding to Fig. 3A. Predicted allergens with weak evidence are shown in grey. The proteins with evidence for allergenicity all belong to the Gal d3 allergen from chicken eggs, and were all based on an identity score of >35 % using an 80 amino acid sliding window (win80lin>35%).

pred. Protein	length (aa)	UniProt (best hit)	allergen information					AllerCatPro 2.0 results			
			allergen	Allergome #	evidence (0–9)	allergen species and tissue	exposure	% ID (80 aa)	% ID (3D)	evidence	comment
peg.3447	305	M0TBU2	*Mus a 4*	8199	2	*Musa acuminata (Banana)*	Ingestion	45.8	25	weak	3Depi ≤ 93%
peg.5162	2376	B6CQU1	*Pru p 2*	5977	4	*Prunus persica (Peach)*	Ingestion	51.2	52.9	weak	3Depi ≤ 93%
peg.5163	256	B6CQU1	*Pru p 2*	5977	4	*Prunus persica (Peach)*	Ingestion	54.4	77.3	weak	3Depi ≤ 93%
peg.4638	368	L7QH52	*Ara h 5*	54	3	*Arachis hypogaea (Peanut)*	Ingestion	47.5	70.6	weak	3Depi ≤ 93%
peg.7179	126	L7QH52	*Ara h 5*	54	3	*Arachis hypogaea (Peanut)*	Ingestion	45	70.6	weak	3Depi ≤ 93%
peg.9961	305	M0TBU2	*Mus a 4*	8199	2	*Musa acuminata (Banana)*	Ingestion	45.8	25	weak	3Depi ≤ 93%
peg.9136	183	F1NL17	*Gal d 3*	361	4	*Gallus gallus (Chicken egg)*	Contact_Skin, Ingestion, Inhalation	36.4	–	strong	win80lin >35%
peg.16,632	1938	A0A2L1FDX2	*Sac g 1*	12,101	0	*Saccostrea glomerata (Sydney rock oyster)*	Ingestion	40.8	53.8	weak	Q-repeats and 3Depi ≤ 93%
peg.16,262	181	F1NL17	*Gal d 3*	361	4	*Gallus gallus (Chicken egg)*	Contact_Skin, Ingestion, Inhalation	35.1	–	strong	win80lin >35%
peg.13,026	181	F1NL17	*Gal d 3*	361	4	*Gallus gallus (Chicken egg)*	Contact_Skin, Ingestion, Inhalation	35.1	–	strong	win80lin >35%
peg.10,216	661	F1NL17	*Gal d 3*	361	4	*Gallus gallus (Chicken egg)*	Contact_Skin, Ingestion, Inhalation	37.5	–	strong	win80lin >35%
peg.10,214	247	F1NL17	*Gal d 3*	361	4	*Gallus gallus (Chicken egg)*	Contact_Skin, Ingestion, Inhalation	36.2	–	strong	win80lin >35%

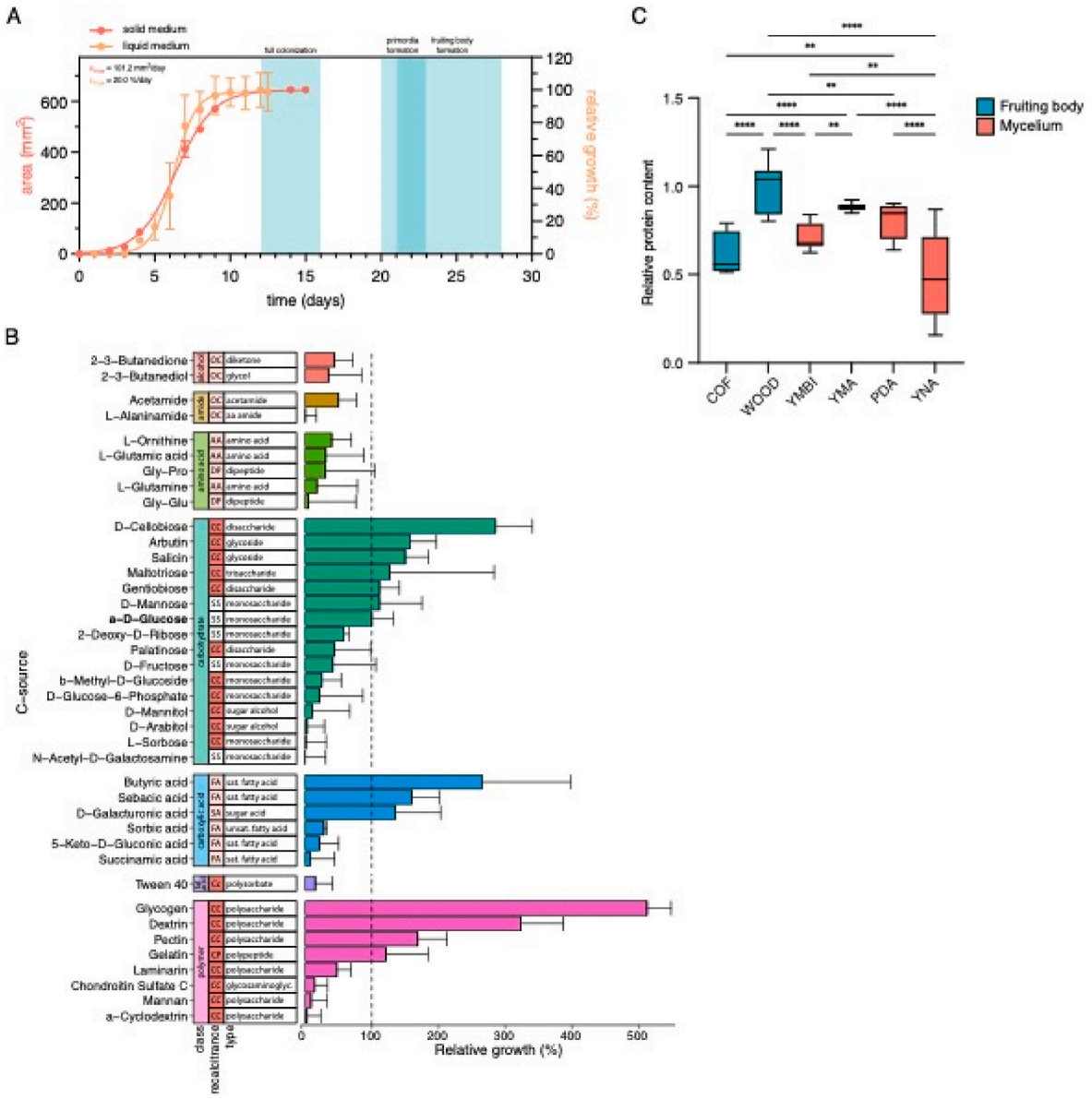


Fig. 4. *P. ostreatus* mycelium as a sustainable protein source

A. Growth curves for *P. ostreatus* M2191 on solid medium (YMA) and liquid medium (Biolog). The mycelial growth rate (mm^2/day) was obtained from the slope of the linear function considering the time interval from 4 to 8 days. The average growth rate values of 4 replicates were reported for each sample. For the liquid medium, relative growth was calculated using the OmniLog units normalized to 100% for n = 2 biological replicates. For reference, the three phases (full substrate colonization, pinhead formation and first fruiting body formation) which are important in the industrial cultivation of *P. ostreatus* are indicated. **B**. Relative growth of *P. ostreatus* M2191 on Biolog PM01 and PM02A plates with different carbon sources. Shown a growth rates relative to glucose (100%), using the average Y-value with a parametric background subtraction of the control wells and corrected for T0 to adjust for the starting value of the medium. The complexity (recalcitrance) of the carbon source as well as the classification of the compound is indicated. OC, organic compound; AA, amino acid; DP, dipeptide; CC, complex carbohydrate; SS, simple sugar; FA, fatty acid; SA, sugar acid; Cc, complex carbon; CP, complex protein polymer. **C.** Protein content of *P. ostreatus* M2191 mycelium and fruiting bodies on different substrates. Box and whiskers plot representing 25th to 75th percentiles and whiskers represent min to max values for n = 4 biological and 2 technical replicates. One-way ANOVA p-values: *) ≤0.05, **) ≤0,01, ***) ≤0.001 and ****) ≤0.0001. Protein content is calculated using a BSA calibration curve using the Amide II region (1605-1485 cm-1) of the FTIR spectrum, and corrected for the negative control (water).

fermentors can reach 33–39 g per liter per day, assuming a water content of 90% (Berger et al., 2022; Hadar and Cohen-Arazi, 1986). These findings suggest that while industrial mushroom cultivation can produce substantial yields, mycelium cultivation in bioreactors may offer even higher biomass production rates, highlighting the potential of mycelium as an efficient and sustainable source of mycoprotein for food applications.

Fungi, and especially xylophilic saprophytes like *P. ostreatus*, are extremely adaptable to their environment. They grow on many different substrates by rapidly adapting their protein expression and biochemistry. To test strain M2191's ability to use different carbon sources, we performed a Phenotype MicroArray (PM) using 190 different carbon sources. We identified 40 carbon sources used by M2191 in the presence of minimal nitrogen (Fig. 4B), including 20 complex carbohydrates, 5 simple sugars, and 15 amino acids, organic compounds or fatty acids.

Several of these carbon sources outperformed glucose as substrates for M2191 growth, including the carbohydrates cellobiose and maltotriose; the glucosides salicin and arbutin, the carboxylic acids butyric acid, sebacic acid and D−galacturonic acid; and the polymers glycogen,

dextrin and pectin (Fig. 4B). The ability of M2191 to grow on substrates like pectin and D−galacturonic acid, both major constituents of plant cell walls, suggests the presence of enzymes enabling the use of citrus or cassava peels (Kortei et al., 2014). Additionally, the growth of M2191 on laminarin and glutamic acid suggests that it could grow well on kelp or other brown seaweeds. Moreover, M2191 is able to grow on other complex carbon sources that require a high energy input such as mannan (found in hemicellulose), Tween80, Tween 40, α-cyclodextrin, and glycogen. Lignocellulosic substrates were not directly included in this array. These findings match those observed in earlier studies demonstrating successful submerged growth of *Pleurotus* species on rice and sunflower side streams, barley straw, wheat bran, and sesame oil press cake (Ahlborn et al., 2019; Pinela et al., 2020; Radhika et al., 2013; Vlasenko and Kuznetsova, 2020). These results suggests that M2191 has the capacity to metabolize complex and more recalcitrant carbon sources and agro-industrial waste streams.

Growth on a wide variety of different substrates is expected to result in wide variation in biomass makeup, including the concentration of protein, a desirable food component. Protein content in fruiting bodies of *Pleurotus* spp. Is known to range from 15 to 33% in dry matter (Ahlborn et al., 2019; Onuoha et al., 2021; Valenzuela-Cobos et al., 2020). Using Fourier-transform infrared spectroscopy (FTIR), we evaluated the protein content mycelium and fruiting bodies of strain M2191 grown on different substrates. We found that the protein content of *P. ostreatus* varied substantially depending on the substrate, suggesting that not only the morphological state, but also substrate defines protein

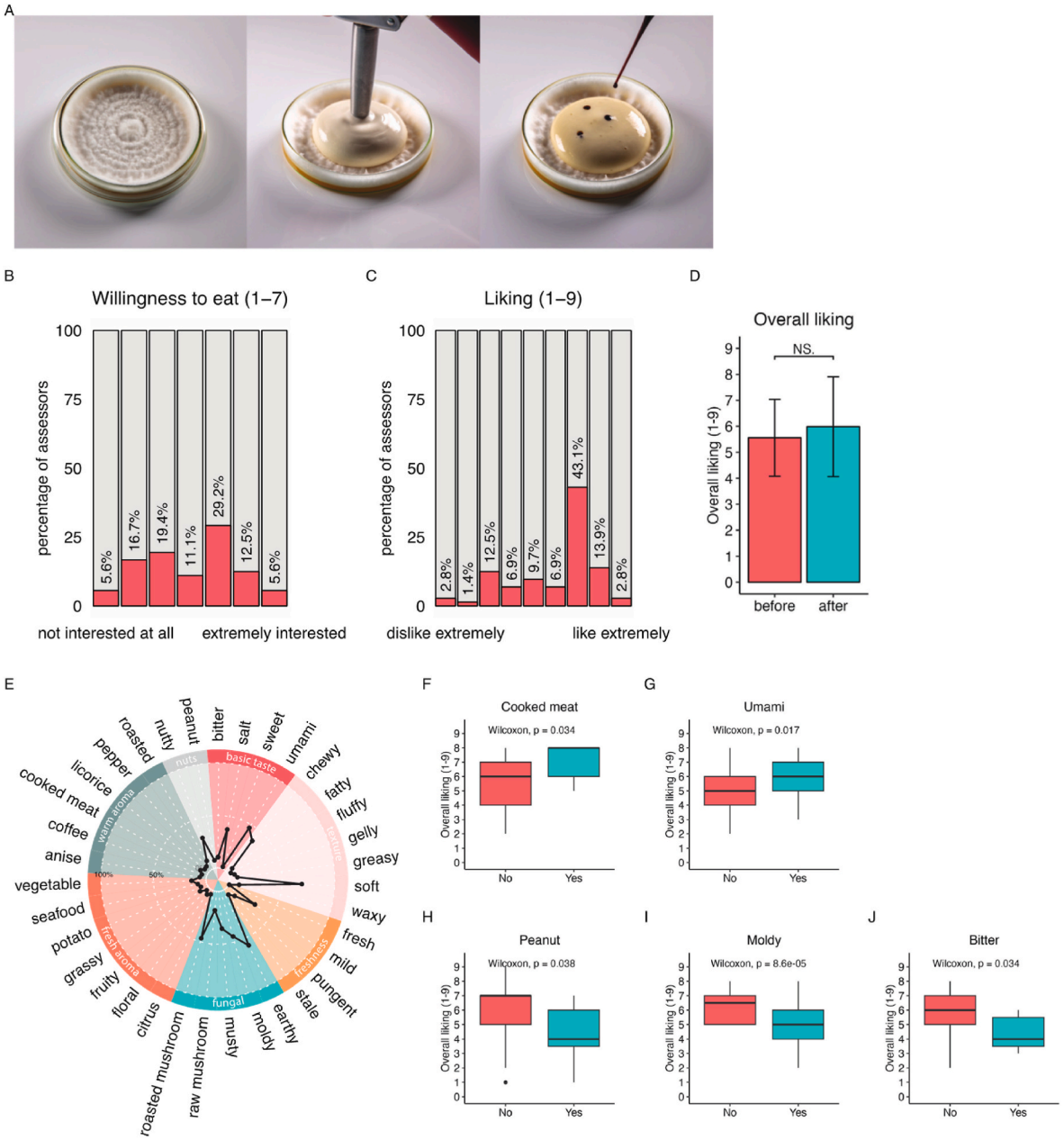


Fig. 5. Sensory analysis: consumer openness

A. "Mycelium" dish on YMBI, as developed and served in restaurant the Alchemist. For serving, a foam sauce of mushrooms was added on top and seasoned with dots of thickened apple balsamic vinegar. The basic version of the dish as depicted on the left was used for the sensory analysis. Photo by Søren Gammelmark. **B.** Willingness to eat and **C.** overall liking of the sensory analysis. **D.** Overall liking score (± SD) before and after tasting the sample. N.S.; non-significant in a students' t-test **E.** Spider plot of attributes attributed to the sample. **F-G.** Increased overall liking scores for associated specific attributes (yes). **H-J.** Decreased overall liking scores for associated specific attributes (yes).

content. Wood substrates supported the highest protein content in fruiting bodies, and fruiting bodies from coffee substrates contained about 40% less protein (Fig. 4C). Although the initial protein and nitrogen content in coffee is higher than in wood chips (Ballesteros et al., 2014), this result is not surprising, since wild *Pleutorus ostreatus* is a saprophytic species that decomposes hardwood trees. The preference for low nitrogen content is also supported in a study where the addition of spent coffee grounds to wheat straw led to a lower protein content in fruiting bodies of oyster mushroom (Alsanad et al., 2021). In mycelium, the complete media Yeast Malt Agar (YMA) and Potato Dextrose Agar (PDA) yielded the highest relative protein content in mycelium, and the minimal medium YNA the lowest with 47% less protein than YMA, consistent with previous observations (e.g. (Elkanah et al., 2022)). Previous studies also reported differences between mycelium and fruiting bodies of *P. ostreatus* in the composition of vitamins, monosaccharides, as well as in the concentration and structure of polysaccharides (Wang et al., 2017; Zajul, 2017). Strain, developmental stage, and the associated microbial community are all also known to affect protein content of mycelia (Berger et al., 2022).

3.6. Culinary application and sensory analysis of fungal mycelium suggest consumer openness

The primary obstacles for the consumption of meat alternatives are unfamiliarity and inferior sensory quality (Hartmann and Siegrist, 2017; Hoek et al., 2013). Therefore, positive sensory experiences play a crucial role in the acceptance of novel foods (Tan et al., 2016). For this reason, we set out to develop and serve a dish labeled "Mycelium" at the two-Michelin-star restaurant the Alchemist in Copenhagen, Denmark. The *P. ostreatus* M2191 mycelium was presented in the main dining room at the restaurant to showcase the growth of the mycelium, after which toppings were added (Fig. 5A).

To gain insights into consumer liking and sensory attributes, the bare mycelium, grown on edible YMBI (as presented in the left panel of Fig. 5A) was assessed by an untrained consumer panel ($n = 72$). Subjects were asked to rate their willingness to eat the sample before tasting, and its likeability before and after tasting. Most subjects rated the sample a 5 for willingness to eat (1–7 scale), and a 7 for overall liking (1–9 scale), indicating a general openness to the sample (Fig. 5B–C). Consumer liking is not significantly affected by the tasting of the sample, suggesting overall consumer acceptance (Fig. 5D).

The last part of the sensory analysis was carried out by a check-all-that-apply (CATA) assessment with 30 attributes chosen from literature (Misharina et al., 2009; Tagkouli et al., 2021). The CATA assessment shows a relatively high score for fungi-related attributes (Fig. 5E). Other descriptors include umami and salty, chewy, soft, mild, and nutty.

Next, we aimed at relating attributes to consumer liking. Interestingly, we identified two attributes with increased overall liking, namely 'cooked meat' and 'umami' (Fig. 5F–G). Conversely, 'peanut', 'moldy' and 'bitter' were attributes that are associated with a decreased overall liking (Fig. 5H–J). The results of our sensory analysis indicate that there is no food neophobia of mushroom mycelium among consumers, supported by a general willingness to eat it and overall liking. Nonetheless, consumers could become more receptive to tasting novel foods if they are visually attractive and presented as a familiar product, and the development of products in this direction may help to lower the initial barriers of consumption. As such, we provide some indications of attributes that can positively contribute to this ('cooked meat' and 'umami'), as well as attributes that should be avoided ('peanut', 'moldy' and 'bitter') as they lead to decreased appreciation of the product. Given that the sensory analysis was conducted on the mycelium in a neutral form, there is significant potential for improvement in this area, for example by improving the visual appearance to make it not look like a mold. Additionally, providing consumers with extra information on concerns and expectations could further success of the product (Tan et al., 2016). This sensory analysis indicates an overall liking and openness to this novel food, and provides insights into attributes that are associated with overall liking.

4. Discussion

Feeding the growing global population requires the exploration of alternative protein sources to meet the rising protein demand. Agriculture and food production contribute about a quarter of all global greenhouse gases, with around half of this generated by the production of livestock (Crippa et al., 2021). A recent study suggested an 80% reduction in global warming potential if all animal-derived products are replaced with novel foods, including microbial protein (Mazac et al., 2022). Microorganisms, including fungi, already play a crucial but underappreciated role in our agri-food system, but it is surprising that mycelial biomass is not produced more intensely on a fermenter scale and that microbial biomass is not widely consumed. Basidiomycota like *P. ostreatus* offer an attractive alternative to traditional protein sources with a smaller environmental footprint. These organisms are also known for their efficiency in resource use, their nutritional benefits, and the lack of ethical concerns (Thavamani et al., 2020). Mycelial growth, occurring in a shorter timeframe than fruiting body formation, presents an efficient approach for biomass production particularly beneficial for industrial-scale purposes. Besides a lower environmental impact (Rubio et al., 2020), fungal bioprocesses have the advantage of efficient and consistent production leading to a continuous supply of biomass. These processes can use waste streams and occur in controlled environments that reduce the risk of contamination with microorganisms, heavy metals, or agro-chemicals.

In this interdisciplinary study, we explored oyster mushroom mycelium as a culinary material by comparing the mycelium and fruiting bodies of *P. ostreatus* strain M2191. The findings suggest that this strain's mycelium has great potential as a novel food source. First, using a pan-genome analysis, we identified an open pangenome and high genetic variability in the Pleurotus genus, indicating that different strains may vary in gastronomical characteristics. Second, none of the known peptide toxins were expressed at high levels in M2191, and none of the regulated mycotoxins were found in this strain. Instead, several secondary metabolites with potential health benefits were annotated. We also report a relatively low allergenicity of the strain, although proteomics or other techniques are required for confirmation. Third, strain M2191 was characterized as a sustainable protein source with the capacity to metabolize complex and more recalcitrant carbon sources. Protein content is defined mostly by substrate, which is important from a nutritional perspective. Mycelium exhibited rapid growth compared to traditional mushrooms, which potentially allows for faster production cycles and higher yields. Finally, we developed a dish based on mycelium at the two-Michelin-star restaurant the Alchemist in Copenhagen, Denmark, to showcase its culinary potential. A sensory analysis of the mycelium conducted with a consumer panel, revealed an overall positive reception and openness to mycelium as a novel food. Additionally, the panel provided valuable insights into fungal attributes associated with both liking and disliking the mycelium. Based on these data, we propose that mycelium of edible filamentous fungi can be used as a more sustainable and delicious food source.

Edible fungi offer distinct advantages over conventional protein due to their long history of safe consumption, and their reputation for their nutritious, flavorful, and health-promoting traits. Using the mycelium of edible mushrooms, such as that of *P. ostreatus*, presents many benefits over using their fruiting bodies.

Mycelium produced under controlled conditions poses lower consumption risks from unwanted or toxic compounds, including mycotoxins and allergens, compared to fruiting bodies. This advantage extends especially to mycelium from edible basidiomycetes, in contrast to ascomycetes like *Fusarium* species (Quorn).

Our findings show that mycelium accumulates a high protein content similar to fruiting bodies. Depending on the literature source, cultivation

conditions, and strain, the total protein concentration of fruiting bodies for *Pleurotus* spp. Is up to 33% in dry matter (Ahlborn et al., 2019; Onuoha et al., 2021; Valenzuela-Cobos et al., 2020), and 31.1% in fruiting bodies of strain M2191 (Gebeyehu and Tesfaw, 2024), which approaches meats, fish and eggs (41–52%, 61–75% and 53% respectively) (Parodi et al., 2018). Additionally, mycoprotein is considered high-quality protein, containing a complete amino acid profile and with a Protein Digestibility-Corrected Amino Acid Score (PDCAAS) of 0.91 (Miller and Dwyer, 2001), similar to that of animal proteins like casein, egg white, and beef, and better than plant-based protein (Singh et al., 2008).

Exploration of the genetic diversity in the *Pleurotus* genus is crucial for developing strategies in strain selection, cultivation, and the creation of a diverse range of mycelium-based protein products with unique sensory and nutritional attributes. This can contribute to the overall goal of diversifying the alternative protein market and meeting the growing demand for sustainable protein sources. Conversely, research on a strain from this genus may not fully apply to other strains, which could have potential implications for strain selection, cultivation, safety, and the development of mycelium-based food products. Our findings highlight the need for further research to understand and harness the genetic diversity of oyster mushroom mycelium, such as using a comparative genomics study to identify genetic markers associated with desirable culinary traits. Doing so helps to develop strategies for strain selection, cultivation, and the development of mycelium-based food products.

Fungal mycelium has the potential of providing potentially health-promoting secondary metabolites and micronutrients. For example, prior studies have noted that *P. ostreatus* mycelium contains higher levels of the lipid-lowering compound lovastatin compared to the fruiting bodies (Krakowska et al., 2020), highlighting its potential as a nutraceutical source. Likewise, comparative research shows that mycelia produce lower levels of uric acid-inducing purines compared to their fruiting body counterparts (Huang et al., 2017), which is reflected in our data where adenosine, a purine compound, was found less frequently in mycelium compared to fruiting bodies. Furthermore, we annotated the essential micronutrient vitamin B5 (pantothenic acid, the precursor of coenzyme A) and ergosterol, a vitamin D2 precursor, in our strain M2191. UV-B treatment of mycelia would open a simple way to generate vitamin D2-enriched mycoprotein (Krings and Berger, 2014).

The health-promoting effects of fungal mycelium can be harnessed through biofortification and nutraceutical approaches. By manipulating cultivation conditions, one could supply additional nutrients during growth to address possible deficiencies in human nutrition. For example, one can supply metal salt solutions during growth to enhance the availability of essential ions like Fe, Zn and Li (Falandysz et al., 2022; Muszyńska et al., 2015). Moreover, submerged growth allows for sterile culturing conditions that enable medicinal applications. Our results also underscore the potential of *Pleurotus* sp. Mycelium as a valuable source of bioactive ingredients, with applications in the food- and pharmaceutical industries.

This idea can be extended to control flavor. Secondary metabolites like aroma compounds are strongly influenced by growth substrates and growth stage (Vlasenko and Kuznetsova, 2020). Volatiles with a typical mushroom odor, such as octan-3-one and octan-3-ol, are known to be present in aerial mycelia and in the fruiting body, but less so in submerged grown mycelia (Kabbaj et al., 2002). Thus, fermenter-cultured mycelia can be grown in chemically-defined, optimized media to enhance their sensory characteristics.

An important observation from our study is that nutritional composition, growth, and metabolite production are highly dependent on substrate. Fungi are extremely adaptable to their environment by rapidly adjusting their protein expression, allowing them to grow on many different substrates (Fig. 4B). Consequently, the makeup of their biomass varies more than any other food, depending on factors such as the growth substrate, strain, developmental stage, and the associated microbial community (Berger et al., 2022). This variety is also reflected in our measurements of protein content which suggest that the substrate is most defining for the nutritional composition. Likewise, there are also reported differences between substrates in the composition of monosaccharides, as well as in the concentration and structure of polysaccharides (Wang et al., 2017; Zajul, 2017). Nutritional composition can be improved and tailored to specific needs. The production of secondary metabolites, including mycotoxins, is also strongly connected to environmental conditions.

For this reason, we analyzed the mycelium and fruiting bodies of *P. ostreatus* on different growth substrates and found that they do not produce known regulated mycotoxins in detectable amounts. These findings, however, may be limited by our use of only one extraction method and the untargeted LC-MS/MS approach, which may fail to pick up uncharacterized mycotoxins or low concentrations of compounds. Also, our approach did not allow us to detect two non-regulated nematocidal compounds reported in *P. ostreatus*, linoleic acid hydroperoxide (Satou et al., 2008) and trans-2-decenedioic acid (Kwok et al., 1991). While their effects in humans have not been thoroughly researched, they could potentially be toxic. These compounds are not included in the library and are better detected using GCMS approaches. The variability observed in detection across biological replicates underscores the context and substrate-dependent nature of secondary metabolite production, as supported by Sarris et al. (2020). Studies on growth substrates demonstrate large intra-strain differences in growth rates and biochemical compositions, emphasizing the complexity inherent in fungal metabolic pathways (Leong et al., 2021; Omarini et al., 2014). The safety of mycelium grown on agro-industrial waste streams must be evaluated not only for the mycelium itself but also for the substrate used, as it may influence nutritional content and potential contaminants.

An additional complicating factor is that many bioactive compounds produced by filamentous fungi are not characterized at all, and it is unknown whether their effects on human health are neutral, positive or negative. It is also known that many secondary metabolites are specific to certain growth stages such as defense compounds that have been shown to be increased in fruiting bodies, presumably because they are threatened by predators (Kabbaj et al., 2002; Speroni et al., 2016; Vetchinkina et al., 2022). Therefore, while our study provides valuable insights into the absence of known regulated mycotoxins in *P. ostreatus*, further research is needed to fully understand the potential presence and toxicity of uncharacterized compounds.

The safety of foods, including the mycelium of edible fungi, is subject to legal regulations worldwide. Legal authorities will question whether the chemical safety of mycelia is different compared to their corresponding fruiting bodies that are generally considered safe for consumption. Our data emphasize the safety and nutritional value of mycelia from oyster mushrooms under controlled cultivation conditions, though further research is needed.

Our data also underscore the need for policy and regulation adaptation within the novel foods framework. With fermenter-grown mycelium, cultivation conditions are more controlled and defined than for industrial fruiting body cultivation. As such, the risk associated with consuming undesired or toxic constituents in mycelia of edible basidiomycetes appears lower than their fruiting body counterparts, and even lower than with sources such as Quorn, an ascomycete-based product. Additionally, commercially available mushrooms, even when properly produced, may contain contaminants like microorganisms, insect eggs, agro-chemicals, and heavy metals (Kokkoris et al., 2019). Mycelium contains lower concentrations of heavy metals compared to fruiting bodies (Muszyńska et al., 2015), suggesting a potential advantage for mycelium-based products in terms of heavy metal accumulation. Our results suggest that rather than assessing safety based on a history of safe use in the EU prior to May 15, 1997, safety should be assessed based on scientific evaluation of the composition and levels of undesirable compounds in edible filamentous fungi. This is also reflected in the EFSA's opinion on *Lentinula edodes* mycelium, which argues that although it is

treated as a novel food, toxicological studies are not required as risks are expected to be equal to the consumption of shiitake fruiting bodies (Turck et al., 2022). This approach would include the need for the implementation of new risk assessment methodologies by the European Food Safety Authority (EFSA). Strategies that can be considered by EFSA include evaluating the history of safe consumption of corresponding fruiting bodies, conducting thorough analyses of known toxins in relevant growth conditions, and performing allergenicity assessments to ensure consumer safety. Of course, this evaluation should also include an afety assessment of the potential substrate the mycelium is grown on or in.

The use of mycelium from edible basidiomycetes like oyster mushroom as a novel food source present a promising yet challenging landscape. Several barriers need to be addressed, including regulatory hurdles, concerns about genetic diversity, strain choice and safety, as well as optimizing growth speed for commercial viability. By conducting an interdisciplinary study that combines gastronomy with microbiology, genetics, analytical chemistry, and sensory analysis, this research aims to provide valuable insights into the potential of using edible filamentous fungi as a more sustainable food source. The findings from this study will contribute to the development of new food products that are not only delicious but also environmentally friendly and safe for human consumption.

CRediT authorship contribution statement

Loes van Dam: conceptualized the project and experimental design, methodology development, data generation, curation and visualization, wrote the original draft with input and feedback from all other authors. **Pablo Cruz-Morales:** methodology development, did the genomics, genome mining and phylogenomics. **Nabila Rodriguez Valerón:** did the sensory analysis and contributed to the analysis of the resulting data, DVP, had the original culinary idea and performed the culinary applications. **Ana Calheiros de Carvalho:** methodology development. **Diego Prado Vásquez:** conceptualized the project and experimental design. **Moritz Lübke:** methodology development. **Line Kloster Pedersen:** methodology development. **Rasmus Munk:** funded and supervised the culinary development. **Morten Otto Alexander Sommer:** developed the project idea, conceptualized the project and experimental design, funded the research activities. **Leonie Johanna Jahn:** developed the project idea, conceptualized the project and experimental design, did the project and collaboration management, funded the research activities, wrote the original draft with input and feedback from all other authors.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Leonie Johanna Jahn reports financial support was provided by The Good Food Institute. Leonie Johanna Jahn reports a relationship with MATR Foods that includes: employment and equity or stocks. Morten OA Sommer reports a relationship with MATR Foods that includes: equity or stocks. Morten OA Sommer reports a relationship with Novonesis AS that includes: board membership. Line Kloster Pedersen reports a relationship with Visibuilt that includes: equity or stocks. Line Kloster Pedersen reports a relationship with MATR Foods that includes: equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100866.

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