Universidade Estadual de Maringá
Centro de Ciências Agrárias
Programa de Pós-graduação em Ciência de Alimentos

RÚBIA CARVALHO GOMES CORRÊA

Aplicações Biotecnológicas e Nutracêuticas de *Pleurotus* spp.

Maringá – 2016

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Aplicações Biotecnológicas e Nutracêuticas de *Pleurotus* spp.

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Orientadora

Tese apresentada ao Programa de Pós-graduação em Ciência de Alimentos da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciência de Alimentos.

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BIOGRAFIA

Rúbia Carvalho Gomes Corrêa, filha de Susy Carvalho Gomes e Pedro Honório Corrêa, nasceu em 26 de junho de 1986 na cidade de Maringá - PR. Em 2004 ingressou no curso de Engenharia de Alimentos da Universidade Estadual de Maringá, graduando-se em 2009.

No ano de 2010, trabalhou como bolsista recém-formada no Projeto de Extensão Universitária “Suporte Técnico à Agroindústria Familiar de Conservas”, financiado pela Fundação Araucária do Paraná. No ano seguinte, concluiu o "Curso de Especialização em Biotecnologia Aplicada à Agroindústria", do Departamento de Biologia Celular e Genética – UEM.

No mês de fevereiro de 2012, concluiu o Mestrado no Programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, com a dissertação intitulada “Propriedades antioxidantes e reológicas de geleia de goiaba com adição de suco de uva concentrado”, sob orientação da Profa. Dra. Angélica Marquetotti Salcedo Vieira. O trabalho de dissertação gerou o artigo *Antioxidant and rheological properties of guava jam with added concentrated grape juice*, publicado no periódico Journal of the Science of Food and Agriculture (Fator de impacto JCR: 1.714).

Em março de 2012, iniciou o Doutorado no Programa de Pós-Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, sob a orientação da Profa. Dra. Rosane Marina Peralta.

Durante o ano de 2015 foi bolsista da CAPES no Programa de Doutorado Sanduíche no Exterior (PDSE), tendo realizado estágio no grupo Biochemcore da Escola Superior de Agrárias do Instituto Politécnico de Bragança (Portugal), sob a orientação da Profa. Dra. Isabel Cristina Fernandes Rodrigues Ferreira.

Em paralelo à sua tese de doutorado, colaborou com outros projetos que geraram os seguintes artigos publicados em periódicos e livros científicos:

1) Corrêa RCG, Rhoden SA, Mota TR, Azevedo JL, Pamphile JA, De Souza CGM, Polizeli MLTM, Bracht A, Peralta RM. Endophytic fungi: expanding the arsenal of

industrial enzyme producers. Journal of Industrial Microbiology and Biotechnology 2014, 41, 1467-1478; doi:10.1007/s10295-014-1496-2. Fator de impacto JCR: 2.439.

2) Castoldi R, Bracht A, De Morais GR, Baesso ML, Correa RCG, Peralta RA, Moreira RFPM, Polizeli MLTM, De Souza CGM, Peralta RM. Biological pretreatment of *Eucalyptus grandis* sawdust with white-rot fungi: Study of degradation patterns and saccharification kinetics. Chemical Engineering Journal 2014, 258, 240-246; doi:10.1016/j.cej.2014.07.090. Fator de impacto JCR: 4.321.

3) Souza AHP, Corrêa RCG, Barros L, Calhelha RC, Santos-Buelga C, Peralta RM, Bracht A, Matsushita M, Ferreira ICFR. Phytochemicals and bioactive properties of *Ilex paraguariensis*: An *in-vitro* comparative study between the whole plant, leaves and stems. Food Research International, 2015, 78, 286-294; doi:10.1016/j.foodres.2015.09.032. Fator de impacto JCR: 2.818.

4) Da Silva BP, Corrêa RCG, Kato CG, Seixas FAV, Bracht A, Peralta RM. *Enzymes from basidiomycetes — peculiar and efficient tools for biotechnology*, do livro *Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications* (ELSEVIER), editado por Goutam Brahmachari, Arnold Demain e Jose Adrio, com publicação prevista para 2016.

Tem experiência nas áreas de Ciência e Tecnologia de Alimentos, atuando principalmente nos seguintes temas: Bioquímica e Química de Alimentos e Produtos Naturais.

Dedico

À Deus

Aos meus amados pais Susy e Pedro

Aos meus irmãos Susana, Pedro e Raísa

// AGRADECIMENTOS

A Deus, pela sua abundante graça, infinita misericórdia e maravilhoso amor.

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À Dra. Lillian Barros, investigadora do grupo Biochemcore, pela essencial e brilhante colaboração nos experimentos realizados durante o estágio.

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APRESENTAÇÃO

Em consonância com as regras do Programa de Pós-graduação em Ciência de Alimentos, esta tese está apresentada na forma de artigos científicos.

ARTIGO 1

Corrêa RCG, Brugnari T, Bracht A, Peralta RM, Ferreira ICFR. Biotechnological, nutritional and therapeutic uses of *Pleurotus* spp. (Oyster mushroom) related with its chemical composition: A review on the past decade findings. Trends in Food Science & Technology 2016, 50, 103-117; doi: 10.1016/j.tifs.2016.01.012. Fator de impacto JCR: 4.651.

ARTIGO 2

Corrêa RCG, De Souza AHP, Calhelha RC, Barros L, Glamoclija J, Sokovic M, Peralta RM, Bracht A, Ferreira ICFR. Bioactive formulations prepared from fruiting bodies and submerged culture mycelia of the Brazilian edible mushroom *Pleurotus ostreatoroseus* Singer. Food & Function 2015, 6, 2155–2164; doi: 10.1039/c5fo00465a. Fator de impacto JCR: 2.791.

ARTIGO 3

Corrêa RCG, da Silva BP, Castoldi R, de Sá-Nakanishi AB, Peralta RA, Bracht A, Peralta RM. Spent mushroom substrate of *Pleurotus pulmonarius*: a source of easily hydrolyzable lignocellulose. Folia Microbiologica 2016, *in press*, doi: 10.1007/s12223-016-0457-8. Fator de impacto JCR: 1.000.

GENERAL ABSTRACT

INTRODUCTION AND AIMS – The genus *Pleurotus* is a cosmopolitan group of mushrooms with high nutritional value and therapeutic properties, besides a wide array of biotechnological and environmental applications. The most important *Pleurotus* species cultivated in large scale are *P. ostreatus* and *P. pulmonarius*. However, the detection of novel bioactives in less explored *Pleurotus* species, together with the determination of their chemical structures and mechanisms of action, are demands that science is seeking to accomplish. *P. ostreatoroseus* is an example of a Brazilian edible mushroom whose chemical characterization and bioactivity remain underexplored. Besides, another current and important issue is the fact that mushroom processing industries generate an unused surplus of pretreated lignocellulosic fibers called spent mushroom substrate (SMS). This pretreated lignocellulosic biomass can be used to produce ethanol, a promising alternative energy source for the environmentally unfriendly crude oil. The cost of ethanol production from lignocellulosic materials is relatively high based on current technologies, and the main challenges are the low yield and high cost of the hydrolysis process. Therefore, the principal aims of the three articles that comprise this thesis were: **1)** To provide a critical review on aspects related to chemical compounds isolated from the genus *Pleurotus* with possible biotechnological, nutritional and therapeutic uses. In the review, only reports published after 2005 were considered. **2)** To prepare bioactive formulations of the fruiting body and mycelium of *P. ostreatusroseus* (ethanolic extracts), to characterize them in terms of hydrophylic and lipophilic compounds, to evaluate their antioxidant, anti-inflammatory and antimicrobial potential, and to confirm their non-toxicity in a primary cell culture of porcine liver cells. **3)** To investigate the potential of SMS of *P. pulmonarius* in the generation of fermentable reducing sugars through saccharification of its polysaccharides by a mixture of commercial cellulase and β-glucosidase.

METHODS –1) The bioactive formulations of *P. ostreatoroseus* were obtained by extracting its mycelium (produced by submerged culture) and basidioma with an ethanolic (70:30 in water) solution. The soluble materials were firstly concentrated with a rotary vacuum evaporator at 40 °C, and freeze-dried. Free sugars were determined by high performance liquid chromatography (HPLC) coupled to a refraction index detector (RI detector). Organic acids were determined by ultra-fast liquid chromatography (UFLC) coupled with a photodiode array detector (PDA). Phenolic acids determination was performed using UFLC. Double online detection was carried out in the DAD using 280 nm as the preferred wavelength and in a mass spectrometer (MS) connected to a HPLC system via the DAD cell outlet. Tocopherols were determined by HPLC coupled to a fluorescence detector. Regarding the antioxidant activity assays, the sample concentrations ($mg \cdot mL^{-1}$) providing 50% antioxidant activity or 0.5 absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against the sample concentrations. Trolox was used as a positive control. For the anti-inflammatory activity assay, the bioactive formulations were subjected to further dilutions from 8 $mg \cdot mL^{-1}$ to 0.125 $mg \cdot mL^{-1}$ and the tests were performed in mouse macrophage-like cell line RAW264.7. The effect of the samples on the growth of porcine liver primary cells (PLP2), according to methodology established by the group, was evaluated by the sulforhodamine B (SRB) colorimetric assay. For the antibacterial activity assay, |Gram-

negative and Gram positive bacteria were used. The Gram-negative bacteria were: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterobacter cloacae*; the Gram-positive were: *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus*, and *Listeria monocytogenes*. The minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations were determined by the microdilution method. For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus niger*, *Trichoderma viride*, *Penicillium funiculosum*, *Penicillium ochrochloron*, and *Penicillium verrucosum* var. *cyclopium*. The minimal inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtitre plates. **2)** *P. pulmonarius* was cultivated on corn cob solid state cultures. During 30 days of vegetative growth, at intervals of 5 days, samples of SMS were collected from the cultivation bags and analyzed in order to investigate the transformations that occurred until fruiting bodies formation. Evaluations of the mycelial growth and consumption of reducing sugars and determinations of enzyme activities (laccase, catalase, Mn peroxidase and superoxide dismutase, SOD) were done in the crude water extracts obtained from each sample. The hydrogen peroxide content was quantified via the 2′–7′-dichlorofluorescein-diacetate (DCFH-DA) assay. The corn cob fibers obtained before and after fungal cultivation were subjected to enzymatic hydrolysis using cellulase from *Trichoderma reesei* ATCC and β-glucosidase from *Aspergillus niger*. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of product per minute. Also, the characterization of corn cob before and after *P. pulmonarius* cultivation was performed by scanning electron microscopy and Fourier transform infrared spectroscopy. The influence of the growth of *P. pulmonarius* on the substrate was analyzed in terms of the percent diminutions in intensity of the lignin (1427 and 1515 cm^{-1}) and carbohydrate peaks (1395, 1098 and 898 cm^{-1}).

MAIN RESULTS AND DISCUSSION – 1) The bioactive formulations contain at least five free sugars, four organic acids, four phenolic compounds and two tocopherols. The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates than the mycelium-based preparation, as well as higher anti-inflammatory and antimicrobial activities. The absence of hepatotoxicity was confirmed in porcine liver primary cells. These functional responses can be related to the levels of bioactive components including phenolic acids, organic acids and tocopherols. **2)** After mushroom harvesting, the SMS was hydrolyzed with commercial cellulase and β-glucosidase resulting in 300 g reducing sugars/kg dry SMS, being more than 60% glucose. Our data suggest that the SMS obtained from *P. pulmonarius* cultured on corn cob is a promising pretreated lignocellulose fiber for the obtainment of cellulosic ethanol.

CONCLUSIONS – 1) A series of compounds have already been precisely defined in *Pleurotus* spp., including polysaccharides, phenolics, terpenes and sterols. However, intensification of structure determination is highly desirable and demands considerable efforts. Further studies including clinical trials need to be carried out to ascertain the safety of these compounds as adequate alternatives to conventional drugs. Not less important is to extend the search for novel bioactives to less explored *Pleurotus* species. **2)** Overall, and to the best of our knowledge, this was the first report of anti-inflammatory properties of *P. ostreatoroseus* fruiting body and mycelium extracts, and from the results obtained, a clear anti-inflammatory and antimicrobial potential of the tested samples can

be inferred. Therefore, these formulations can be used to prepare dietary supplements for nutraceutical purposes. **3)** This study reveals the potential of the SMS from *P. pulmonarius* cultivation as a source of biologically pretreated lignocellulosic fibers, useful for the obtainment of cellulosic ethanol. Nowadays, the cost of production of cellulosic ethanol is relatively high. The use of SMS could be a strategy for improving the yield and reducing the costs of the process.

KEY-WORS: *Pleurotus ostreatoroseus*, bioactive formulations, anti-inflammatory potential, chemical composition, biological pretreatment, enzymes, hydrogen peroxide, *Pleurotus pulmonarius*, saccharification, spent mushroom substrate.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – O gênero *Pleurotus* é um grupo cosmopolita de cogumelos que apresenta alto valor nutritivo e propriedades terapêuticas, além de uma ampla gama de aplicações biotecnológicas e ambientais. As mais importantes espécies de Pleurotus cultivadas em grande escala são *P. ostreatus* e *P. pulmonarius*. No entanto, a detecção de novos bioativos em espécies Pleurotus menos exploradas, em conjunto com a determinação de suas estruturas químicas e mecanismos de ação, são demandas que a ciência está buscando realizar. *P. ostreatoroseus* é um exemplo de cogumelo comestível brasileiro cuja caracterização química e bioatividade permanecem pouco explorados. Além disso, outra atual e importante questão é o fato de que as indústrias de cogumelos geram um excesso de oferta não utilizada de fibras lignocelulósicas pré-tratadas, chamado de "*Spent Mushroom Substrate*" (SMS). Esta biomassa lignocelulósica pré-tratada pode ser utilizado para produzir etanol, uma promissora fonte de energia alternativa ao petróleo bruto que é prejudicial ao meio ambiente. O custo de produção de etanol a partir de materiais lignocelulósicos baseado em tecnologias atuais é relativamente elevado, e os principais desafios são o baixo rendimento e o alto custo do processo de hidrólise. Portanto, os principais objetivos dos três artigos que compõem esta tese foram: **1)** Produzir uma análise crítica sobre os aspectos relacionados aos compostos químicos isolados do gênero *Pleurotus* com possíveis aplicações biotecnológicas, nutricionais e terapêuticas. Na revisão, apenas artigos publicados após 2005 foram considerados. **2)** Preparar formulações bioativas formulações bioativas do corpo de frutificação e micélio de *P. ostreatusroseus* (extratos etanólicos), para caracterizá-los em termos de compostos hidrofílicos e lipofílicos, para avaliar suas propriedades antioxidantes, anti-inflamatória e potencial antimicrobiano, e para confirmar a sua não toxicidade numa cultura celular primária de células de fígado de porco. **3)** Investigar o potencial do SMS de *P. pulmonarius* na geração de açúcares redutores fermentáveis, através da sacarificação de seus polissacarídeos por uma mistura de celulase e *β*-glucosidase comerciais.

METODOLOGIA – 1) As formulações bioativas de *P. ostreatoroseus* foram obtidas através da extração de seu micélio (produzido por cultivo submerso) e basidioma com uma solução etanólica (70:30 em água). Os materiais solúveis foram primeiramente concentrados em um rotavapor a vácuo a 40 ° C, e liofilizados. Os açúcares livres foram determinados por cromatografia líquida de alta eficiência (HPLC) acoplada a um detector de índice de refracção (detector RI). Os ácidos orgânicos foram determinados por cromatografia líquida de ultra eficiência (UPLC) acoplada a um detector de arranjo de fotodiodos (PDA). A determinação dos ácidos fenólicos foi realizada utilizando UPLC. Uma linha de detecção dupla foi realizada no DAD com a adoção de 280 nm como o comprimento de onda preferido e num espectrômetro de massas (MS) ligado a um sistema de HPLC através da saída de células DAD. Os tocoferóis foram determinados por HPLC acoplado a um detector de fluorescência. Com relação aos ensaios de atividade antioxidante, as concentrações da amostra (mg.mL^{-1}) proporcionando 50% de atividade antioxidante ou 0,5 de absorbância (EC_{50}) foram calculados a partir dos gráficos de percentagens de atividade antioxidante (DPPH, β-caroteno / linoleato e TBARS) ou ensaios de absorbância a 690 nm (ensaio do azul ferricianeto / prussiano) contra as concentrações das amostras. Trolox foi usado como controle positivo. Para o ensaio de atividade anti-inflamatória, as formulações bioativas foram submetidas a diluições de 8 mg.mL^{-1} a 0,125 mg.mL^{-1} e os testes foram realizados com a linha celular RAW264.7 de

macrófagos de rato. O efeito das amostras no crescimento das células primárias de fígado de suíno (PLP2), segundo metodologia estabelecida pelo grupo, foi avaliada por ensaio colorimétrico com sulforrodamina B (SRB). Para o ensaio de atividade antibacteriana, foram utilizadas bactérias Gram-positivas e Gram-negativas. As bactérias Gram-negativas foram: *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterobacter cloacae*; as Gram-positivas foram: *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus flavus*, e *Listeria monocytogenes*. As concentrações inibitória mínima (MIC) e bactericida mínima (MBC) foram determinadas pelo método de microdiluição. Para os bioensaios antifúngicos, foram usados os seguintes microfungos: *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus niger*, *Trichoderma viride*, *Penicillium funiculosum*, *Penicillium ochrochloron*, e *Penicillium verrucosum* var. *cyclopium*. A determinação da concentração inibitória mínima (MIC) foi realizada por uma técnica de diluição em série usando placas de microtitulação de 96 poços. 2) *P. pulmonarius* foi cultivado em cultura em estado sólido com sabugo de milho. Durante 30 dias de crescimento vegetativo, em intervalos de 5 dias, amostras de SMS foram coletados a partir dos sacos de cultivo e analisados a fim de investigar as transformações ocorridas até a formação dos corpos de frutificação. As avaliações do crescimento micelial, consumo de açúcares redutores e determinações de atividades enzimáticas (lacase, catalase, peroxidase e Mn superóxido dismutase, SOD) foram realizadas em extratos brutos aquosos obtidos a partir de cada amostra. O teor de peróxido de hidrogénio foi quantificado através do ensaio de 2'-7'-diclorofluoresceína-diacetato (DCFH-DA). As fibras do sabugo de milho obtidas antes e após o cultivo do fungo foram sujeitas a hidrólise enzimática utilizando celulase de *Trichoderma reesei* ATCC e *β*-glucosidase de *Aspergillus niger*. Uma unidade de atividade enzimática foi definida como a quantidade de enzima capaz de libertar 1 nmol de produto por minuto. Além disso, a caracterização do sabugo de milho antes e depois do cultivo de *P. pulmonarius* foi realizada por microscopia eletrônica de varredura e espectroscopia de infravermelho com transformada de Fourier. A influência do crescimento de *P. pulmonarius* sobre o substrato foi analisada em termos das diminuições percentuais na intensidade dos picos de lignina (1427 e 1515 cm^{-1}) e de hidratos de carbono (1395, 1098 e 898 cm^{-1}).

PRINCIPAIS RESULTADOS E DISCUSSÃO – 1) As formulações bioativas contém pelo menos cinco açúcares livres, quatro ácidos orgânicos, quatro compostos fenólicos e dois tocoferóis**.** A formulação a base de basidioma revelou maior poder redutor, maior atividade de eliminação do DPPH, maior inibição da peroxidação em sistema *β*-caroteno e maior inibição da peroxidação lipídica em homogenatos de cérebro, quando comparada à preparação a base de micélio, bem como superiores atividades anti-inflamatória e antimicrobiana. A ausência de hepatotoxicidade foi confirmada em células primárias de fígado suíno. Essas respostas funcionais podem ser relacionadas com os níveis de componentes bioativos, incluindo os ácidos fenólicos, ácidos orgânicos e os tocoferóis. **2)** Após a colheita dos cogumelos, o SMS foi hidrolisado com celulase e *β*-glicosidase comerciais, resultando em 300 g de açúcares redutores / kg de SMS seco, sendo mais de 60% de glicose. Nossos dados sugerem que o SMS obtido a partir de *P. pulmonarius* cultivado em espiga de milho é uma fibra lignocelulósica pré-tratada promissora para a obtenção de etanol celulósico.

CONCLUSÕES – 1) Uma série de compostos já foram precisamento definidos em *Pleurotus* spp., incluindo polissacarídeos, fenólicos, terpenos e esteróides. No entanto, a intensificação da determinação da estrutura é altamente desejável e exige esforços

consideráveis. Outros estudos, incluindo ensaios clínicos, devem ser realizados para verificar a segurança destes compostos como alternativas adequadas às drogas convencionais. Não menos importante é estender a busca de novos bioativos para espécie Pleurotus menos explorados. **2)** Considerando todo o contexto, e até onde temos conhecimento, este foi o primeiro relato de propriedades anti-inflamatórias de extratos dos corpos de frutificação e micélio de *P. ostreaturoseus* e, a partir dos resultados obtidos, um claro potencial anti-inflamatório e antimicrobiano das amostras testadas pode ser inferido. Por isso, estas formulações podem ser usadas para o preparo de suplementos dietéticos para fins nutracêuticos. **3)** Este estudo revela o potencial do SMS obtido a partir do cultivo de *P. pulmonarius* como uma fonte de fibras lignocelulósicas pré-tratadas biologicamente, úteis para a obtenção de etanol celulósico. Hoje em dia, o custo de produção de etanol de celulose é relativamente alto. O uso de SMS pode ser uma estratégia para melhorar o rendimento e reduzir os custos do processo.

KEY-WORS: *Pleurotus ostreatoroseus*, formulações bioativas, potencial antiinflamatório, composição química, pré-tratamento biológico, enzimas, peróxido de hidrogênio, *Pleurotus pulmonarius*, sacarificação, *spent mushroom substrate*.

ARTIGO 1

Biotechnological, nutritional and therapeutic uses of *Pleurotus* spp. (Oyster mushroom) related with its chemical composition: A review on the past decade findings

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Abstract

Background: The particular characteristics of growth and development of mushrooms in nature result in the accumulation of a variety of secondary metabolites, several of them with biological activities. The genus *Pleurotus* is a cosmopolitan group of mushrooms with high nutritional value and therapeutic properties, besides a wide array of biotechnological and environmental applications.

Scope and approach: The present report aims to provide a critical review on aspects related to chemical compounds isolated from the genus *Pleurotus* with possible biotechnological, nutritional and therapeutic uses. Investigations on the genus have immensely accelerated during the last ten years, so that only reports published after 2005 have been considered.

Key findings and conclusions: The most important *Pleurotus* species cultivated in large scale are *P. ostreatus* and *P. pulmonarius.* However, more than 200 species have already been investigated to various degrees. Both basidiomata and mycelia of *Pleurotus* are a great renewable and easily accessible source of functional foods/nutraceuticals and pharmaceuticals with antioxidant, antimicrobial, anti-inflammatory, antitumor and immunomodulatory effects. A series of compounds have already been precisely defined including several polysaccharides, phenolics, terpenes and sterols. However, intensification of structure determination is highly desirable and demands considerable efforts. Further studies including clinical trials need to be carried out to ascertain the safety of these compounds as adequate alternatives to conventional drugs. Not less important is to extend the search for novel bioactives to less explored *Pleurotus* species.

Keywords: β-glucan, functional foods, mushrooms, natural products, submerged cultures.

1. Introduction

Mushrooms have been regarded as gourmet cuisine across the globe since antiquity for their unique taste and subtle flavor. They are considered as sources of important nutrients including dietary fiber, minerals, and vitamins, in particular, vitamin D (He, Perera & Hemar, 2012). More than 2,000 species of mushrooms exist in nature, but only around 25 are widely accepted as food and few are commercially cultivated (Valverde et al., 2015). Recently, they have become increasingly attractive as functional foods due to their potential beneficial effects on human health. Hence, food industry is especially interested in both cultivated and wild edible mushrooms. The most extensively cultivated mushroom worldwide is *Agaricus bisporus* (J. E. Lange) Emil J. Imbach., followed by *Lentinula edodes* (Berk.) Pegler and *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm. Mushrooms production is continuously increasing. The commercial production in 2012 hit 7,959,979 tonnes of mushrooms, with China accounting for most of the production (5,150,000 tonnes), while 1,869,091 tonnes were harvested in Europe (Grujic et al., 2015). Due to the increase in population and consumption, the world demand for mushrooms is projected to grow 15% a year (Kamarudzaman et al., 2015).

The genus *Pleurotus* (Fries) Kummer (Basidiomycota, Agaricales) was defined by Paul Kummer in 1871. It is a cosmopolitan group of mushrooms with high nutritional value and therapeutic properties, besides a wide array of biotechnological and environmental applications (Knop, Yarden & Hadar, 2015). Usually regarded as oyster mushrooms, these edible basidiomycetes are among the most popular worldwide, as much as they achieved the third position in the production of edible mushrooms, behind the species of the genus *Agaricus* and *Lentinula* (Fernandes et al., 2015). The most important *Pleurotus* species cultivated in large scale are *P. ostreatus* and *P. pulmonarius (*Fr.) Quél. (Bazanella et al.,

2013). *P. pulmonarius* has been often marketed by spawn manufacturers and cultivators under the incorrect name "*Pleurotus sajor-caju*". The real *Pleurotus sajor-caju* (Fr.) Singer is in fact a separate species of mushroom, which was returned to the genus *Lentinus* by Pegler (1975), and is correctly named *Lentinus sajor-caju* (Fr.) Fries (Buchanan, 1993).

Since the first report of hypotensive activity of the *Pleurotus* mushroom in a mouse model in 1986, many researchers have demonstrated their medicinal potentialities and classified them as 'mushroom nutraceuticals'; that were posteriorly added to the group of functional foods (Patel, Narain & Singh, 2012). In the last decade, the number of patents and scientific articles regarding the genus *Pleurotus* has exponentially increased, with an increment of more than 2-fold in the total of scientific research/review articles in the last 5 years (**Figure 1**).

Extensive research on cultivation techniques (Gregori, Svagelj & Pohleven, 2007; Carvalho, Sales-Campos & Andrade, 2010), chemical composition and nutritional profile (Reis et al., 2012; Atri et al., 2013; Maftoun et al., 2015) has been done in the last ten years, along with a comprehensive account of the biotechnological capabilities of the genus *Pleurotus* including enzyme production (Inácio et al., 2015a; Knop, Yarden & Hadar, 2015) (**Figure 2**). More recently, the scientific reports referring to *Pleurotus* species have also focused on novel approaches for taxonomic issues (Menolli Jr., Breternitz & Capelari, 2014; Maftoun et al. 2015), isolation and characterization of new functional compounds, besides the in depth-study of their medicinal properties (Khan & Tania, 2012; Patel, Narain & Singh, 2012; Yahaya, Rahman & Abdulhah, 2014).

In view of the above, this review aims to summarize and evaluate the past decade findings related to biotechnological, nutritional and therapeutic uses of *Pleurotus* sp. with special

attention to novelties regarding their chemical composition. This includes discussion of the main isolated and identified compounds or fractions and their corresponding bioactivities.

2. Biodiversity and Taxonomy

As of 2015 the Index Fungorum lists 202 species in the *Pleurotus* genus. **Table 1** presents the most studied species in the past ten years, the main areas of publications regarding these mushrooms, as well as their geographical distribution worldwide.

Species delimitation within the *Pleurotus* genus has been a complex issue for decades (Menolli Jr., Breternitz & Capelari, 2014). Years ago, Kitamoto et al. (2004) pointed out the main causes of the taxonomic controversy involving *Pleurotus* species: initial misidentification, absence of type specimens, instability of morphological characters due to environmental changes, limited reports on physiological characteristics, and the lack of mating compatibility studies. Fortunately, in recent years the adoption of biochemical and molecular approaches has brought some clarifications for species delimitation in the genus, mainly when combined with morphology and sexual compatibility (Menolli Jr., Breternitz & Capelari, 2014). The currently adopted methodologies of identification include isozyme electrophoresis, sequence analysis of ribosomal DNA, internal transcribed spacer region (ITS), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and mating compatibility testing (Maftoun et al., 2015). Recently, molecular approaches made it possible to confirm the taxonomic status of some important *Pleurotus* varieties

such as *P. eryngii*, *P. ferulae*, and *P. elaeoselini*. It also enabled reclassifications and the identification of new species (Zervakis et al., 2014).

In 2009, sequencing of the *P. ostreatus* genome was completed. Thanks to this accomplishment, a broad picture of the ligninolytic peroxidase gene family has been obtained. Besides, molecular techniques have also enabled progresses such as targeted gene replacement, RNAi-based gene silencing, and overexpression of genes in *P. ostreatus*. By this way, the recent information of the genomics of *P. ostreatus* secondary metabolism will allow an upgrade in the production of these compounds (Knop, Yarden & Hada, 2015).

As a more affordable option to expensive molecular techniques, the diffuse reflectance infrared Fourier transform (DRIFT) has also been used for studying the molecular composition and for identifying biological samples. It consists in a fast, reagent-free, noninvasive and highly specific approach (Movasaghi et al., 2008). Zervakis et al. (2012) used the DRIFT spectroscopy to exam 16 taxa of the genus *Pleurotus*, concluding that it was a fast, reliable, and cost-efficient methodology for the classification of pure cultures from closely related mushroom species.

3. Nutritional Aspects

Pleurotus spp are famous for owning all three properties expected from a food — nutrition, taste, and physiological functions —being thus appreciated for both their sensory characteristics and outstanding nutritional profile. The terpenes, lactones, amino acids, and carbohydrates of their composition determine a range of precious aromas and flavor characteristics to their fruiting body and mycelial biomass (Smiderle et al., 2012).

P. ostreatus, the most popular species of the genus, is commonly used in the preparation of soups, in stir-fry recipes with soy sauce or eaten stuffed. *P. eryngii* (DC.) Quél., another species with gastronomic prestige, is considered ideal for vegetarian dishes (consumed fresh), being served sautéed, grilled, braised, stewed, or boiled (Reis et al., 2012).

Concerning the amount of crude protein, mushrooms are ranked below animal meats, but well above most other foods, including milk, which is an animal product. Not to mention the fact that mushroom proteins contain all nine essential amino acids required by humans, enabling their use as a substitute for meat diet (Kakon, Karim & Sah, 2012). However, their nutritional supremacy in relation to the vegetarian diet is also virtue of their chitin rich cell wall that acts as a source of dietary fiber, along with their vitamin content (including thiamine, riboflavin, ascorbic acid, ergosterine, and niacin), considerable contents of micro and macro-elements as phosphorus and iron, carbohydrates and very low fat tenor (Maftoun et al., 2015).

Fresh fruiting bodies of *Pleurotus* spp contain 85–90% moisture (Khan & Tania, 2012), and the moisture percentage depends on the mushroom species besides other parameters related to harvest, growth, culinary and storage conditions (Reis et al., 2012). Atri et al. (2012) investigated the nutritional composition of *P. floridanus* Singer, *P. pulmonarius, P. sapidus* Quél., *P. cystidiosus* O. K. Mill. and *P. sajor-caju* (Fr.) Sing and reported, on dry weight basis, contents of carbohydrates of 85.86–88.38%, proteins 0.98–2.17%, crude fat 0.62–0.84%, crude fibers 2.76–3.12% and ash 1.03–2.20%. In turn, Khan & Tania (2012) found some diverse values in their review study on the nutritional value of *P. ostreatus, P. sajor-caju, P. florida* (Mont.) Singer, *P. cystidiosus, P. geesteranus* Singer, *P. eryngii, P. tuber-regium* (Fr) singer and *P. flabellatus* (Berk. & Br.) Sacco. They found, on dry

weight basis, contents of carbohydrates ranging from 36 to 60%, proteins from 11 to 42% and lipids from 0.2 to 8%.

According to Khan & Tania (2012) the carbohydrates in *Pleurotus* spp. are mainly in the form of polysaccharides or glycoproteins. The most abundant polysaccharides are chitin, α- and β-glucans, and other hemicelluloses (e.g., mannans, xylans, and galactans). The glucans present various types of glycosidic linkages, such as branched (1→3), (1→6)-β-glucans and linear (1→3)-α-glucans. The contents of these polysaccharides in the fruiting bodies range from 36 to 60 g/100 g dry weight. Total dietary fiber (mainly chitin) in *Pleurotus* mushrooms ranges from 10 to 31 g per 100 g dry weight, glucans being also components of soluble or insoluble dietary fibers.

Reis et al. (2012), in an inter-species comparative study on the most widely cultivated and appreciated mushrooms, found that *P. ostreatus* and *P. eryngii* had higher levels of monounsaturated fatty acids compared to *Agaricus bisporus*, *Lentinula edodes* and *Flammulina velutipes* (Curtis) Singer. Atri et al. (2013) reported that, among the fatty acids, the monounsaturated are present in a higher proportion (37.17–68.29%) than the saturated ones (26.07–47.77%) in *Pleurotus* spp. Maftoun et al. (2015), in their broad compilation data of the nutritional composition of *Pleurotus* mushrooms, reported that oleic acid (C18:1) was the major monounsaturated fatty acid while linoleic acid (C18:2n–6c) was the major polyunsaturated fatty acid in *P. ostreatus*. They also found that the most common monounsaturated fatty acid present in *P. sajor caju*, *P. cystidiosus*, *P. pulmonarius*, *P. floridanus* and *P. sapidus* was oleic acid. Among the saturated fatty acids (20.2%), the main contributers were palmitic acid (C16:0; 11.2%), followed by pentadecanoic acid (C15:0; 2.55%) and stearic acid (C18:0; 2.53%). Among the

polyunsaturated fatty acids (69.1%), linoleic acid (68.1%) was the most common and abundant.

Atri et al (2012) detected three main sugars including sucrose (0.338–2.011 %), glucose (0.553–0.791%) and xylose (0.01%) when analyzing *Pleurotus* spp. They also found ascorbic acid content ranging from 0.46 to 0.49 mg/100 g, total phenolics ranging from 6.76 to 16.92 mg of gallic acid equivalents/100 g, β–carotene ranging from 0.134 to 0.221 μg/100 g and lycopene from 0.055 to 0 .075 μg/100 g.

For detailed information about essential amino acids, fatty acids, minerals, vitamins, soluble sugars and volatile compounds profiles of the most studied *Pleurotus* species, the recent review of Maftoun et al. (2015) might be consulted.

4. Cultivation and Post-Harvest of *Pleurotus* spp.

4.1. Mushroom Production

The production of mushrooms with better flavor, appearance, texture, nutritional qualities, and medicinal properties at a sustainable cost constitutes a challenge for both industry and independent farmers, since many important operations are involved in this biotechnological process (Sanchéz, 2004). **Table 2** summarizes the main cultivation techniques, postharvest treatments and industrial applications of *Pleurotus* spp. during the last decade.

Numerous articles have reported the viability of producing the *Pleurotus* spp. basidiome using a wide range of byproducts as substrates, e.g., elephant grass, coast-cross, cotton

waste textile, rice straw, by-products of corn production, sawdust, husk of coffee, wheat straw, crushed sugarcane and stalks (banana tree, pea, peanut). Further, several kinds of materials were applied as supplementation, with high biological efficiency being obtained with wheat bran and rice bran (Carvalho, Sales-Campos & Andrade, 2010). However, in the past years, a wide range of alternative, sustainable and green substrates were used for *Pleurotus* mushrooms production, such as casing material in a compost mixture (Mishra et al. 2013), handmade paper and cardboard industrial wastes (Kulshreshtha et al. 2013), agro-residues combined with biogas digester residues (Chanaky, Malayil & Vijayalakshmi, 2015) and blank/printed paper (Fernandes et al., 2015).

Several studies on the role of the culture medium on mushrooms growth yield and nutritional quality have been done, but Ryu et al. (2014), in an innovative study, have investigated media combinations and components responsible for producing fruiting bodies with a long shelf life. They developed a cultivation medium for extending the shelf life and improving yield of *P. eryngii* mushrooms, increasing the viability of the export procedures. This medium contained 4.5% of crude protein and 15% of nitrogen free extracts.

The cultivation of *Pleurotus* spp. at high temperatures has been studied for a number of mushroom producers and scientists. Considering that some *Pleurotus* species are unable to develop mushrooms at temperatures of more than 28ºC, the most important step in the cultivation of these mushrooms under high-temperature conditions (usual condition in tropical countries) is the cold stimulation of the mature mycelium (Chen, 2007). Yingyue et al. (2014) evaluated the effect of cold in the production of *P. pulmonarius* mushrooms. They found that time and the interaction of temperature *versus* time of the cold stimulation

treatment were the two major factors influencing density of pinheads, yield per bag and number of mushrooms per bag. Meanwhile, temperature was the major factor influencing the yield per bag and stability. The best performance was recorded following a 12 h cold stimulation at 5ºC, suggesting that an appropriate cold stimulation may enhance the performance of the primordial initiation and yield of *P. pulmonarius* cultivation during the summer season.

Dulay, Ray & Hou (2015) investigated the optimal liquid culture conditions for producing *P. cystidiosus* with reference to the nutritional and physical growth factors, as well as with respect to lipid composition. They reported Sabouraud dextrose broth (SDB) as the most suitable culture medium, with maximum mycelial biomass favorably produced in SDB at pH 7.6 when incubated at 28°C. Agitation did not improve mycelial growth of mushrooms.

In the production of *Pleurotus* mushrooms, every ton of mushroom produced generates about five tons of dry spent residual material. This spent mushroom substrate (SMS) has been under-exploited in the past decades, sometimes being used for land filling and crop production only. However, as the correct disposal of SMS is one of the main environmental issues for the mushroom industry, new alternatives for the biotechnological application of this abundant by-product have been explored. Newly, the *Pleurotus* SMS was identified as a low-cost biosorbent for heavy metals removal, and as an effective degradation agent of organochlorine pesticides (Juárez et al., 2011; Kamarudzaman et al., 2015).

4.2. Submerged cultivation

Ten years ago about 80% to 85% of all edible-medicinal mushroom products were derived from the fruiting bodies and only 15% proceeded from mycelia extracts (Lindequist, Niedermeyer & Julich, 2005). However, the process of producing fruiting bodies or basidiomata is effortful and time-consuming, as it demands large volumes of substrate, space, and qualified labor, factors that hinder research in the laboratory. Cultivations that are performed in the vegetative phase are much more interesting and functional for research considering that they can be kept in the laboratory, performed on a small and medium scale, and important parameters such as temperature, humidity, pH and aeration can be easily controlled (Inácio et al, 2015a). Thus, submerged cultivation is a promising and still under-explored alternative for the extraction of bioactive molecules in short time, which also allows the mycelia storage for a long period without genetic alterations, beneficiating the conservation of biodiversity (Zilly et al., 2011). However, for using the mycelial biomasses, it is necessary to prove that they are similar to fruiting bodies (Soares et al., 2013). Submerged fermentation is also proper for enzyme production and waste bioconversion. With respect to submerged liquid fermentation with *Pleurotus* spp., recent studies reported the use of potato dextrose broth, amino acids, liquor maiz, reducing sugars (mainly glucose and xylose), casein hydrolyzate, soybean cake, yeast extract and peptone as the main carbon and nitrogen sources. The culture conditions reported refer to temperatures of 25-30 °C and culture pH of 4-6, in addition to the use of static culture or agitation ranging from 100 to 160 rpm (Arango & Nieto, 2013). Most recent publications aimed substrate optimization for maximal production of hydrolytic and oxidative ligninolytic extracellular enzymes.

As members of the white-rot fungi (WRF), *Pleurotus* spp. present the ability to grow on a variety of lignocellulosic biomass substrates and degrade both natural and anthropogenic aromatic compounds. This occurs by virtue of the presence of non-specific oxidative enzymatic systems, which consist mainly in laccases, manganese peroxidases (MnPs) and versatile peroxidases (VPs) (Hofrichter et al., 2010), besides the newly explored dye decolorizing peroxidases (DyPs) and heme-thiolate peroxidases (HTPs). A lot of information has been accumulated in the past decade concerning the biochemistry, structure and function of the *Pleurotus* ligninolytic peroxidases (Knop, Yarden & Hadar, 2015).

Recently, the possibility of extending the liquid culture technology for the production of mycelia to the mushroom spawn industry has been studied. Generally the edible mushroom cultivation industry utilizes grain spawn for this purpose. However, it is already known that preparation of grain spawn requires a longer growth period and poses higher risk of contamination compared to liquid spawn (Confortin et al., 2008). Abdulla et al. (2013) investigated the alternative of producing liquid spawn of *P. pulmonarius* by submerged fermentation in a 2-L stirred-tank bioreactor under controlled conditions and assessed its ability to colonise rubber wood sawdust substrate for sporophore production. The ideal liquid spawn cultivation medium contained 20 g L^{-1} of brown sugar, 4 g L^{-1} of rice bran, 4 g L^{-1} of malt extract, and 4 g L^{-1} of yeast extract (BRMY) with an initial pH of 5.5 and was incubated at 28 $^{\circ}$C with agitation speed of 250 rpm and oxygen partial pressure of 30–40%. The maximal dry biomass production of 11.72 ± 5.26 g L^{-1} was observed after 3 days of fermentation. The authors concluded that liquid spawn has the ability to colonise sterile rubber wood-sawdust as fruiting substrates in a shortened time

and to produce a higher yield of sporophores in comparison with the regularly used grain spawn.

4.3. Post Harvested Treatment

The commercial value of mushrooms falls due to quality loss during postharvest storage because the storage conditions are quite different from the growing conditions. This provokes changes in the physiological and molecular mechanisms that lead to deterioration (Li et al., 2013). In the past years, diverse post-harvest treatments have been investigated in an effort to discover new alternatives for extending the mushroom shelf life: cold storage (Dama et al., 2010), modified atmosphere packaging (MAP) (Guillaume et al., 2010), gamma and electron beam irradiation (Xiong et al., 2009; Fernandes et al., 2012), and coating (Jiang, Feng, & Li, 2012) treatments.

Li et al. (2013) investigated the high carbon dioxide and low oxygen treatment on the sensory characteristics, MDA (malondialdehyde) content, O_2^- production rate, and enzyme activities of SOD (superoxide dismutase), POD (peroxidase), CAT (catalase), and CCO (cytochrome C oxidase) in *P. eryngii*. They reported that 2% O_2 + 30% CO_2 treatment could maintain sensory characteristics of the mushroom and significantly prolong its shelf life.

In turn, Zhang et al. (2015) investigated the activity and molecular mechanisms of serine proteinase (Spr) during storage of *P. eryngii*. The activity of Spr in 2% O_2 + 30% CO_2-treated mushrooms was notably lower than in the controls. The spatio-temporal expression of PeSpr1 in the ambient air and 2% O_2 + 30% CO_2 storages correlated with the Spr

activity. Thus, the authors concluded that PeSpr1 plays an important role in post harvested *P. eryngii*, information that is valuable for post harvest investigation.

Newly, Huang, Lin & Tsai (2015) studied the effect of ultraviolet-B (UV-B) light irradiation on the vitamin D2 content of edible fruiting bodies and mycelia of *P. eryngii*, *P. citrinopileatus* Singer, *P. ferulae* Lanzi., *P. ostreatus* and *P. salmoneostramineus* L. Vass., and their antioxidant properties. The vitamin D2 content of irradiated fruiting bodies significantly increased from 0–3.93 to 15.06–208.65 mg/g, Vitamin D2 content in irradiated mycelia of *P. citrinopileatus*, *P. ostreatus* and *P. salmoneostramineus* mushrooms increased from 0.28–5.93 to 66.03– 81.71 mg/g, respectively. The three irradiated mycelium polysaccharide contents decreased from 1.3% to 24.6%. Despite the fact that UV-B irradiation affects the content of ergothioneine, flavonoids and total phenols, the irradiated samples still contained a sufficient amount of these antioxidant components.

5. Isolated Compounds and Bioactivity

Demand is growing in the food industry for new functional ingredients or bioactive compounds from natural sources, as they are widely applied in the formulation of functional foods. This has promoted, especially in the past years, an increasing interest in extracting ingredients from foods such as mushrooms and in developing functional foods (Li & Shah, 2015).

Numerous bioactive compounds, namely polysaccharides, peptides, glycoproteins, phenolics, lipids and hydrolytic and oxidative enzymes have been extracted from crude extracts, mycelia, and basidioma of *Pleurotus* spp. for investigation purposes. Two of the

most interesting bioactive compounds produced by *Pleurotus* mushrooms are the immune stimulant polysaccharides and the natural statins. The latter are hypocholesterolemic and with higher activity than the synthetic ones due to their milder side effects (Inácio et al., 2015a). Patel, Naraian & Singh (2012) published a comprehensive account on the medicinal properties of extracts of both fruiting bodies and mycelium of *Pleurotus* mushrooms. Their list includes antihypercholesterolemic, antihypertensive, antidiabetic, antiobesity, antiaging, antimicrobial, and antioxidant activities in addition to a hepatoprotective action. Also, different types of extracts from *Pleurotus* mushrooms have been reported as potential anticancer agents in several tumor cell lines, acting through distinct mechanisms. Clear clinical evidence of anticancer activities of *Pleurotus* mushrooms, however, is still not available (Khan & Tania et al. 2012).

Table 3 presents a compilation of the last decade most important studies on *Pleurotus* spp. mushroom fractions and isolated/identified compounds, including high (e.g. polysaccharides, small peptides and proteins) and low (e.g. terpenes, fatty acid esters and polyphenols) molecular weight compounds, as well as their corresponding bioactivities.

5.1. High molecular weight compounds

Several polysaccharides have been isolated from the fruiting bodies, cultured mycelia and culture filtrates of various mushrooms (Ren, Pereira & Hemar, 2012). Those polysaccharides showing antitumor activity have a great variety of chemical composition and structure, with different types of glycosidic linkages, such as (1,3)-, (1,6)- β-glucans and (1,3)- α-glucans (**Figure 3A**). In what refers to the polysaccharides from *Pleurotus* sp, Facchini et al. (2014) reported the efficacy of a polysaccharide fraction obtained from the

mycelium of *P. ostreatus* with NH_4-oxalate at 100 °C in inhibiting the development of Ehrlich Tumor (ET) and Sarcoma 180 (S-180). Also, Llauradó et al. (2015) examined the *in vitro* antimicrobial and the complement/macrophage stimulating effects of a hot water extract from the mycelium of *Pleurotus* sp. The extract activated the microbial autolytic system of both bacterial and yeast strains, acting also on innate immunity by triggering the complement system via the alternative pathway (and presumably the classical pathway of adaptive immunity) and by enhancing macrophage functions. The authors suggested the polysaccharide-rich extract as an accessible and innovative antimicrobial food ingredient. Recently, Li & Shah (2015) added a polysaccharide extracted from *P. eryngii* (PEPS) to milk before its fermentation process. They found that the addition of PEPS had a considerable effect on bacterial growth, texture properties, and proteolytic and ACE inhibitory activities of fermented milk during refrigerated storage and proposed its use as a nutritional and functional additive.

Zhang et al. (2014) performed the purification and measured the antioxidant activities of intracellular zinc polysaccharides (IZPS) from *P. cornucopiae*. IZPS subfractions, separated chromatographically by means of a DEAE-52 cellulose anion-exchange column, showed higher antioxidant activities *in vitro* and *in vivo*. Rhamnose and glucose were the predominant monosaccharides in IZPS, which also contains in its structure, xylose, mannose, and galactose. In turn, Li & Shah (2014) performed the sulphonation of polysaccharides from *P. eryngii* and reported that their antioxidant and antibacterial properties had improved due to the sulphonation process.

The following bioactive polysaccharides and proteins isolated/identified from *Pleurotus* spp., along the past years should be remarked: (1) a (1→3),(1→6)-linked β-glucan isolated from *P. pulmonarius* with proven anti-inflammatory and analgesic properties in a rodent

model (Smiderle et al., 2008); (2) nebrodeolysin, a novel hemolytic protein isolated from *P. nebrodensis* that induced apoptosis in L929 and HeLa cells and presents anti-HIV-1 activity in CEM cell culture (Ly et al, 2009); (3) a RNase purified from *P. djamor* that inhibits the proliferation of hepatoma cells and breast cancer cells (Wu et al., 2010).

More recently, Silveira et al. (2014) made the first report of a linear (1 → 3)-*β*-D-glucan isolated from the fruiting bodies of *P. sajor-caju.* Its bioactivities were evaluated *in vitro*, using THP-1 macrophages, and *in vivo*, through formalin and peritonitis tests in mice. The glucan was able to inhibit the inflammatory phase of nociception induced by formalin at a low dose and reduced the number of total leukocytes and myeloperoxidase (MPO) levels induced by LPS. Shortly after, the same group purified and identified a mannogalactan constituted by a main chain of (1→6)-linked *α*-D-Gal*p* and 3-O-methyl-*α*-D-Gal*p* units (Silveira et al., 2015), that was obtained from *P. sajor-caju.* The mannogalactan was able to reduce the nociception, *in vivo*, in the writhing and in formalin tests and reduced the carrageenan-induced paw edema, indicating that it could be an effective antinociceptive and anti-inflammatory agent.

Freshly, Cui et al. (2015) purified and characterized a novel *P. nebrodensis* polysaccharide (PN-S), and evaluated its immune-stimulating activity in RAW264.7 macrophages. They observed that PN-S effectively modulated phagocytosis levels and enhanced the immune activity of murine peritoneal macrophages. Yan, Jing & Wang (2015) also isolated and characterized a polysaccharide (PNPA) from the fruiting bodies of *P. nebrodensis*, and further examined its effect on myocardial ischemia–reperfusion (I/R) injury in rats and elucidated the underlying mechanism. The PNPA had a backbone consisting of 1,3-linked-D-glucopyranosyl and 1,3,6-linked-D-galactopyranosyl residues, which was terminated with a 1-linked-D-mannopyranosyl terminal at *O*-3 position of a 1,3,6-linked-D-

galactopyranosyl unit along the main chain in the ratio of 4:1:1. According to the authors, PNPA exerted a protective effect on myocardial I/R injury in part through improving endogenous antioxidants and suppressing myocardial cell apoptosis. Finally, Ren et al. (2015) performed the isolation of polysaccharides (PAP) from the fruiting bodies of *P. abalonus*, and evaluated their antiproliferative activity in human colorectal carcinoma LoVo cells. HPLC analysis showed that PAP consisted of D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-glucose and D-galactose, and that their corresponding mole percentages were 3.4%, 1.1%, 1.9%, 1.4%, 87.9% and 4.4%, respectively. The authors reported that the PAP has anti-proliferative effects against human colorectal carcinoma LoVo cells via cell cycle arrest at the S-phase and cellular apoptosis, and that the generation of ROS is a critical mediator in PAP-induced LoVo cancer cell growth inhibition.

Hagiwara et al. (2005) demonstrated the antihypertensive effect of a D-mannitol isolated from *P. cornucopiae*, through the inhibition of an angiotensin I converting enzyme (ACE), in spontaneously hypertensive rats (SHR) by oral administration. Later, in another *in-vivo* study, Jang et al. (2011) described the characterization of a new angiotensin I-converting enzyme (ACE) inhibitory peptide isolated from the basidioma of *P. cornucopiae*. In their study, two types of the purified ACE inhibitors were obtained and posteriorly analyzed. Amino acid sequences of the two purified oligopeptides were found to be RLPSEFDLSAFLRA and RLSGQTIEVTSEYLFRH. The water extracts of the *P. cornucopiae* fruiting body showed antihypertensive effect on spontaneously hypertensive rats at the dose of 600 mg/kg. Yahayaa, Rahmana & Abdullah (2014), in a recent review, reported the therapeutic potential of mushrooms in preventing and ameliorating

hypertension, and listed the mostly noted *Pleurotus* species having antihypertensive effects: *P. ostreatus*, *P. cornucopiae*, *P. nebrodensis*, and *P. cystidiosus.*

5.2. Low molecular weight compounds

Menikpurage et al. (2009) investigated the activity of chemical components in *P. cystidiosus* against *Colletotrichum gloeosporioides*, with the purpose of developing a novel method to control anthracnose. The antifungal activity was investigated by fractionating the mushroom with acetone (A), dichloromethane (D), and hexane (H). After antifungal assay and normal phase chromatography, the fraction with the highest inhibitory activity was separated using the Chromatotron and a single compound (A2-3-13) was isolated. Using NMR spectroscopy they found it was 3β, 5α, 6β-trihydroxyergosta-7,22-diene (**Figure 3B**), an oxidized ergosterol active against *C. gloeosporioides*.

Later, Suseem & Saral (2013) performed a complete analysis of the essential fatty acid esters of *Pleurotus eous* (Berk.) Sacc. and investigated its antibacterial activity. A petroleum ether extract of the *P. oeus* fruiting bodies was analysed by CG-MS and 5 compounds were identified: cyclopentanetridecanoic acid, methyl ester; tartronic acid, (*p*-ethoxyphenyl), diethyl ester; 7, 10-Octadecadenoic acid, methyl ester; Heptadecanoic acid, 16-methyl, methyl ester and 9-Octadecenoic acid [*Z*]-, 2-hydroxyl-1-[hydroxymethyl] ethyl ester. Among several crude extracts tested, only the petroleum ether extract showed strong antibacterial activity by inhibiting the growth of both Gram positive and Gram negative bacterial isolates. The authors suggested that *P. eous* could be

added as an extra nutrient to food products as it constitutes a new potential source of natural antibacterial agents.

Wang et al. (2013) reported the isolation, identification, and bioactivity of monoterpenoids and sesquiterpenoids isolated from the mycelia of *P. cornucopiae* (**Figure 4**). In their work, four new monoterpenoids (1−4) and one new sesquiterpenoid (6) were obtained from the solid culture of *P. cornucopiae* fermented on rice. Compound 1 presented an unusual spiro [benzofuran-3,2′-oxiran] skeleton. Compounds 1−5, 7, and 8 showed moderate inhibitory activity against nitric oxide production in lipopolysaccaride-activated macrophages. Compounds 6 and 7 exhibited slight cytotoxicity against HeLa and HepG2 cancer cells. Compounds 1−8 were isolated for the first time from *P. cornucopiae*, what advances the understanding of the secondary metabolism of this fungus.

A few years ago, Lee et al. (2007) investigated the antioxidant properties of ethanolic, cold and hot water extracts prepared from *P. citrinopileatus* fruiting bodies, mycelia and fermentation filtrate. They found that all the extracts had antioxidant properties. However, three extracts from the fruiting bodies were more effective than those of the mycelia and filtrate. Ethanolic extracts were more effective as antioxidants, except for the hydroxyl radicals scavenging ability. The contents of total phenols were higher in three extracts from the fruiting bodies (8.62–12.38 mg/g). In addition, the contents of total phenols were moderately to highly (0.425–0.948 mg/g) associated with antioxidant properties.

In a recent study of our group, we investigated and compared the hydrophilic and lipophilic compounds as well as the antioxidant, anti-inflammatory and antimicrobial activities of formulations (ethanol extracts) prepared with fruiting bodies and submerged culture mycelia of *P. ostreatoroseus* Singer (Corrêa et al., 2015). We found that the bioactive formulations contain at least five free sugars, four organic acids, four phenolic

compounds and two tocopherols. The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates than the mycelium-based preparation, as well as higher anti-inflammatory and antimicrobial activities. In addition, the absence of hepatotoxicity was confirmed in porcine liver primary cells. We concluded that these functional responses are related to the levels of bioactive components including phenolic acids, organic acids and tocopherols.

6. Concluding Remarks

In the last years, several research groups described pharmacological effects from both fruiting bodies and mycelia extracts of *Pleurotus* spp. The present review proposes that not only *Pleurotus* basidiomata but also their mycelia should being explored as a great renewable and easily accessible resource for developing functional foods/nutraceuticals and even pharmaceutical agents with antioxidant, antimicrobial, anti-inflammatory, antitumor and immunomodulatory effects. Unfortunately, precise identifications of specific molecules involved in the bioactivity of mushroom extracts are not very abundant. This is clearly an area still demanding considerable efforts. Chemically defined molecules isolated from *Pleurotus* spp may represent an exciting advance for their characterization as functional foods and as source of new innovative drugs. Further studies including clinical trials need to be carried out to ascertain the safety of these compounds as adequate alternatives to conventional drugs. The detection of novel bioactives in less explored *Pleurotus* species, together with the determination of their chemical structures and mechanisms of action, are demands that science might seek to accomplish in the near future.

Conflict of interest

The authors declare no competing financial interest.

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Table 1. Main focuses of the last ten year publications and geographical distribution of most known *Pleurotus* spp*..

Species	Main areas of publications in the last decade	Geographical distribution	References
P. calyptratus	Industrial dyes decolourization, taxonomy	Central and Eastern Europe, Asia	Maftoun et al. (2015), Zervakis et al. (2012), Eichlerová, Homolka & Nerud (2006)
P. cornucopiae	Isolation and characterization of bioactive compounds, bioactive properties	Europe, Asia	Zhang et al. (2014), Wang et al. (2013), Jang et al. (2011), Hagiwara et al. (2005)
P. cystidiosus	Isolation and characterization of bioactive compounds, aroma extracts	Europe, Asia, North America, South America	Maftoun et al. (2015), Usami et al. (2014), Menikpurage et al. (2009)
P. dryinus	Enzyme production	Europe, Asia, North America, North Africa	Maftoun et al. (2015), Yoon et al. (2014), Elisashvili et al. (2008)
P. eryngii	Polysaccharides with bioactive properties, new technologies to improve production/extend mushroom shelf-life, antioxidant potential	Europe, Asia, Africa	Ryu et al. (2015), Li & Shah (2015), Li & Shah (2014), Mishra et al. (2013)

P. opuntiae	Taxonomy, basic investigation and consumption (mainly in Mexico)	Mediterranean Europe, America, Africa, Asia	Maftoun et al. (2015), Fuentes (2014), Mora & Martinez-Carrera (2007)
P. ostreatus	Isolation and characterization of bioactive compounds, bioactive properties, enzyme production, biotransformation, nanoparticles (most studied *Pleurotus* species)	Widespread around the world	Maftoun et al. (2015), El-Batal et al. (2015), Facchini et al. (2014), Purmono et al. (2013)
P. djamor	Bioactive properties, applications, enzyme production, nanoparticles	Indonesia, Malaysia, Japan, Mexico	Ramam et al. (2015), Velioglu &Urek (2015), Wu et al. (2010)
P. pulmonarius	Isolation and characterization of bioactive compounds, bioactivities, enzyme production, biotransformation, spent mushroom substrate	Widespread around the world	Inácio et al. (2015b), Silveira et al. (2015), Juaréz et al. (2011), Smiderle et al. (2008)
P. nebrodensis	Polysaccharides with bioactive properties, new technologies to improve production/extend mushroom shelf-life	China, Southern Europe, Central Asia	Yan, Jing & Wang (2015), Cui et al. (2015), Lv et al. (2009), Xiong et al. (2009)
P. citrinopileatus	Compounds with bioactive properties, cultivation techniques, antioxidant potential	Asia, Southern United States, Mexico	Huang, Lin & Tsai (2015), Kulshreshtha (2013), Liu et al. (2012), Lee et al. (2007)

*All species are saprotrophic and edible.

Table 2. Cultivation techniques, postharvest handling and main industrial applications of *Pleurotus* spp. in the last decade.

Species	Cultivation or special postharvest techniques	Application	Novelty, main contribution	Ref.
P. calyptratus	Static or shaken submerged culture with N-rich and N-limited Kirk media	Decolourization of Orange G and Remazol Brilliant Blue R	Orange G decolorization in *P. calyptratus* was caused mainly by laccase, while RBBR decolorization was effected by manganese peroxidase (MnP).	Eichlerova´ et al. (2005)
P. dryinus	Submerged cultures with mandarin peels and tree leaves	Production of lignocellulolytic enzymes	A simple and inexpensive medium containing only mandarin peels and yeast extract as sole carbon and nitrogen sources was developed. This medium allowed simultaneous production of high levels of both hydrolases and oxidative enzymes by *P. dryinus*. By adding Mn^{2+} to the medium it was possible to control the ratio between laccase and MnP.	Elisashvili et al. (2006)
P. calyptratus	Static cultivation with N-limited Kirk medium or malt extract medium	Decolourization of industrial dyes and enzymes production	*P. calyptratus* was able to decolorize efficiently several synthetic dyes, especially Orange G and RBBR. A more rapid Orange G decolorization in Kirk medium was detected, while RBBR was decolorized to a higher extent in Malt extract medium. The strain produced a relatively high amount of Lac, MnP and also aryl-alcohol oxidase.	Eichlerova´, Homolka & Nerud (2006)

Pleurotus spp.	Not available	Characterization of non-volatile components	Four *Pleurotus* species, including *P. djamor*, *P. ferulae*, *P nebrodensis* and *P. sapidus* were studied. Glutamic acid, aspartic acid, leucine and arginine were the major amino acids in these four species. Their palatable amino acid contents were high in *P. ferulae* , moderate *in P. nebrodensis* and *P. sapidus*, and low in *P. djamor* (15.8 mg/g). The four *Pleurotus* species studied were distinctly different in non-volatile components.	Guo, Lin & Lin (2007)
P. ostreatus and *P. cornucopiae* var. *'citrino-pileatus'*	Solid state cultivation (SSC) in cottonseed hulls	Environmental manipulation of fatty acid (FA) profiles in *Pleurotus* mushrooms	Variations in the growth temperature influenced the FA profiles in both tested mushrooms. Lowering the growth temperature below 17 °C provided an expected increase in FA unsaturation in polar and non-polar lipids of *P. ostreatus*. Therefore, it may be possible to manipulate environmentally lipid unsaturation in *Pleurotus* spp. through modified growth temperature.	Pedneault et al. (2007)
Pleurotus spp.	Submerged and solid-state fermentation in several lignocellulosic wastes	Production of lignocellulolytic enzymes	The study pointed out that the nature of lignocellulosic material and the method of fungi cultivation are factors determining the expression of lignocellulolytic potential of fungi as well as the ratio of individual enzymes in enzyme complexes. SSF of tree leaves is favorable for laccase and MnP secretion by the majority of the *Pleurotus* strains, whereas SF provides better production of hydrolytic enzymes.	Elisashvili et al. (2008)
P. nebrodensis	Postharvest irradiation with ^{60}Co	γ-irradiation as a strategy for extending mushrooms shelf life	An irradiation dose of 1.2 kGy significantly delayed the onset of fruiting body softening, splitting and browning compared with non-irradiated controls and test samples subjected to lower or higher irradiation doses. It also had a positive effect on other indicators of mushroom tissue senescence, resulting in smaller decreases in soluble protein levels and more protracted	Xiong et al. (2009)

			increases in proteinase activity.	
P. pulmonarius	SSC in pangola grass	Spent Mushroom Substrate (SMS) used in treatment of chlorothalonil containing wastewater	Freshly obtained SMS extract was able to reduce 100% of the initial concentration of chlorothalonil (2 mg/l) after 45 min of reaction. Storage time had a negative effect on the stability of the enzymatic activity. Cooling and freezing the SMS extract also had a negative effect on chlorothalonil degradation.	Juárez et al. (2011)
P. citrinopileatus	SSC in handmade paper and cardboard industrial wastes	A sustainable and green proposal for mushroom cultivation	*P. citrinopileatus* was cultivated on a sludge of handmade paper and cardboard industrial waste. Protein content, carbohydrate content and fat content of all carpophores were found to significantly decrease over control. Besides, carpophores were found to possess frameshift mutagens from the sludge. However, the use of a combination of sludge and wheat straw not only increased the biological efficiency but also provided less mutagenic carpophores.	Kulshreshtha et al. (2013)
P. eryngii	SSC in casing materials	A sustainable and green proposal for mushroom cultivation	Enhanced yield of *P. eryngii* was achieved on spent compost casing material. Use of casing materials enhanced the yield by 21–107% over non-cased substrate. Casing of substrate using locally available materials to maximise bioconversion efficiency of *P. eryngii* constitutes a relatively easy, feasible and low-cost practice.	Mishra et al. (2013)
P. ostreatus	Not available	*P. ostreatus* nano-particles as a new	The use of *P. ostreatus* nano-particles (PONP) as a new nano-adsorbent to remove Mn(II) from aqueous solution was investigated. The maximum Mn(II) adsorption capacity of	Ma et al. (2013)

		nano-biosorbent	PONP was 130.625 mg/g at 298.15 K, which was higher than many other adsorbents.	
P. ostreatus	Liquid stationary cultures with potato dextrose broth (PDB) or high nitrogen (HN) media	Biotransformation of synthetic insecticide	The ability of *P. ostreatus* to transform heptachlor as well as heptachlor epoxide was investigated. Heptachlor was eliminated by this fungus in PDB and HN media during a 14d incubation period. *P.ostreatus* was also capable of degrading heptachlor epoxide, which is a recalcitrant metabolite of heptachlor.	Purmono et al. (2013)
P. pulmonarius	Seed culture in brown-sugar:ricebran:malt :yeast extract medium (BRMY) and SSC in rubber wood sawdust	Optimization of mushroom commercial cultivation	A high amount of P. *pulmonarius* liquid spawn was produced in BRMY medium using an automated bioreactor. High yield, uniform, small pellets were obtained in just three days. The liquid inoculum had the ability to colonise sterile rubber wood sawdust as fruiting substrates in a shortened time suggesting that the mycelium was dispersed more efficiently as opposed to grainspawn.	Abdullah et al (2013)
P. cystidiosus	Liquide culture of basidioma in Sabouraud dextrose broth (SDB)	Production of bioactive lipids	This innovative study reports the successful cultivation of mushrooms in liquid medium. SDB was the most suitable culture medium and the maximal mycelial biomass of *P. cystidiosus* was obtained in SDB at pH 7, when incubated at 28°C and 30°C. Agitation did not improve mycelial growth. Cholesterol, triglycerides, free fatty acids, and polar lipids were detected in *P. cystidiosus* mushrooms.	Dulay, Ray & Hou at al. (2014)
P. eryngii var. *tuoliensis* and *P.*	Not available	Characterization of odor components of the volatile oil from	The main components of the *P. eryngii* var. *tuoliensis* oil were palmitic acid, oleic acid and linoleic acid. The main components of the *P. cystidiosus* oil were palmitic acid, indole	Usami et al.

cystidiosus		*Pleurotus* spp.	and myristic acid. The results of the sniffing test, odor activity value (OAV) and flavor dilution (FD) factor indicate that methional, 1-octen-3-ol and nonanal are the main aroma-active components of *P. eryngii* var. *tuoliensis* oil, while dimethyl trisulfide and 1-octen-3-ol were estimated as the main aroma-active components of the *P. cystidiosus* oil.	(2014)
P. pulmonarius	SSC in several lignocellulosic wastes	Cold stimulation as a strategy for improving mushroom yield	This study assessed the performance of mushroom initiation and yield by cold stimulation of *P. pulmonarius*. Various combinations of temperature and time were examined in a factorial design, in order to determine the most appropriate cold stimulation treatment. The best performance among the 12 treatments was recorded following a 12 h cold stimulation at 5°C.	Yingyue et al. (2014)
P. eryngii	Not available	Strategy for prolonging the shelf life of postharvest mushrooms	The high activity of serine proteinase (Spr) was one of the key factors causing deterioration of mushroom fruiting bodies. To investigate the activity and molecular mechanisms of Spr during storage in *P. eryngii*, the mushrooms were stored under high carbon dioxide and low oxygen treatment (2% O_2 + 30% CO_2), which was proved to significantly prolong mushroom shelf life.	Zhang et al. (2015)
P. ostreatus	Solid state fermentation in wheat flour and malt extract	Enzyme production and synthesis of gold nanoparticles	Optimization of production conditions yielded an enzyme with activity over 32,450 IU/g of fermented substrate. Factorial design was capable of establishing the conditions that multiplied the activity of the enzyme several fold. The partially purified enzyme was capable of decolorizing several dyes with	El-Batal et al. (2015)

			over 80% reduction in color. The enzyme was also used in the synthesis of gold nanoparticles.	
P. djamor var. *roseus*	SSC in paddy straw substrate	Mycosynthesis and characterization of silver nanoparticles	The present study reports the biological synthesis of silver nanoparticles (AgNPs) using an aqueous extract of *P. djamor* var. *roseus* and its cytotoxicity against human prostate carcinoma (PC3) cells. Nanoparticle formation was confirmed by UV–visible (UV–vis) spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analysis.	Raman et al. (2015)
P. eryngii	SSC in several lignocellulosic wastes	Strategy for extending the shelf life and improving yield	Among all tested mushroom medias, the one that produced the longest shelf life and highest yield contained 4.5% of crude protein and 15% of nitrogen free extracts. Regression analysis supported CP and CaO presented synergistic effects on shelf life. These results might be used by mushroom farmers to produce long shelf life and high yield mushrooms.	Ryu et al. (2014)
P. djamor	SSF with various industrial wastes	Optimization of cultural conditions for biosurfactant production	This study demonstrated an economical biosurfactant production by *P. djamor* in SSF in determined the optimum condition. In this condition 10.205 g/l biosurfactant was produced which reduced water surface tension to 28.82 mN/m. In laboratory's large-scale production 8.9 g/l biosurfactant was produced, which was carried out in a tray bioreactor. With regard to dual product strategies, a lipase enzyme was simultaneously produced.	Velioglu & Urek (2015)
P. florida and	SSC in agro-residues combined	Cultivation on a combination of	The study investigated the effects of the addition of biogas digester residue (BDR) to paddy straw (PS) and coir pith (CP),	Chanaky, Malayil &

P.flabellatus	with biogas digester residue	anaerobically digested plant material and agro-residues	used as substrates for growing mushroom. The substrate that produced higher yields and biological efficiency was PS mixed with BDR followed by CP with BDR. Addition of BDR with agro-residues could increase the mushroom yield by 20–30%.	Vijayalakshmi (2015)
P. pulmonarius	SSC in orange waste	Enzymes production and biotransformation of orange waste	Pectinase was the main hydrolytic enzyme produced by the fungus, with the highest enzymatic activity of 9.4 U/mL after 35 days of cultivation. Laccase was the main oxidative enzyme produced with maximal activity of 12.2 U/mL obtained after 20 days of cultivation. There was no lignin degradation during the cultivation and the fungus culture promoted a protein enrichment in the substrate.	Inácio et al. (2015b)
P. ostreatus	SCC in blank and printed paper substrates	A sustainable proposal for mushroom cultivation and a profitable means to recycle paper	The objective of this work was to evaluate the chemical composition of fruiting bodies of *P. ostreatus* grown on blank and printed paper substrates, in comparison with samples grown on oat straw (control). The nutritional properties of the control sample were similar to values reported in the literature, while the chemical composition of the samples obtained using paper scraps, either blank or printed, was highly satisfactory.	Fernandes et al. (2015)
P. ostreatus	Not available	Improvement of antioxidant ability and rheological properties in yogurts	The multiplication of fermentative bacteria was greater in yogurts supplemented with *P. ostreatus* aqueous extract (POE). The utilization of POE in yogurts improves rheological properties and texture characteristics (lower firmness but higher cohesiveness, adhesive, springiness and less syneresis).The supplemented yogurts with POE contained more total phenolics and exhibited higher antioxidant activity than controls.	Vital et al. (2015)

Table 3. Chemical compounds in *Pleurotus* spp. and their correspondent bioactivities reported in the past ten years.

***Pleutorus* spp.**	**Compound and Bioactivity**	**Novelty, main contribution**	**Ref.**
P. cornucopiae	D-mannitol, ameliorates hypertension	The antihypertensive effect, induced by the inhibition of an angiotensin I converting enzyme (ACE), was demonstrated in spontaneously hypertensive rats (SHR) by oral administration.	Hagiwara et al. (2005)
P. citrinopileatus	Polyphenols, antioxidant effect	The ethanolic, cold and hot water extracts of *P. citrinopileatus* fruiting bodies, mycelia and fermentation filtrate were evaluated for their antioxidant properties. Overall, extracts from fruiting bodies presented a superior antioxidant potential than those from mycelia and filtrate.	Lee et al. (2007)
P. pulmonarius	β-glucan, anti-inflammatory and analgesic properties	A glucan extracted from the basidioma was tested for its effects on the acetic acid-induced writhing reaction in mice, a typical model for quantifying inflammatory pain. The great anti-inflammatory and analgesic activities observed were possibly by the inhibition of pro-inflammatory cytokines.	Simiderle et al. (2008)
P. nebrodensis	Nebrodeolysin, antitumoral and anti-HIV-1 effects	A novel hemolysin was isolated from *P. nebrodensis* by ion exchange and gel filtration chromatography. It exhibited haemolytic activity towards rabbit erythrocytes and caused efflux of potassium ions from erythrocytes, with strong cytotoxicity against Lu-04, Bre04, HepG2, L929 and HeLa cells, besides anti-	Lv et al. (2009)

		HIV1 activity in CEM cell culture.	
P. cystidiosus	Ergosterol, antifungal activity	The antifungal activity was investigated by fractionating the mushroom to acetone (A), dichloromethane (D), and hexane (H). After antifungal assay and normal phase chromatography, the fraction with the highest inhibitory activity was separated using the Chromatotron and a single compound (A2-3-13) was isolated. Using NMR spectroscopy they found it was 3*β*, 5*α*, 6*β*-trihydroxyergosta-7,22-diene.	Menikpurage et al. (2009)
P. djamor	Ribonuclease, antiproliferative activity	A 15-kDa RNase was purified from *P. djamor* using ion exchange chromatography and gel filtration. The RNase exhibited maximal RNase activity at pH 4.6 and 60 °C. It inhibited proliferation of hepatoma cells and breast cancer cells.	Wu et al. (2010)
P. sajor-caju	Polysaccharides, antineoplasic Effect	Female Swiss mice were inoculated with the Ehrlich ascitic tumor and the polysaccharidic fractions of *P. sajor-caju* were administered intraperitoneally, during a 6-day period. Two fractions presented a lower volume of ascitic liquid and a higher reduction in the number of neoplasic cells, when compared to the positive control. Glucose was the major component detected in the fractions, followed by galactose and mannose.	Dalonso et al. (2010)
P. cornucopiae	Peptide, anti-hypertensive effects	This study describes the characterisation of a new angiotensin I-converting enzyme (ACE) inhibitory peptide from the basidioma of *P. cornucopiae*. Two types of the purified ACE inhibitors were obtained and posteriorly analysed, showing two types of oligopeptides. The amino acid sequences of the two purified oligopeptides were found to be RLPSEFDLSAFLRA and RLSGQTIEVTSEYLFRH. Water extracts of *P. cornucopiae* fruiting body showed antihypertensive effect on spontaneously hypertensive rats at a dosage of	Jang et al. (2011)

		600 mg/kg.	
P. cornucopiae	Monoterpenoids and sesquiterpenoids, anti-inflammatory and antitumoral potencial	Four new monoterpenoids (1−4) and one new sesquiterpenoid (6) were isolated from the mycelia fermented on rice. Compound 1 possesses a spiro[benzofuran-3,2′-oxiran] skeleton. The absolute configuration of the 6,7-diol moieties in compounds 1, 2, and 6 was assigned. Compounds 1−5, 7, and 8 showed inhibitory activity against nitric oxide production in lipopolysaccaride-activated macrophages while compounds 6 and 7 exhibited cytotoxicity against HeLa and HepG2 cells.	Wang et al. (2013)
P. eous	Fatty acid esters, antibacterial activity	Petroleum ether extract of the *P. oeus* fruiting bodies were analysed by CG-MS and 5 compounds were identified: cyclopentanetridecanoic acid, methyl ester; tartronic acid, (p-ethoxyphenyl), diethyl ester; 7, 10-octadecadenoic acid, methyl ester; heptadecanoic acid, 16-methyl, methyl ester and 9-octadecenoic acid [Z]-, 2-hydroxyl-1-[hydroxymethyl] ethyl ester. Among several crude extracts tested, only the petroleum ether extract showed strong antibacterial activity by inhibiting the growth of both gram positive and gram negative bacterial isolates.	Suseem & Saral (2013)
P. cornucopiceae	Polysaccharides, antioxidant activities in vitro and in vivo	Intracellular zinc polysaccharides (IZPS) were extracted and purified, and three subfractions (IZPS-1, IZPS-2, and IZPS-3) were separated by anion-exchange column chromatography. They showed certain scavenging effects on superoxide anion ($O2^{\bullet-}$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and positive rising of reducing power *in vitro*. All the subfractions were found able to act as upregulators of the superoxide dismutase, GSH peroxidase and catalase and significantly decreased the contents of malondialdehyde and lipid peroxidation *in vivo*.	Zhang et al. (2014)

P. eryngii	Sulphated polysaccharides, antioxidant and antibacterial activities	Polysaccharides from *P. eryngii* (PEPS) and exopolysaccharides from *Streptococcus thermophilus* ASCC 1275 (ST1275 EPS) were sulphated, with degrees of sulphonation of 0.69 and 0.31, respectively. Antioxidant activities of both PEPS and ST1275 EPS were significantly improved after sulphonation. Overall, sulphated PEPS presented a superior antibacterial potential when compared with sulphated ST1275 EPS.	Li & Shah et al. (2014)
Pleurotus spp.	D-mannitol and Oligo peptides, anti-hypertensive effects	The mostly noted species having antihypertensive effects include *P. ostreatus*, *P. cornucopiae*, *P. nebrodensis*, and *P. cystidiosus*. Their ameliorating effect on elevated blood pressure has been attributed to their inhibitory effect on angiotensin converting enzyme (ACE).	Yahayaa, Rahmana & Abdullah (2014)
P. sajor-caju	A Linear β-D glucan, anti-inflammatory activity	*P. sajor-caju* fruiting bodies cultivated in banana straw, produced a linear β-D-d-glucan (1→3)-linked. This is the first report of such a structure isolated from the *Pleurotus* genus. An immunomodulatory effect was observed when THP-1 macrophages were treated with the β-D-glucan. Also, the β-D-glucan was able to inhibit the inflammatory phase of nociception induced by formalin in a low dose and reduced the number of total leukocytes and myeloperoxidase (MPO) levels induced by LPS.	Silveira et al. (2014)
P. ostreatus	Polysaccharide fractions, antitumor activity	The efficacy of polysaccharidic fractions extracted from the mycelial biomass of *P. ostreatus* DSM 1833 in inhibiting the development of Ehrlich Tumor (ET) and Sarcoma 180 (S-180) was tested. The fraction obtained by extraction with NH4-oxalate at 100 ◦C, for 3 h, 4 times, was the one that presented the best results, being effective against both tumors and, at the concentration of 30 mg/kg, showed no toxic effects on healthy animals.	Facchini et al. (2014)

Pleurotus sp.	Polysaccharide fractions, antimicrobial activity and complement/macrophage stimulating effects	The extract activated the microbial autolytic system of eight strains: seven autolyzing strains with intensity values ranging from 2.7% in *Candida* sp. to 36.1% in *Saccharomyces cerevisiae*, while autolysis was of 1.8% in one non-autolyzing strain (*Bacillus cereus*). The extract (5–100 µg/well) enhanced the acid phosphatase activity in murine peritoneal macrophages by 133–184% compared to controls. The findings introduce a novel "bifunctional" approach (antimicrobial-immunomodulatory) to the nutraceutical potential of the *Pleurotus* hot-water mycelial extract.	Llauradó et al. (2015)
Pleurotus spp.	*β*-glucans, immunomodulatory activity	The use of edible fungi has not been explored for the production and delivery of low cost vaccines, despite these organisms' attractive features. These include the fact that edible biomass can be produced at low costs in a short period of time, its high biosynthetic capacity, its production of immunomodulatory compounds, and the availability of genetic transformation methods. Perspectives associated to this biotechnological application are identified and discussed in this review that proposes *Pleurotus* fungus as a convenient host for the development of innovative vaccines.	Pérez-Martínez et al. (2015)
P. sajor-caju	Exopolysaccharide (EPS), anti-inflammatory activity	The mannogalactan was purified by freeze-thawing and dialysis, and it was characterized by GC-MS analysis and NMR spectroscopy as a main chain of (1→6)-linked *α*-D-Gal*p* and 3-O-methyl-*α*-D-Gal*p* units. This is the first report of a methylated polysaccharide on EPS of *P. sajor-caju*. The mannogalactan was able to reduce the nociception, *in vivo*, in the writhing and formalin tests and also reduced the carrageenan-induced paw edema, which indicates that it could be an antinociceptive and anti-inflammatory agent.	Silveira et al. (2015)
P. nebrodensis	Polysaccharide, immune-stimulating activity	A novel *P. nebrodensis* polysaccharide (PN-S) was purified and characterized, and its immune-stimulating activity was evaluated in RAW264.7 macrophages. After exposure to PN-S, the phagocytosis of the macrophages was significantly	Cui et al. (2015)

		improved. PN-S treatment enhanced the productions of interleukin-6 (IL-6), nitric oxide (NO), interferon gamma (INF-γ), and tumor necrosis factor-α (TNF-α) in the macrophages, with up-regulation of mRNA expressions of interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), interferon gamma(INF-γ) and tumor necrosis factor-α (TNF-α) being observed in a dose-dependent manner, as measured by qRT-PCR.	
P. nebrodensis	Polysaccharide, car diac protection against ischemia–reperfusion injury	A polysaccharide (PNPA) from the fruiting bodies of *P. nebrodensis* was isolated, characterized and the effect of PNPA on myocardial ischemia–reperfusion (I/R) injury in rats was further investigated. Pretreatment with PNPA for 30 days attenuated myocardial infarct size as compared to I/R model group. A decrease in superoxide dismutase, catalase and glutathione levels, as well as an increased malondialdehyde content were observed in both myocardial serum and tissues of control I/R group, whereas pretreatment with PNPA markedly restored these changes, and also relieved myocardial cell apoptosis.	Yan, Jing & Wang (2015)
P. abalonus	Polysaccharides, antioxidant and antitumor effects	Polysaccharides (PAP) from the fruiting bodies of *P. abalonus* were isolated, and the antiproliferative activity of the polysaccharides in human colorectal carcinoma LoVo cells were evaluated. HPLC analysis showed that PAP consisted of D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-glucose and D-galactose. PAP was shown to exert a high antioxidant activity *in vitro* and a dose-dependent antiproliferative effect against LoVo cancer cells. Flow cytometry analysis demonstrated that PAP exhibited a stimulatory effect on apoptosis of LoVo cells, and induced the cell-cycle arrest at the S phase.	Ren et al. (2015)

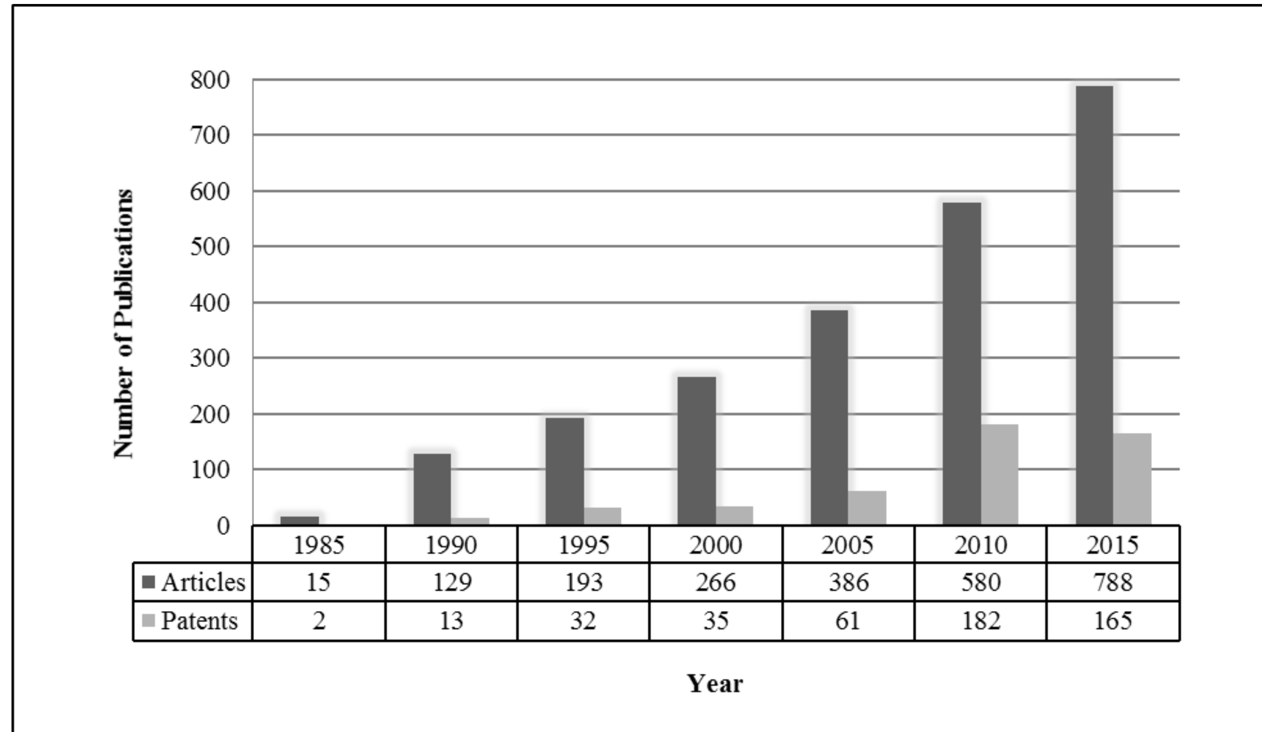


Figure 1. Number of research articles and reviews, and patents published in the period from 1985 to 2015 regarding the *Pleurotus* genus (obtained on Web of Science, August 2015; keyword restrict to the title: *Pleurotus*)

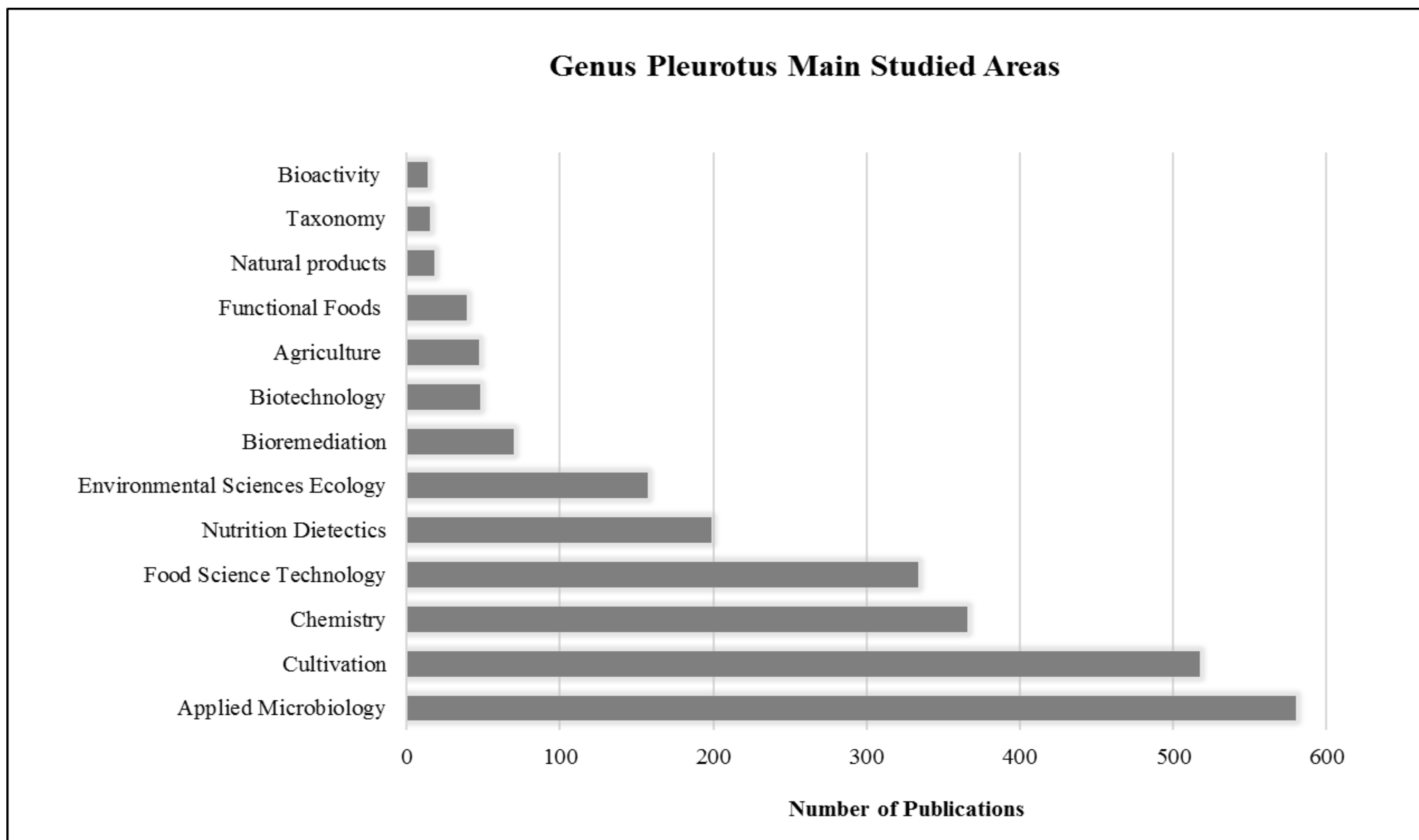
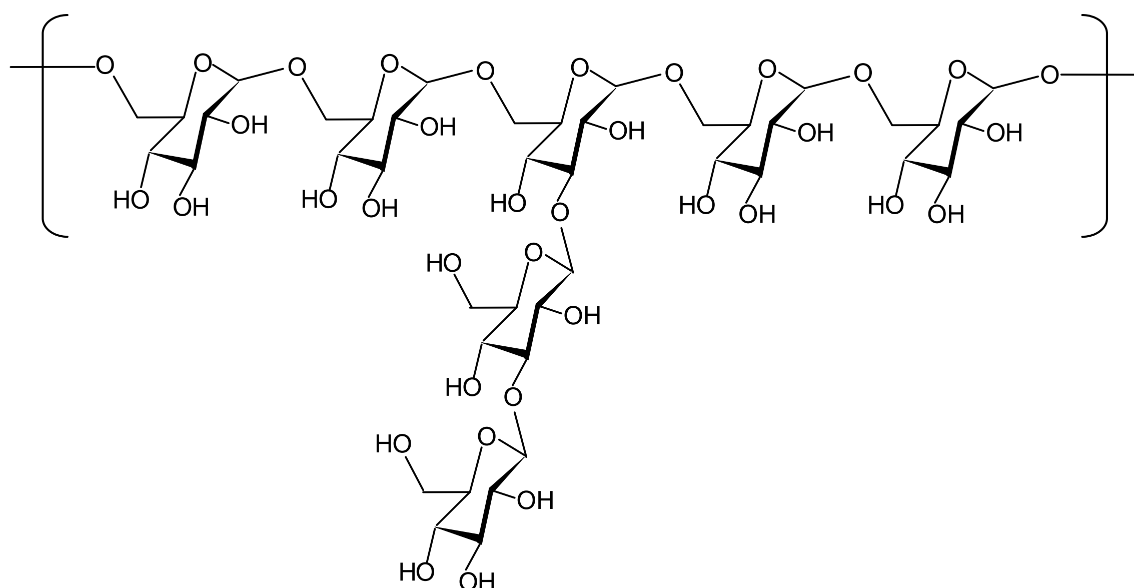


Figure 2. Distribution of research articles reviews and patents published in the period from 1985 to 2015 regarding the *Pleurotus* genus according to the main studied areas (obtained on Web of Science, August 2015; keyword restrict to the title: *Pleurotus*).

A



B

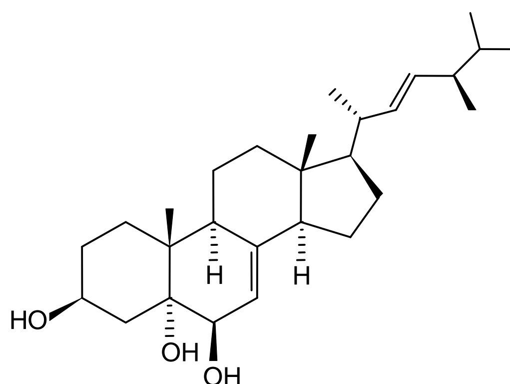
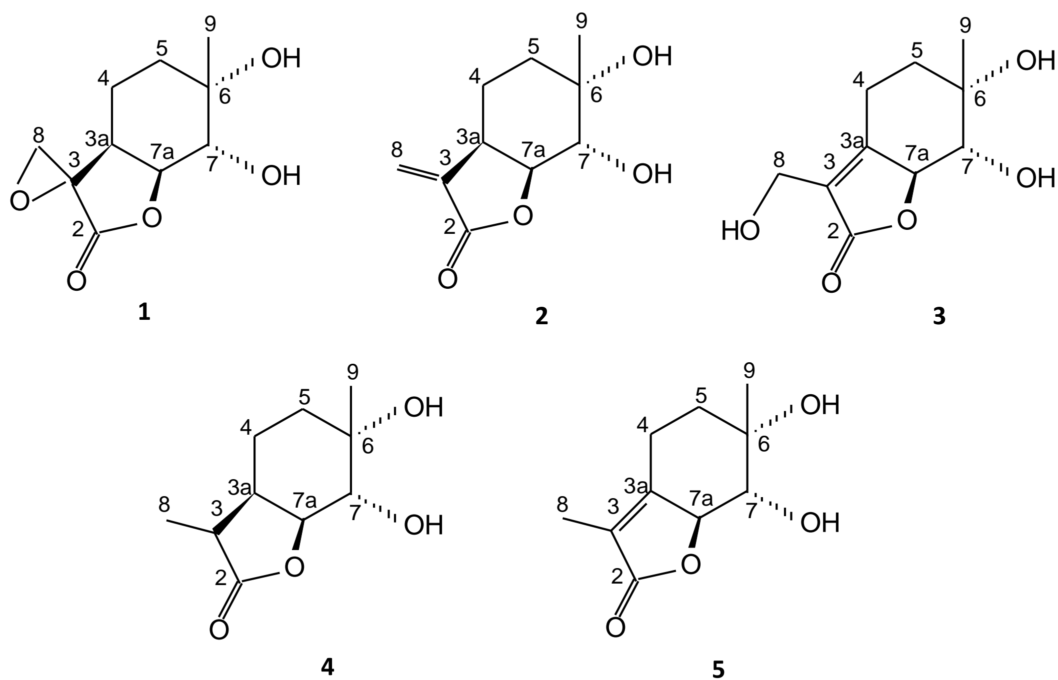


Figure 3. (A) Polysaccharide repeating unit purified from fruiting bodies of *Pleurotus citrinopileatus* **(B).** An oxidized ergosterol 3*β,* 5*α,* 6*β*-trihydroxyergosta-7,22 diene, identified in *Pleurotus cystidiosus* acetone dichloromethane extract. The chemical structures were drawn using the ChemWindow software (Soft Shell International Ltd) based on originals presented by Liu et al. (2012) (panel A) and Menikpurage et al. (2009) (panel B).

A



B

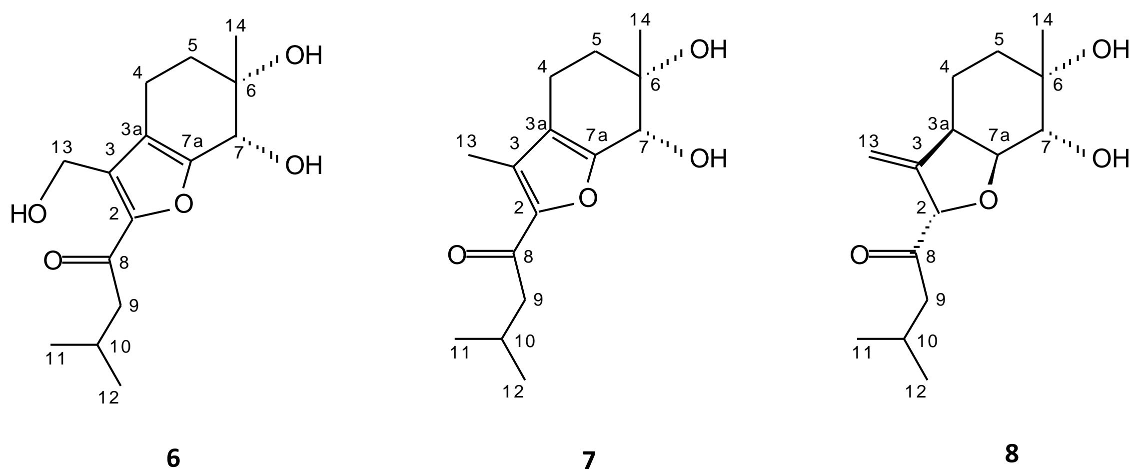


Figure 4. Monoterpenoids (A) and sesquiterpenoids (B) from the mycelia of *Pleurotus cornucopiae* ethyl acetate extract. Details of biological activities of compounds (1-8) are described in the text. The chemical structures were drawn using the ChemWindow software (Soft Shell International Ltd) based on originals presented by Wang et al. (2013).

ARTIGO 2

Bioactive formulations prepared from fruiting bodies and submerged culture mycelia of the Brazilian edible mushroom *Pleurotus ostreatoroseus* Singer

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ABSTRACT

Pleurotus ostreatoroseus is a Brazilian edible mushroom whose chemical characterization and bioactivity still remain underexplored. In this study, the hydrophilic and lipophilic compounds as well as the antioxidant, anti-inflammatory and antimicrobial activities of formulations (ethanol extracts) prepared with its fruiting bodies and mycelium, obtained from submerged cultivation, were compared. The bioactive formulations contain at least five free sugars, four organic acids, four phenolic compounds and two tocopherols. The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates, besides higher anti-inflammatory and antimicrobial activities, than the mycelium-based preparation. The absence of hepatotoxicity was confirmed in porcine liver primary cells. These functional responses can be related to the levels of bioactive components including phenolic acids, organic acids and tocopherols.

Keywords: anti-inflammatory activity, antimicrobial activity, bioactive formulations, submerged cultures.

Introduction

Besides their worldwide-appreciated flavour, mushrooms own exceptional nutritional value, with low lipid content and large amounts of carbohydrates and proteins, in addition to essential amino acids and fat-soluble vitamins (vitamins A, D, E and K) (Furlani and Godoy, 2005). Moreover, mushrooms are increasingly attractive as functional foods and as potential sources for the development of new drugs (Ferreira et al., 2014).

Among the bioactive compounds found in mushrooms, tocopherols (Heleno et al., 2010), phenolic compounds (Vaz et al., 2011) and some organic acids (Barros et al., 2013) have been implicated in their nutraceutical potential (Barros et al., 2007, 2008) and bioactivity such as antioxidant (Elmastas et al., 2007; Ferreira et al., 2009) and antimicrobial (Alves et al., 2012) effects.

Pleurotus is an important genus of basidiomycetes, especially those occurring in the subtropics and tropics, which occupy the third position in the production of edible mushrooms (Cardoso et al., 2013; Fernandes et al., 2015). *Pleurotus* spp. can be easily cultivated due to their ability to colonise and degrade a wide variety of substrates containing cellulose, hemicellulose and lignin, using them in their own development (Bazanella et al., 2013; Fernandes et al., 2015). Furthermore, these species have a quick mycelium growth and fruiting, and a low cost of culture (Ramos et al., 2011; Pokhrel et al., 2013). For these reasons, as also for their well-known nutritional and functional characteristics, *Pleurotus* spp. have become very interesting from a commercial point of view (Fernandes et al., 2015).

Pleurotus ostreatoroseus Singer is an edible Brazilian mushroom that stands out for its characteristic rosy coloration and delightful flavour of the fruiting bodies. It was firstly described by Singer (1961) from sample material collected at *Dois Irmãos* Park (Brazilian Atlantic Forest), Recife, PE, Brazil. This species is included among the white rot fungi for

its excellent potential in lignin degradation and is considered an autochthonous mushroom in the tropics, which grows quite well in tropical temperatures (Rosado et al., 2002).

Although a few studies have covered the chemical characterization and antitumor and immunomodulatory effects of polysaccharides isolated from *P. ostreatoroseus* fruiting bodies (Carbonero et al., 2006; Carbonero et al., 2008; Gracher et al., 2010), the chemical elucidation and bioactivity of other molecules, such as phenolic compounds, still remain unknown.

Regarding antioxidant, anti-inflammatory and antimicrobial effects of *Pleurotus* spp., there are some studies with *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm, *Pleurotus cystidiosus* O.K. Mill, *Pleurotus pulmonarius* (Fr.) Quél, *Pleurotus djamor* (Rumph. ex Fr.), *Pleurotus citrinopileatus* Singer and *Pleurotus eryngii* (DC.) Quél (Ferreira et al., 2009; Alves et al., 2012; Lin et al. 2014; Silveira et al. 2014; Silveira et al. 2015). Nevertheless, to the author's knowledge, there are no previous reports on antioxidant, anti-inflammatory and antimicrobial activities of *P. ostreatoroseus*.

In the present work, an study was performed with fruiting body and mycelium of *P. ostreatoroseus*, by preparing bioactive formulations (ethanolic extracts) that were further characterized in terms of hydrophilic and lipophilic compounds. The antioxidant, anti-inflammatory and antimicrobial potential of the prepared extracts were evaluated and compared, along with confirmation of non-toxicity tested in a primary cell culture of porcine liver cells.

Material and Methods

Fruiting body selection and nutritional characterization

Fruiting bodies (basidiocarps) of *P. ostreatoroseus* were obtained from a local producer in Maringá, PR, Brazil, in Spring 2014. The fruiting bodies were selected in accordance with

the commercial requirements in Brazil, i.e., before the rupture of the veil (closed cap), in order to preserve the sensory characteristics as well as firmness, which latter reduces fragmentation during processing.

The fruiting bodies were nutritionally characterized regarding moisture, proteins, fat, carbohydrates and ash, by using the standard procedures (AOAC, 1995). The crude protein content (N×4.38) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600±15ºC. Total fibre was determined by enzymatic-gravimetric method (AOAC, 1995). Carbohydrates value was calculated by difference.

Production of the P. ostretatoroseus mycelia

A commercial isolate of *P. ostreatoroseus* was obtained from a local producer. The stock culture was maintained on wheat bran extract agar slants (**Figure 1**) and sub-cultured every month. The slants were incubated at 28 °C for 7 days and then stored at 4 °C in a refrigerator for up 30 days. The inocula were prepared by adding actively growing mycelia from a newly prepared slant culture (5 mycelial agar discs with 0.5 cm of diameter) into 50 mL medium in a 250 mL Erlenmeyer flask that were incubated for 5 days at 28 °C on a rotary shaker at 160 rpm. The wheat bran extract medium was prepared with 100 g of wheat bran that were boiled in 1 L of distilled water, then the mixture was filtered in gauze and mineral solution (Vogel, 1956) at final concentration of 2% was added to the filtrate. For the submerged culture, 150 mL of the same medium were prepared in a 500 mL flask, and pre-culture broth was inoculated (at 1.0 mL/L). The flasks were incubated at 28 °C on a rotary shaker at 160 rpm for up to 7 days. The mycelia were recovered from

the liquid medium by filtration, washed with distilled water, immediately stored in freezer and posteriorly freeze-dried.

Preparation of the bioactive formulations

The extraction procedure followed the methodology proposed by Carvajal et al. (2012), with the choice of ethanol as extractor solvent due to its low cost, abundance and lower toxicity in comparison with other organic solvents. Fruiting bodies were dried and milled to a fine powder (40 mesh) while previously freeze-dried mycelium was milled to the same granulometry. The samples (5 g) were extracted by stirring with 100 mL of ethanol 70:30 (in water) at 25 °C and at 130 rpm for 3 h and filtered through Whatman n° 1 paper. The extraction procedure was repeated twice. The combined filtrates were concentrated with a rotary vacuum evaporator at 40 °C in order to eliminate the solvent and posteriorly freeze-dried. The freeze-dried powders were stored in freezer until use. The extraction yield was about 20% for both basidioma and mycelium samples.

Standards and Reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sugar, organic acid and phenolic compound standards were from Sigma (St. Louis, MO, USA). Racemic tocol (50 mg/mL) and tocopherols, were purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent in antimicrobial assays. Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/ mL, respectively) were

purchased from Gibco Invitrogen Life Technologies (California, USA). Sulforhodamine B, trypan blue, trichloro acetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, USA). RAW264.7 cells were purchased from ECACC ("European Colletion of Animal Cell Culture") (Salisburg, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and dexamethasone from Sigma. Ethanol and all other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Chemical characterization of the P. ostreatosroseus formulations

Free sugars. The extracts (500 mg) were spiked with the Internal Standard, IS (raffinose, 5 mg/mL), re-dissolved in water (5 mL) and defatted three times with 10 mL of ethyl ether, successively. After ethyl ether removal, the residues were filtered through a 0.22 µm disposable LC filter disk and transferred into an injection vial. Analysis was performed by a high performance liquid chromatograph (HPLC) and the system consisted of an integrated system with a pump (Knauer, Smartline system1000, Berlin, Germany), degasser system (Smartline manager 5000) and an auto-sampler (AS-2057 Jasco, Easton, MD, USA), coupled to a refraction index detector (RI detector Knauer Smartline 2300), as previously described by the authors (Reis et al., 2012b). The chromatographic separation was achieved with an Eurospher 100-5 NH_2 column (4.6 mm × 250 mm, 5 µm, Knauer) operating at 35 ºC (7971R Grace oven). The mobile phase used was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal

response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from the commercial standards of each compound. The results were expressed in mg per g of extract.

Organic acids. The extracts (50 mg) were re-dissolved in meta-phosphoric acid (2 mL) and subsequently filtered through Whatman nº 4 paper. Organic acids were determined by ultra-fast liquid chromatography (UFLC, Shimadzu 20A series, Shimadzu Corporation, Kyoto, Japan) coupled with a photodiode array detector (PDA) as previously described by the authors (Barros et al., 2013). Separation was achieved on a Sphere Clone (Phenomenex) reverse phase C_{18} column (5 µm, 250 mm × 4.6 mm i.d) thermostatted at 35 ºC. The elution was performed with 3.6 mM sulphuric acid using a flow rate of 0.8 mL/min. The organic acids were quantified by the comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of extract.

Phenolic acids. The extracts were re-dissolved in ethanol:water (20:80, v/v) and filtered through a 0.22 µm disposable LC filter disk for HPLC analysis. Phenolic acids determination was performed using the UFLC mentioned above, as previously described by Reis et al. (2012a). Separation was achieved with a Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm as preferred wavelength and in a mass spectrometer (MS) connected to a HPLC system via the DAD

cell outlet. The phenolic compounds were characterized according to the UV and mass spectra, retention times, and comparison with authentic standards. The identified phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in µg per g of extract.

Tocopherols. BHT solution (10 mg/mL, 100 µL) and IS solution (tocol 2 µg/mL, 250 µL) were added to the extracts (described above) prior to the extraction procedure. The extracts were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, a saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000g) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, re-dissolved in 1 mL of hexane, dehydrated with anhydrous sodium sulfate, filtered through a 0.22 µm disposable LC filter disk, and transferred into a dark injection vial. Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm, as previously described by the authors (Reis et al., 2012b). The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters operating at 35 ºC. The mobile phase used was a mixture of hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in µg per g of extract.

Bioactivity of the P. ostreatosroseus formulations

Antioxidant activity

Successive dilutions of the stock solution were made and used for *in vitro* assays already described by Reis et al. (2012a), to evaluate their antioxidant activity of the samples. The sample concentrations (mg/mL) providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (ferricyanide/Prussian blue assay) against the sample concentrations. Trolox was used as a positive control.

Ferricyanide/Prussian blue assay. The extract solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50°C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured into the 48 wells plate in addition to deionized water (0.8 mL) and ferric chloride (0.1% *w/v*, 0.16 mL), and the absorbance was measured at 690 nm in an ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

DPPH radical-scavenging activity assay. This methodology was performed using the Microplate Reader mentioned above. The reaction mixture on the 96 well plate consisted of extract solutions with different concentrations (30 µL) and a methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as percentage of the DPPH discoloration using the equation: $\%RSA=[(A_{DPPH}-A_S)/A_{DPPH}]\times100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution.

Inhibition of β-carotene bleaching or β-carotene/linoleate assay. A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing extract solutions with different concentrations (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation: (Absorbance after 2h of assay/ initial absorbance)×100.

Thiobarbituric acid reactive substances (TBARS) assay. Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 *w/v* brain tissue homogenate which was centrifuged at 3000*g* for10 min. An aliquot (100 μL) of the supernatant was incubated with the different concentrations of the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 500 μL), followed by thiobarbituric acid (TBA, 2%, *w/v*, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%)=[(A−B)/A]×100%, where A and B were the absorbances of the control and the sample solution, respectively.

Anti-inflammatory activity

The extracts were dissolved in water, initially concentrated at 8 mg/mL and then further dilutions were prepared from 8 mg/mL to 0.125 mg/mL. The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum and glutamine at 37°C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. Under our experimental cell density (5 x 10^5 cells/mL), the proportion of dead cells was less than 1%, according to Trypan blue dye exclusion tests. Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Then, cells were treated with the different concentrations of each of the extracts for 1 h. Dexamethasone (50 µM) was used as a positive control for the experiment. The following step was stimulation with LPS (1 µg/mL) for 18 h. The effect of the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in NO basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM. For the determination of nitric oxide, a Griess Reagent System kit (Promega) was used, which contains sulfanilamide, NED and nitrite solutions. Hundred microliters of the cell culture supernatant was transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, 5-10 minutes each, at room temperature. The nitrite produced was determined by measuring the optical density at 515 nm, in the microplate reader referred above, and was compared to the standard calibration curve.

Antibacterial activity

The following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter*

cloacae (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Siniša Stanković", University of Belgrade, Serbia.

The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method (Espinel-Ingroff, 2001). The fresh overnight culture of bacteria was adjusted spectrophotometrically to a concentration of 1×10^5 CFU/mL. The requested CFU/mL corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm (OD625). Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of the inoculum. Different solvent dilutions of the ethanolic extract were added to the wells containing 100 µL of Tryptic Soy Broth (TSB) and afterwards, 10 µL of inoculum was added to all wells. The microplates were incubated for 24 h at 37 °C. The MIC of the samples was detected following the addition of 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The minimum inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to tested extracts were determined also by a colorimetric microbial viability assay based on reduction of a INT color and compared with positive control for each bacterial strains (CSLI, 2006; Tsukatani et al., 2012). MBC was determined by serial sub-cultivation of 10 µL into microplates containing 100 µL of TSB. The lowest concentration that shows no growth after this sub-culturing was read as the MBC. Standard drugs, namely streptomycin and ampicillin were

used as positive controls. 5% DMSO was used as negative control. Samples were tested in duplicates and experiments were repeated three times.

Antifungal activity

For the antifungal bioassays, the following microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum* var. *cyclopium* (food isolate). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-cultured once a month (Booth, 1971).

The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (*v/v*). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 to a final volume of 100 µL/well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. The investigated extract was dissolved in a 5% solution of DMSO and added to broth malt medium with fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µL in microtitre plates containing 100 µL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating

99.5% killing of the original inoculum. 5% DMSO was used as a negative control, while bifonazole and ketokonazole were used as positive controls. Samples were tested in duplicates and experiments were repeated three times.

Toxicity for liver cells

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house. It was designated as PLP2. Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin and 100 μg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^3 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2-3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Abreu et al., 2011). Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for SRB assay was followed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

Statistical analysis

Three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). Results were compared by means of a Student's t-test to determine the significant difference among samples, with $p \leq 0.05$.

The analysis was carried out using the SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

Results and discussion

Chemical characterization of the P. ostreatoroseus formulations

The *P. ostreatoroseus* fruiting body was nutritionally characterized and the results are presented in **Table 1**. The sample showed regular contents of moisture and ash, besides an exceptional content in total fibre. The basidioma also presented high content of protein and low fat levels. Patil et al. (2010) reported a similar value of protein content (21 g/100 g) for *Pleurotus ostreatus*. Fernandes et al. (2015) found very distinct nutritional values referring to protein and carbohydrate contents for *P. ostreatus*, but similar results referring to moisture and ash contents.

The herein characterized fruiting bodies and the mycelia produced by submerged culture were submitted to an ethanolic extraction in order to prepare bioactive formulations, which were characterized in terms of hydrophilic and lipophilic compounds (**Table 2**). Regarding free sugars composition, it was possible to quantify five distinct compounds, namely fructose, mannitol, sucrose, trehalose, and melezitose (**Figure 2**). The mycelium-based formulation revealed higher contents of all identified sugars than the basidioma-based preparation, with a total content of free sugars more than 2-fold higher. This notable difference between the total sugar contents presented by the studied formulations could be explained, among other reasons, by the utilization of a sugar-rich wheat bran extract medium to produce the mycelium biomass. Once it is impossible to eliminate all the sugar provided by the culture medium through the filtration and washing processes previously described, the mycelium sample might have assimilated some free sugars from the

medium. Reis et al. (2012b) reported lower contents of fructose (0.1 ± 0.00 mg/g extract and 0.3 ± 0.00 mg/g extract) and mannitol (5.4 ± 0.04 mg/g extract and 6.0 ± 0.00 mg/g extract) for ethanolic extracts of *Pleurotus ostreatus* and *Pleurotus eryngii*, respectively. These authors also found a lower content of sucrose (0.3 ± 0.00 mg/g extract) in the ethanolic extract of *P. eryngii*. Beluhan and Ranagajec (2011) reported a much higher content of mannitol (98.20 ± 0.55 mg/g extract) and a similar content of trehalose (17.9 ± 0.12 mg/g extract) for an ethanolic extract prepared from basidiomas of *P. ostreatus*.

It was possible to quantify four different organic acids (**Table 2**), namely oxalic, malic, citric and fumaric acids. The mycelium-based formulation revealed the highest concentration in oxalic acid, while the fruiting body-based formulation presented the highest contents of malic and citric acids. Fumaric acid was only found in the fruiting body extract, which also presented the highest content in total organic acids. The profile of organic acids described by Fernandes et al. (2015) for *P. ostreatus* was slightly different, since the authors detected quinic instead of malic acid.

The prepared formulations revealed the presence of *p*-hydroxybenzoic, *cis p*-coumaric and *trans p*-coumaric acids, as also cinnamic acid (**Table 2**). *p*-Hydroxybenzoic, *cis p*-coumaric and *trans p*-coumaric acids were only found in the fruiting body-based formulation. Cinnamic acid was found in both formulations, presenting the fruiting body extract the highest content of this compound. The fruiting body-based formulation was clearly richer in phenolic acids comparing with the mycelium-based one. Reis et al. (2012a) also reported the presence of *p*-hydroxybenzoic in basidioma and mycelium of *P. ostreatus* (1.56 ± 0.06 and 0.05 ± 0.00 1 μg/g dw, respectively), besides cinnamic acid (0.23 ± 0.02 and 9.65 ± 0.86 μg/g dw, respectively).

α-Tocopherol and β-tocopherol were found in both formulations, with no statistical differences between them regarding the contents of each one of the compounds. These two

vitamers of tocopherols were present in the profile of *Pleurotus* species reported by Reis et al. (2012b) and Lin et al. (2014).

Bioactivity of the P. ostreatoroseus formulations

The fruiting body-based formulation revealed higher reducing power, DPPH scavenging activity, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates, than the mycelium-based preparation (**Table 3**). Reis et al. (2012a) reported lower reducing power (EC_{50} values = 3.31 ± 0.03 mg/mL and 3.72 ± 0.09 mg/ml, respectively), DPPH scavenging activity (6.54 ± 0.16 mg/mL and 8.67 ± 0.12 mg/mL, respectively), β-carotene bleaching inhibition (EC_{50} values = 2.74 ± 0.16 mg/mL and 4.68 ± 0.60 mg/mL, respectively) and TBARS formation inhibition (EC_{50} values 2.58 ± 0.86 mg/mL and 3.95 ± 0.58 mg/mL, respectively) for methanolic extracts prepared from basidiomas of *P. ostratus* e *P. eryngii*. Regarding the DPPH scavenging activity, Tsai et al. (2009) reported lower activity in ethanolic extracts of *P. ostreatus* from Taiwan (5.58 ± 0.24 mg/mL). Also, the herein studied *P. ostreatoroseus* mycelium ethanolic extract has higher DPPH scavenging activity than the *P. ostreaus* and *P. eryngii* mycelia methanolic extracts (EC_{50} values 58.13 ± 3.02 mg/mL and 25.40 ± 0.33 mg/mL, respectively; Reis et al., 2012a). The studied ethanolic preparation revealed lower lipid peroxidation inhibition measured by the T-BARS assay than the mycelium methanolic extract of *P. ostreatus* (EC_{50} value = 1.08 ± 0.86 mg/mL), but a much higher inhibition than the mycelium extract of. *P. eryngii* (EC_{50} value = 21.03 ± 0.45 mg/mL), as reported by Reis et al. (2012a).

P. ostreatoroseus formulations revealed a dose-dependent potential anti-inflammatory activity (**Figure 3**), with a relevant decrease of NO production even in the presence of low concentrated extracts (up to 400 μg/mL). The fruiting body-based formulation revealed

higher activity (lower EC_{50} value) than the mycelium-based preparation (**Table 3**). Moro et al. (2012) investigated the anti-inflammatory activity of a methanolic extract of *P. ostreatus* in LPS-activated macrophages and reported no anti-inflammatory activity. Nonetheless, Lin et al. (2014) reported anti-inflammatory effects of ethanolic extracts from *P. eryngii* fruiting bodies and correlated these effects with their contents in antioxidant components. Thus, the higher anti-inflammatory activity revealed by the fruiting body-based formulation, when compared to the correspondent mycelium-based preparation, may be justified by its higher contents in hydrophilic and lipophilic antioxidant compounds, including phenolic acids, as also due to its higher antioxidant capacity. In fact, oxidative stress caused by the production of nitric oxide (NO) during inflammation processes has been related to the occurrence of several diseases such as cancer, diabetes, renal disease and arthritis (Comar et al. 2013; Wendt et al. 2015) . The elimination of NO by NO scavengers or the inhibition of its production by iNOS inactivator alleviates these illness conditions. Thus, the scavenging of NO or suppression of NO production by iNOS are clearly promising indices in screening new functional foods (Tsai et al. 2007; Rebelo et al. 2014).

The studied bioactive formulations exhibited antibacterial activity against all bacteria tested (**Table 4**). The *P. ostreatoroseus* basidioma ethanolic extract presented higher antibacterial activity than the correspondent mycelium extract against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* and *Enterobacter cloacae*. The basidioma-based formulation also presented an antibacterial activity similar to the commercial antibiotic streptomycin (MIC 0.25 mg/mL) against *Staphylococcus aureus*. Both studied formulations possessed the same activity against *Listeria monocytogenes*, and only in the case of *Micrococcus flavus* the mycelium-based preparation presented higher antibacterial activity than the fruiting body-

based formulation. Alves et al. (2012) reported high antibacterial activity of an ether extract of *Pleurotus pulmonarius* against *Staphylococcus aureus*. Tambeker et al. (2011) reported the antimicrobial ability of ethanolic, methanolic and xylene extracts of *P. pulmonarius* against *Escherichia coli* and *Pseudomonas aeruginosa*. Sulphated polysaccharides from *P. eryngii* showed inhibition against *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (Li and Shah, 2014).

Regarding the antifungal activity (**Table 4**), the *P. ostreatoroseus* basidioma ethanolic extract presented higher activity than the correspondent mycelium-based preparation against *Aspergillus versicolor*, *A. ochracues*, *A. niger*, *Trichoderma viride*, *Penicillium funiculosum* and *P. verrucosum*. The studied basidioma ethanolic extract also presented similar antifungal activity similar to that of the commercial antibiotic bifonazole (0.15 mg/mL) against *Aspergillus ochracues* and *Trichoderma viride*. Both bioactive formulations possessed the same activity against *Aspergillus fumigatus*. Only in the case of *Penicillium ochrochloron* the mycelium-based formulation presented higher antifungal activity than the fruiting body-based preparation. Hearts et al. (2009) found no antifungal activity in the aqueous extract of *Pleurotus ostreatus* against *Aspergillus fumigatus*, *A. niger* and *Penicillium* sp. Moreover, Wang et al. (2004) reported the activity of an antifungal peptide isolated from *Pleurotus eryngii* fruiting bodies and Ngai et al. (2004) reported antifungal effects of a ribonuclease isolated from basidiomas of *P. pulmonarius*. In both cases, the inhibition of mycelium growth was against *Fusarium oxysporum* and *Mycosphaerella arachidicola*.

As the *P. ostreatoroseus* bioactive formulations displayed antioxidant, anti-inflammatory and antimicrobial activity, it was important to guarantee an absence of cytotoxicity against liver cells, which are considered the best *in vitro* model for studies of human cytotoxicity. The studied samples revealed no toxicity in liver primary culture PLP2, once the GI_{50}

values obtained were higher than the highest concentration tested (> 400 μg/mL). The positive control ellipticine gave a GI_{50} = 2.29 μg/mL (**Table 3**).

Overall, and to the best of our knowledge, this is the first report of anti-inflammatory properties of *P. ostreatoroseus* fruiting body and mycelium extracts, and from the results obtained, it can be inferred a clear anti-inflammatory and antimicrobial potential of the tested samples. Therefore, these formulations can be used to prepare dietary supplements with nutraceutical purposes.

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Table 1. Nutritionally characterization of *P. ostreatoroseus* fruiting bodies expressed on a dry weight basis (mean ± SD).

	P. ostreatoroseus Fruiting bodies
Fat (g/100 g)	3.0 ± 0.1
Proteins (g/100 g)	26.0 ± 0.2
Ash (g/100 g)	7.6 ± 0.1
Carbohydrates (g/100 g)	18.4 ± 0.1
Total fibre (g/100 g)	45.0 ± 0.2

Table 2. Chemical characterization of *P. ostreatoroseus* fruiting body- and mycelium-based formulations (mean ± SD).

Free sugars	Fruiting body	Mycelium	*t*-Students test *p*-value
Fructose	0.46 ± 0.05	10 ± 1	<0.001
Mannitol	12.0 ± 0.1	15 ± 1	0.006
Sucrose	0.84 ± 0.09	10 ± 1	<0.001
Trehalose	10.3 ± 0.5	15.6 ± 0.3	<0.001
Melezitose	2.44 ± 0.03	7.5 ± 0.4	<0.001
Total (mg/g extract)	26.0 ± 0.3	58 ± 3	<0.001
Organic acids			
Oxalic acid	12 ± 1	39 ± 6	<0.001
Malic acid	95 ± 1	52 ± 1	<0.001
Citric acid	101 ± 26	0.063 ± 0.002	<0.001
Fumaric acid	4.14 ± 0.01	nd	-
Total (mg/g extract)	212 ± 25	91 ± 8	<0.001
Phenolic compounds			
p-hydroxybenzoic acid	0.129 ± 0.001	nd	-
cis p-Coumaric acid	0.03 ± 0.01	nd	-
trans p-Coumaric acid	0.032 ± 0.003	nd	-
Cinnamic acid	0.050 ± 0.002	0.0065 ± 0.0005	<0.001
Total (μg/g extract)	0.24 ± 0.01	0.0065 ± 0.0005	<0.001
Tocopherols			
α-Tocopherol	0.08 ± 0.00	0.09 ± 0.01	0.083
β-Tocopherol	0.45 ± 0.02	0.41 ± 0.01	0.026
Total (μg/g extract)	0.53 ± 0.02	0.50 ± 0.01	0.044

nd- not detected.

Table 3. Bioactivity of *P. ostreatoroseus* fruiting body- and mycelium-based formulations (mean ± SD).

	Fruiting body	Mycelium	*t*-Students test *p*-value
Antioxidant activity (EC_{50} values, mg/mL)			
Reducing power	1.79 ± 0.01	nd	-
DPPH scavenging activity	4.78 ± 0.02	15.62 ± 0.13	<0.001
β-carotene beaching inhibition	0.40 ± 0.01	7.62 ± 0.25	<0.001
TBARS inhibition	0.29 ± 0.00	2.34 ± 0.08	<0.001
Anti-inflammatory activity (EC_{50} values, µg/mL)			
NO production	229.75 ± 4.25	261.23 ± 8.44	0.011
Hepatotoxicity (GI_{50} values, µg/mL)			
PLP2 growth inhibition	>400	>400	-

Results of antioxidant activity are expressed in EC_{50} values: sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power. Trolox EC_{50} values: 41 µg/mL (reducing power), 42 µg/mL (DPPH scavenging activity), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). Results of anti-inflammatory activity are expressed in EC_{50} values: sample concentration providing 50% of inhibition in production of NO. Dexamethasone EC_{50} value 16 µg/mL. Results of hepatotoxicity are expressed in GI_{50} values: sample concentration providing 50% of inhibition of the net cell growth. Ellipticine GI_{50} value 2.3 µg/mL.

Table 4. Antibacterial and antifungal activities of *P. ostreatoroseus* fruiting body- and mycelium-based formulations.

Bacteria	Fruiting body MIC/MBC	Mycelium MIC/MBC	Streptomycin MIC/MBC	Ampicillin MIC/MBC
Staphylococcus aureus	0.30/0.60	0.40/0.75	0.25/0.50	0.10/0.15
Bacillus cereus	0.15/0.30	0.40/0.75	0.05/0.10	0.10/0.15
Listeria monocytogenes	0.60/1.20	0.60/1.20	0.15/0.30	0.15/0.30
Micrococcus flavus	0.60/1.20	0.45/0.60	0.13/0.25	0.10/0.15
Pseudomonas aeruginosa	0.30/1.20	0.60/1.20	0.05/0.10	0.10/0.20
Escherichia coli	0.60/1.20	0.45/0.60	0.05/0.10	0.30/0.50
Salmonella typhimurium	0.15/0.30	0.45/0.60	0.05/0.10	0.15/0.20
Enterobacter cloacae	0.30/0.60	0.60/1.20	0.05/0.10	0.15/0.20
Fungi	Fruiting body MIC/MFC	Mycelium MIC/MFC	Bifonazole MIC/MFC	Ketoconazole MIC/MFC
Aspergillus fumigatus	0.60/2.40	0.60/3.60	0.15/0.20	0.20/0.50
Aspergillus versicolor	0.30/2.40	0.60/3.60	0.10/0.20	0.20/0.50
Aspergillus ochracues	0.15/0.30	0.60/1.20	0.15/0.20	1.50/2.00
Aspergillus niger	0.30/1.20	1.20/2.40	0.15/0.20	0.20/0.50
Trichoderma viride	0.15/0.30	0.30/0.60	0.15/0.20	1.0/1.0
Penicillium funiculosum	0.30/ 0.60	0.60/1.20	0.20/0.25	0.20/0.50
Penicillium ochrochlron	0.60/3.60	0.30/0.60	0.20/0.25	2.50/3.50
P. verrucosum	0.60/3.60	1.20/2.40	0.10/0.20	0.20/0.30

MIC- minimum inhibitory concentration; MBC- minimum bactericidal concentration; MFC- minimum fungicidal concentration.

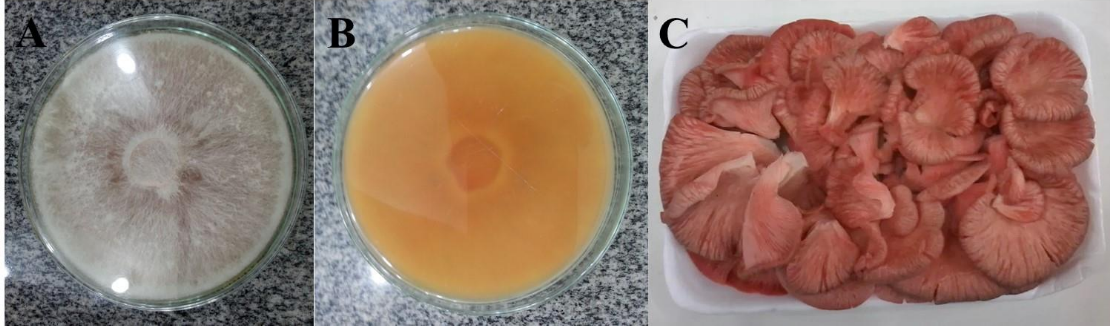

Figure 1. Morphological characteristics of *P. ostreatoroseus*. **a.** Mycelium cultivated on wheat bran extract agar slants. **b.** Bottom of the mycelium plate of figure a in which it can be observed the characteristical rosy coloration. **c.** Fruiting bodies of commercial package.

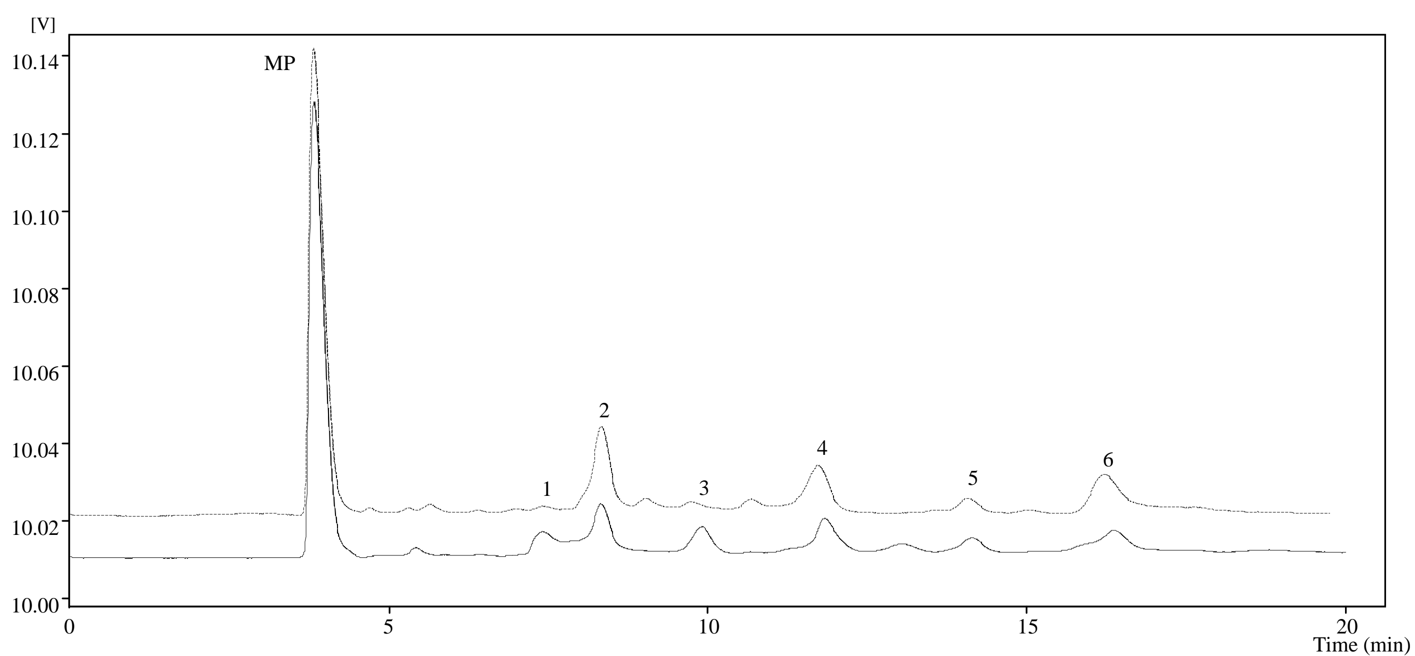

Figure 2. Individual profile of *P. ostreatoroseus* fruiting body (----) and mycelium (—) in sugars: 1- fructose, 2- mannitol, 3- sucrose, 4- trehalose, 5- melezitose, 6- raffinose (IS) and MP- mobile phase.

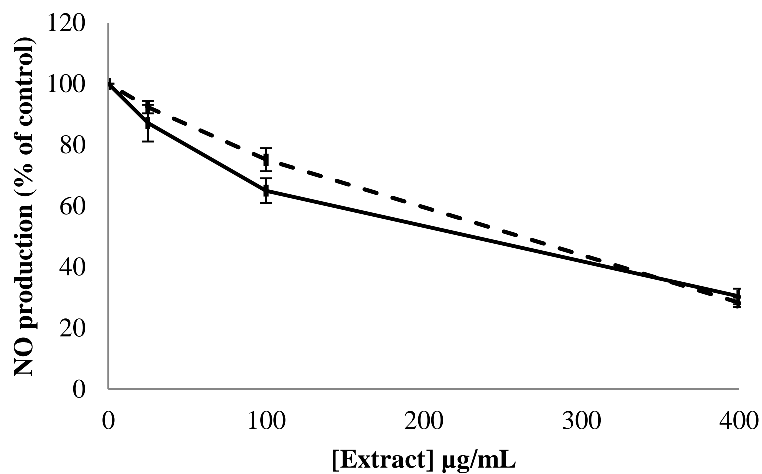

Figure 3. Nitric oxide production as function of concentration of *P. ostreatoroseus* fruiting body (——) and mycelium (----) based formulations. As the production of oxide nitric is proportional to the inflammatory process, a decrease in the nitric oxide concentration corresponds to potential anti-inflammatory activity.

ARTIGO 3

Spent mushroom substrate of *Pleurotus pulmonarius*: a source of easily hydrolysable lignocellulose

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Abstract

Pleurotus pulmonarius was cultivated on corn cob in order to charaterize the transformation of the lignocellulosic substrate during the development of the mycelial biomass and mushroom. Associated events, such as the release of enzymes and the H_2O_2 generation were also followed. High concentrations of H_2O_2 were found in the media, with maximal values at the 10th day (30 µmoles DCF/L). The peaks of laccase and catalase occurred at the 5th day and that of Mn peroxidase at the 30th day, simultaneously with a high activity of superoxide dismutase. Improvements in the endocellulase and xylanase activities were observed after 10 days, with maximal activities during the 20-30 day period. Electron microscopy and FTIR spectroscopy showed strong alterations in lignin, cellulose and hemicellulose. The uncultivated and the cultivated substrate at different times were hydrolysed with cellulase and β-glucosidase. Highest values of reducing sugars (110.5±5.6 µmol/mL), being 65% glucose, were obtained using the 20 days cultivated substrate. After the fruiting stage (first flush), enzymatic hydrolysis of the spent substrate yielded 53.0±2.8 and 77.5±4.0 µmol/mL of glucose and total reducing sugars, respectively. Although the release of reducing sugars of the *P. pulmonarius* SMS was lower than that obtained after 20 days of cultivation, it was still 50% higher than that obtained using the uncultured substrate. This observation, combined with the fact that SMS constitutes a residue generated as a byproduct of the depletion of an agro-industrial

residue, allows the conclusion that this material offers an interesting economic perspective for the obtainment of cellulosic ethanol.

Key words: biological pretreatment, enzymes, hydrogen peroxide, *Pleurotus pulmonarius*, saccharification, spent mushroom substrate

Introduction

Pleurotus spp are edible white-rot fungi produced on a commercial scale using as substrate numerous lignocellulosic wastes (corn cob, sugarcane bagasse, cotton seed hull, cotton and beet pulp, and sawdust) supplemented with rice bran, wheat bran and soybean meal. Under these conditions *Pleurotus* spp are characterized by rapid growth and high biological efficiency (percentage of conversion of dry substrate to fresh basidiomas) (Alananbeh et al. 2014). After the rmushroom harvest, a surplus of an unused source of waste product called spent mushroom substrate (SMS) is discarded.

The SMS is composed of fungal mycelia, extracellular hydrolytic and oxidative enzymes secreted from mushrooms for the degradation of substrates, and unused, but partially modified, lignocellulosic substrates (Phan and Sabaratnam et al. 2012). SMS is usually pasteurized and either discarded or sold as compost, especially as agricultural fertilizer, but its high content of mineral salts limits its use for the fertilization of salt-sensitive plants (Finney et al. 2009). The environmentally correct disposal of this residue incurs in significant costs for the industry, added to the fact that the rate of SMS production simply exceeds by far its demand for existing applications (Kapu et al. 2012). For every kg mushroom, around 5 kg SMS is produced (Williams et al. 2001). The evident solution for this issue seems to be the increase of the SMS demand which requires the development of new technological applications (Finney et al. 2009; Phan and Sabaratnam et al. 2012). SMS has been evaluated for the production of value-added products such as biogas (Lin et al. 2014), bulk enzymes, especially cellulases, hemicellulases and lignin degrading enzymes (Lau et al., 2003; Chiu et al., 2009; Singh et al. 2003; Ko et al. 2005; Mayolo-Deloisa et al. 2009), and animal feed supplements (Fazaeli and Talebian-Masoodi, 2006; van Kuijk et al., 2015). Several additional applications have been suggested, as for example in the preparations of chairs and activated carbon (Ma et al. 2014), in bioremediation (Lau et al., 2003; Chiu et al., 2009), in dye decolourization (Singh et al. 2010; Singh et al. 2011). Other suggested applications include its use as renewable fuel (Finney et al. 2009; Ribas et al. 2009), as energy feedstock (Williams et al. 2001), as biosorbent for dyes and heavy metals (Chen et al. 2005; Toptas et al. 2014), bioremediation of organic pollutants (Juárez et al. 2011), as a mushroom substrate when appropriately supplemented (Rinker 2002), and as a growing medium for nursery seedlings (Zhang et al. 2012).

In the last three decades, extensive research has been conducted on the conversion of lignocellulosic materials to ethanol. The conversion includes two processes, the hydrolysis of cellulose of lignocellulosic materials into fermentable reducing sugars, and the fermentation of these sugars into ethanol (Sun and Cheng 2002). Enzymatic hydrolysis of lignocelluloses is the key step in the production of cellulosic ethanol thanks to two factors, the recalcitrance of the crystalline cellulose itself and the highly protective lignin surrounding it which acts as a physical barrier against enzymatic attack (Castoldi et al. 2014). In some cases, to overcome the problems caused by biomass recalcitrance and improve the saccharification process, pretreatments for removing lignin and hemicellulose and breaking down the cellulosic crystalline structure are required (Himmel et al. 2007). Several chemical and physicochemical pretreatment processes including acid, alkali, organic solvents, steam explosion, ionizing radiation, ionic liquids or combined processes have been used to facilitate the enzymatic hydrolysis of lignocellulose. In general, these processes usually require high amounts of energy, resulting in high costs, undesirable products and generation of inhibitors which will affect enzymatic hydrolysis and fermentation (Mosier et al. 2005, Himmel et al. 2007).

Biological pretreatments using white-rot fungi can be a promising technology due to several advantages such as minimal waste production, no generation of toxic compounds and low energy requirement (Sindhu et al. 2016). However, they also have drawbacks, the most important being the prolonged treatment times and the low digestibility achieved in most cases. The utilization of SMS as a source of pretreated lignocellulosic fibers for biofuel production has been suggested by some authors (Phan and Sabaratnam 2012; Sun and Cheng 2002; Wu et al. 2013). SMS has less lignin than the former lignocellulosic material due to the digestion process catalysed by extracellular ligninolytic enzymes (laccases and peroxidases) during mushroom production. Low lignin content in the lignocellulosic fibers facilitates the enzymatic hydrolysis of polysaccharides, increasing the production of fermentable reducing sugars (Simmons et al. 2010). Although the genus *Pleurotus* also expresses polysaccharide hydrolases such as cellulases and xylanases, they only scarcely degrade the polysaccharide fibers during mycelial growth (Castoldi et al. 2014). Additionally, the capability of the filamentous fungi hyphae to grow on the particles surface and to penetrate the intra-particle spaces to colonize the solid substrates facilitates the subsequent access of hydrolytic enzymes during the enzymatic saccharification process. Based on these considerations, the main

objective of this study was to investigate the potential of SMS of *Pleurotus pulmonarius* in the generation of fermentable reducing sugars through saccharification of its polysaccharides by a mixture of commercial cellulase and β-glucosidase. An attempt was done to identify the enzymatic and non-enzymatic transforming agents of corn cob fibers, the substrate used for the production of mushrooms.

Material and Methods

Substrate

Corn cob (*Zea mays* spp. mays L.) was dried in an oven with air circulation at 40 °C and milled to particle size of 2-3 mm. The soybean meal and wheat bran used as co-substrates, were obtained from the local market and used without modification.

Microorganism and inoculum

Pleurotus pulmonarius CCB 019 was obtained from the Culture Collection of the São Paulo Botany Institute, and maintained in the laboratory by subculturing on potato dextrose agar (PDA). The microorganism was cultured on potato dextrose agar (PDA) Petri dishes for up to 2 weeks at 28 °C. When the Petri dishes were fully covered with mycelia, plugs measuring 10 mm in diameter were made and used as inoculum (Oliveira et al., 2007). The spawn was prepared with 15 g of pre-cooked whole wheat grains with a moisture content of 50%. This material was sterilized at 121 °C for 20 minutes. One mycelia disc (∅ 10 mm) was inoculated and incubated at 25 ^{o}C for 12 days in a dark room (Silva et al., 2002). After this time, mycelia had completely covered all wheat grains.

Mushroom production

The spawn (3%) was transferred to polyethylene bags (20 x 30 cm) containing sterilized substrate consisting of corn cob (90%), soy bean meal (5%) and wheat bran (5%) with an initial moisture adjusted to 85%. The bags were incubated at room temperature (25˚C) in a room with sufficient light and ventilation and equipped with an air humidifier. After the first sight of fruiting body primordium formation (20th day), the bags were cut open on the sides without disturbing the bed and were sprayed with water twice a day using a hand sprayer. The mass of substrate was kept undisturbed until the complete formation of mushrooms. The first flush of mushrooms was obtained 31-34 days after spawning in the

different bags. Mushrooms were manually harvested. In sequence, the spent substrates were dried in an air-circulating oven at 40 °C until their weight became constant and used as described below.

Monitoring mycelial growth and consumption of reducing sugars

During 30 days of vegetative growth, at intervals of 5 days, samples of colonized substrate were collected from the bags and analyzed in order to investigate the transformations that occurred until mushroom formation. Crude extracts were obtained by adding 20 mL of water to each 5 g of culture sample. The samples were mixed for 10 min at 8 °C and the resulting mixtures filtered on Whatman paper. The soluble materials were centrifuged for 10 min at 9000 rpm and the supernatants were immediately used for further determinations. The mycelial growth (fungal biomass) was indirectly monitored through the quantification of glucosamine in the fungal cell wall (Scotti et al. 2001). The reducing carbohydrates of the extracts were determined by using the dinitrosalicylic method (Miller 1959) and expressed as glucose equivalents.

Determination of enzyme activities

The laccase activity (EC 1.10.3.2) was measured with 2,2'-azino bis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS) in 50 mM sodium acetate buffer (pH 4.5). Oxidation of ABTS was determined by the increase in A_{420nm} (ε=36 mM^{-1} cm^{-1}) (Hou et al. 2004). The Mn peroxidase activity (MnP, EC 1.11.1.7) was assayed spectrophotometrically by following the oxidation of 1 mM $MnSO_4$ in 50 mM sodium malonate, pH 4.5, in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{3+}, form a complex with malonate, which absorbs at 270 nm (ε=11.59 mM^{-1} cm^{-1}; Wariishi et al. 1992). The superoxide dismutase (SOD, EC 1.15.1.1) activity was measured in a reaction medium consisting in 2.8 ml of 50 mM Tris–HCl buffer (pH 9.0) with 1 mM EDTA and 0.1 mL of the enzyme sample incubated at 30 °C for 5 min. The reaction was initiated by the addition of 0.05 ml of 10 mM pyrogallol in 10 mM HCl. The changes in absorbance were recorded at 320 nm for 5 min at room temperature. The SOD activity calculation was based on the percentage of inhibition of pyrogallol autooxidation (1 unit (1U) = 50% of

inhibition) (Marklund and Marklund 1974). The catalase (CAT, EC 1.11.1.6) activity was measured according to the method of Aebi (1984). The reaction mixture in a total volume of 1 mL consisted of 0.98 mL of 50 mM potassium phosphate buffer pH 7.0 containing 10 mM H_2O_2 and 0.02 mL of the enzyme sample. Calculation of the catalase activity was based on the amount of hydrogen peroxide decomposed by the enzyme during the incubation time. The disappearance of H_2O_2 was evaluated by measuring the decrease in absorbance at 240 nm for 1 min at room temperature (25 °C). One unit of catalase was defined as the amount of enzyme that decomposes 1 mmol of H_2O_2 per min at pH 7.0 and 25 °C. Enzyme activities were expressed as international enzymatic units (U, μmol min^{-1}). Xylanase (EC 3.2.1.8) and carboxymethylcellulase (CMCase, EC 3.2.1.4) were assayed by standard IUPAC methods (Ghose 1987) in 50 mM acetate buffer (pH 5.0) at 40°C. One unit of activity of xylanase and CMCase was defined as the amount of enzyme forming 1 μmol of product (xylose or glucose, respectively, from birch xylan and carboxymethylcellulose) per min.

Determination of hydrogen peroxide content.

The hydrogen peroxide content was quantified via the 2′–7′-dichlorofluorescein-diacetate (DCFH-DA) assay as previously described (Siqueira et al. 2005). Acetate groups of DCFH-DA allow it to enter the organelles. These groups are removed by esterases producing the reduced DCFH within the organelle, which can be oxidized by peroxidases to the fluorescent oxidized dichlorofluorescein (DCF). The formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorimeter RF-5301 (Shimadzu) in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) was used to express the results as μmol per liter of extract.

Characterization of corn cob before and after *P. pulmonarius* cultivation by scanning electron microscopy and Fourier transform infrared spectroscopy

Scanning electron microscopy (SEM) was performed with a Shimadzu SS-550 Superscan. For the imaging procedures the samples were sputter coated with gold layers. For the Fourier transform infrared (FTIR) spectroscopy 2 mg of each dried sample were mixed

with 200 mg KBr of spectroscopic grade and compressed into pellets at a pressure of about 1 MPa. Sample spectra were obtained in triplicates using an average of 128 scans in the range between 850 cm^{-1} and 2000 cm^{-1} with a spectral resolution of 2 cm^{-1}. Peak heights and areas of the Fourier transform infrared (FTIR) spectra were determined by means of the Opus software version 6.5, normalized by maximum and minimum peaks (Zhang et al. 2007). The influence of the growth of *P. pulmonarius* on the substrate was analyzed in terms of the percent diminutions in intensity of the lignin (1427 and 1515 cm^{-1}) and carbohydrate peaks (1395, 1098 and 898 cm^{-1}) (Zhang et al. 2007; Pandey et al. 2003).

Saccharification of corn cob before and after *P. pulmonarius* cultivation

The corn cob fibers obtained before and after fungal cultivation were subjected to enzymatic hydrolysis using cellulase from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich C8546) and β-glucosidase from *Aspergillus niger* (Novozyme 188) (Sigma C6105). The activity of the cellulase was previously assayed using 1% carboxymethylcellulose as a substrate in 50 mM sodium acetate buffer at pH 5.0 and 37°C. The released reducing sugars were quantified by the DNS reagent (Miller, 1959) using glucose as standard. The β-glucosidase activity was previously assayed using the synthetic substrate *o*-nitrophenyl-β-glucopyranoside incubated in 50 mM citrate buffer, pH 5.4, along with the filtrate at 50°C for 10 min. The reaction was stopped by the addition of sodium tetraborate and the spectrophotometrical measurements were done at 410 nm (Lenartovicz et al. 2003). One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of product per minute. The saccharification was conducted as previously described (Castoldi et al., 2014), with slight modifications. Corn cob substrate (0.5 g) of each sample was added to a 50 mL Erlenmeyer flask. A volume of 10 mL of 50 mmol/L citrate buffer, pH 5.0 was added to each flask. Cellulase was added to obtain a final activity of 5 U/mL and β-glucosidase was added to obtain a final activity of 10 U/mL. The mixtures were maintained on a rotary shaker at 150 rpm at 37 ^{o}C for up to 48 h. Samples were withdrawn periodically and filtered under vacuum. The total reducing sugars present in the filtrates were estimated by the 3,5 dinitrosalicylic method using glucose as standard (Miller 1959). Total glucose present in the filtrates was measured by the glucose oxidase-peroxidase D-glucose assay kit (Goldanalisa, Brazil).

Statistical Analysis

All experiments were carried out in triplicate. The data were analyzed by Student's unpaired t test ($p<0.05$) using the Graph Pad Prism Program (Graph Pad Software, San Diego, USA).

Results and Discussion

Vegetative growth monitoring

Monitoring of the mycelial growth, consumption of reducing sugars, production of the major ligninolytic enzymes (laccase and Mn peroxidase), polysaccharidase enzymes (endocellulase and xylanase), antioxidant enzymes (catalase and superoxide dismutase) and the presence of H_2O_2 in the cultures was conducted for up to 30 days during the vegetative growth of *P. pulmonarius* before basidioma formation (Figure 1). After 15 days, all media were completely covered by the mycelial biomass. Hydrolysis of fungal chitin into N-acetylglucosamine was used to determine the fungal biomass and the highest values, 53 mg glucosamine/g substrate (Figure 1A), were obtained at the 20th day of growth. This time corresponds also to the time of appearance of the first sight of the mushroom primordia.

High concentrations of H_2O_2 were found in the media, with maximal values at the 10th day (30 µmoles DCF/L), followed by a reduction and stabilization around 15 µmoles DCF/L (Figure 1A). The high activities of superoxide dismutase and catalase, two important defensive enzymes against reactive oxygen species (ROS), could be associated with the neutralization of hydrogen peroxide and other ROS not evaluated in this work such as superoxide, hydroxyl radical, and singlet oxygen. The maximal activity of catalase was observed at the 5th day (45 U/L), followed by a reduction and a new improvement in the activity at the 20th day of cultivation. The highest activities of superoxide dismutase were observed at the 10th (15,758 U/L) and 30th days (14,514 U/L) of growth (Figure 1C).

The peak laccase activity (3100 U/L) occurred at the 5th day of growth, followed by a significant decrease of the enzyme activity with the lowest value of 379 U/L observed

at the 20th day of growth (Figure 1B). Subsequently, a progressive increase in the laccase activity occurred until the end of the monitoring period, very near to the fruiting stage (first flush). Similar results were obtained with *Pleurotus flabellatus* cultured on rice straw (Rajarathnam et al., 1987). Kurt and Buyukalaca (2010) monitored the vegetative growth of *P. pulmonarius* over 20 days of incubation during mushroom production on several substrates and also reported highest laccase activities at the 10th day of mycelial growth, followed by a drastic reduction in the activity at the 15th day of mycelial growth and a discrete improvement after the fruiting stage.

The production of Mn peroxidase by *P. pulmonarius* had a significant and progressive increase starting at the 10th day of culture, with a maximal activity at the 30th day (653 U/L), time at which the mushroom formation occurred (Figure 1B). Previous works have shown low production of Mn peroxidase by *P. pulmonarius* when cultured for 10-14 days on solid state using corn cob and wheat bran as substrates, 250 U/L and 220 U/L, respectively (Bazanella et al. 2013; Zilly et al. 2012). In a previous work, conducted with *Pleurotus ostreatus*, a similar relation between Mn peroxidase and the fruiting stage was observed, with maximal Mn peroxidase activity occurring during the mushroom formation (Elisashvili et al. 2003). It is noteworthy that laccase and catalase presented maximal activities simultaneously at the 5th day of growth. Considering that catalase is able to disproportionate H_2O_2 into water and dioxygen, thus acting as a hydrogen peroxide degrader (Vázquez-Fernández et al. 2011), and that laccase is strongly inhibited by H_2O_2 (Milton and Minteer 2014), it seems logical that the peak activities of these two enzymes had happened concomitantly. On the other hand, the peak activity of Mn peroxidase was observed at the 30th day, simultaneously with a high activity of superoxide dismutase, which catalyses the dismutation of the superoxide anion to hydrogen peroxide and oxygen (Kesheri et al. 2014), therefore a hydrogen peroxide generator.

Improvements in the polysaccharidase activities, endocellulase and xylanase, were observed after 10 days of cultivation, with maximal activities at the 20-30th day-cultures (Figure 1D). These events occurred simultaneously with the depletion of the reducing sugars and with the beginning of the fruiting stage. Maximal polysaccharidase activities have also been associated with the fruiting stage of *P. ostreatus* and *P. sajor-caju* cultured in several agricultural wastes (Elisashvili et al. 2003; Kurt and Buyukalaca, 2010).

An illustrative scheme associating some events involved in the transformation of lignocellulosic substrate and development of mycelial biomass and appearance of

mushrooms was constructed and is shown in Figure 2. Maximal activities of laccase and catalase were the earliest events, followed by an improvement in the activity of superoxide dismutase and consequent increase in the concentration of hydrogen peroxide. The latest events that occurred after the appearance of the fruiting body primordium were the improvement of the activities of Mn peroxidase and of the hydrolytic enzymes CMCase and xylanase.

Comparative evaluation of lignocellulosic fibers before and after *P. pulmonarius* cultivation by scanning electronic microscopy and FTIR spectroscopy

Scanning electron microscopy and FTIR spectroscopy were used in an attempt to detect the main structural transformations caused by the enzymatic and non-enzymatic oxidative action of the fungus in the substrate after the vegetative growth and fruiting stage of *P. pulmonarius* (Figures 3 and 4). The most significant changes verified by the microscopic analysis correspond to visible holes over the extent of the majority of the SMS fibers, besides an evident loosening of the structures. Cracks were produced due to the microorganism growth, which conferred to the SMS a completely different aspect in comparison to the smooth and uniform surface of the corn cob (Figure 3A-B). Sun et al. (2011) reported substantial changes in corn stover treated with *Trametes hirsuta* yj9 for 14 to 42 days, which corresponded to small pores and crevices that increased in number and size over time. Yu et al. (2010) studied the treatment of wheat straw with the white rot fungus *Echinodontium taxodii* and also reported modifications in the structure of the substrate fibers after 30 days of treatment. Similar results were found by Castoldi et al. (2014) for *Eucalyptus grandis* sawdust treated with *P. pulmonarius* during 30 days. According to these authors, the presence of irregular holes in the treated surfaces result from the removal of lignin and the disruption of the lignocellulosic networks and can be interpreted as signalling the increased cellulose surface area that became available for enzymatic attack.

The FTIR analyses of both the former substrate (corn cob) and after mushroom harvesting (SMS) are illustrated by Figure 4. The signals with noticeable differences in peak height and peak area are assigned to the following functional groups belonging to lignin, cellulose and hemicellulose: 1736 cm^{-1} (C=O carbonyl and acetyl group vibration of hemicellulose and lignin), 1658 cm^{-1} (conjugated C=C of coniferyl alcohol or C=O of

coniferyl aldehyde), 1515 cm^{-1} (aromatic skeletal vibrations in lignin), 1427 cm^{-1} (syringyl and guaiacyl condensed nuclei), 1375 cm^{-1} (bending vibration of C-H_2 and C-H in cellulose and hemicellulose), 1164 cm^{-1} (C–O–C asymmetric stretching of hemicellulose and lignin), 1098 cm^{-1} (crystalline cellulose) and 898 cm^{-1} (C–H deformation in amorphous cellulose) (Casas et al. 2012; Siqueira et al. 2010; Yu et al. 2010; Cherian et al. 2008; Pandey and Pitman 2003). Analysis of these spectra in terms of the percent modifications observed in the SMS sample when compared to the corn cob sample are shown in Table 1. The most pronounced decrease was found in the band at 1736 cm^{-1} (C=O carbonyl and acetyl group vibration of hemicellulose and lignin), followed by the band at 1515 cm^{-1} (aromatic skeletal vibrations in lignin), indicating an expressive degradation of lignin and hemicellulose. This conclusion is corroborated by the significant diminution in the 1427 cm^{-1} band (syringyl and guaiacyl condensed nuclei). Besides, the equally important diminution in the 1098 cm^{-1} band (crystalline cellulose) and the minor diminution in the 898 cm^{-1} band (amorphous cellulose) is strong evidence of cellulose degradation. Similar results were obtained when *Eucalyptus grandis* sawdust was treated with *P. pulmonarius* for 30 days. (Castoldi et al. 2014) Interestingly, the FTIR spectra revealed a considerable increase in the band at 1658 cm^{-1}. The ratio of the absorbances at 1736 cm^{-1} and 1658 cm^{-1} for the uncultured corn cob is approximately 1:1. This proportion changes to 1:1.3 after cultivation. Considering that the peak at 1658 cm^{-1} has also been assigned to proteins (C=O stretching in amides) (Chen et al. 2014; Ibarra et al. 2004), this increase could be related to the fungal protein appearing in consequence of the *P. pulmonarius* growth. In summary, the comparative analysis of the substrate before and after cultivation with *P. pulmonarius* by FTIR clearly demonstrates the occurrence of lignin degradation, what should facilitate a subsequent cellulose hydrolysis. However, a considerable reduction in the cellulose and hemicellulose contents can also be noted.

Comparative enzymatic saccharification of corn cob before and after *P. pulmonarius* cultivation.

In order to evaluate the effect of *P. pulmonarius* cultivation on the liberation of reducing sugars from corn cob through enzymatic saccharification, uncultured substrate (time zero of cultivation), cultivated substrate at different times (from 0 to 30 days of cultivation and after the first mushroom flush) were submitted to hydrolysis using a

mixture of commercial cellulase and β-glucosidase. In all experiments, the same initial amounts of the lignocellulosic fibers were incubated with the same amount of cellulolytic enzymes under identical conditions. The results of the experiments are shown in Figure 5 where the concentrations of reducing sugars (μmol/mL) and glucose (μmol/mL) were represented against the time of incubation, which was conducted up to 48 h. Tests of the enzyme activities (cellulase and β-glucosidase) revealed no significant changes during the whole incubation time.

As can be seen in Figure 5, the hydrolysis of cultivated substrate with cellulase and β-glucosidase liberated significantly more reducing sugars in all incubation times when compared to the values obtained in the enzymatic hydrolysis of uncultured corn cob. After 48 h of enzymatic hydrolysis, the uncultured substrate released 43.7±3.3 and 53.3±3.7 μmol/mL of glucose and total reducing sugars, respectively. The highest values were obtained with the substrate cultivated for 20 days, the time corresponding to the maximal fungal vegetative growth (71.9±1.7 and 110.5±5.6 μmol/mL of glucose and total reducing sugars, respectively). The liberation of reducing sugars through enzymatic hydrolysis from the substrate cultivated for 25 and 30 days was reduced by 15 and 25%, respectively. Enzymatic saccharification of the cultivated substrate after the first flush of mushroom produced 53.0±2.8 and 77.5±4.0 μmol/mL of glucose and total reducing sugars, respectively. These values represent a reduction of around 30% compared to the maximal ones obtained with the substrate after 20 days of cultivation. This decrease is probably due to the reduction of the cellulose content of the substrate resulting from the intense degradation of cellulose and hemicellulose by the fungus in the late vegetative growth (Ginterová and Lazarová, 1987). In accordance with our FTIR analyses presented above, it has been calculated (Ginterová and Lazarová, 1987) that for the production of the mushroom by *P. ostreatus*, up to 60% of the cellulose within a substrate like wheat straw will be degraded until completion of the cycle. This fact suggests that for obtaining optimal values for animal nutrition, the fungal treatment of lignocellulosic wastes should be terminated before the fruiting stage. This would allow only vegetative growth of the fungi, a stage when lignin is already significantly degraded, but cellulose only slightly, resulting in a digestible delignified substrate useful for animal feed (van Kuijk et al., 2015). All these apparently unfavourable results for the practical use of the SMS become less impacting if one considers, however, that this material constitutes a residue generated as a byproduct of the depletion of other agro-industrial residues. Moreover, although the

release of reducing sugars of the *P. pulmonarius* SMS was lower than that obtained after 20 days of cultivation, it was still 50% higher than that obtained using the uncultured substrate. If these two factors are thus considered, the SMS can still be considered as offering an interesting economic perspective.

Conclusion

Our results showed that the vegetative growth of *P. pulmonarius* caused significant changes in the lignocellulose fiber of corn cob and significantly improved the enzymatic saccharification of cellulose. After mushroom production, the enzymatic saccharification of the spent mushroom substrate resulted in a diminished reducing sugars production, thanks to the degradation of cellulose by the fungus itself. However, in the latter case, mushroom production can yield two highly valued products: (1) the mushrooms for human consumption; (2) the spent substrate, a source of digestible fibers for the obtainment of reducing sugars, useful in fermentative processes such as those leading to the production of cellulosic ethanol. Nowadays, the cost of production of cellulosic ethanol is relatively high. The use of SMS could be a strategy for improving the yield and reducing the costs of the process.

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production of oxidative and hydrolytic enzymes. Food Bioproc Technol 5: 1573-1580

Figure legends

Figure 1. Time course of mycelial growth and production of enzymes by *Pleurotus pulmonarius*. RS= reducing sugars; Xyl=xylanase activity; Cel=endocellulase activity; The results represent the mean value±standard deviation of three independent experiments.

Figure 2. A tentative scheme associating some events involved in the transformation of the lignocellulosic substrate by *P. pulmonarius*.

Figure 3. Scanning electron microscopy of substrate before (A) and after (B) cultivation of *P. pulmonarius* (first flush). In the images, bars = 10 µm.

Figure 4. FTIR spectra of the lignocellulosic substrate before and after cultivation of *P. pulmonarius* (first flush).

Figure 5. Enzymatic hydrolysis of corn cob before and after cultivation of *P. pulmonarius* for different times.

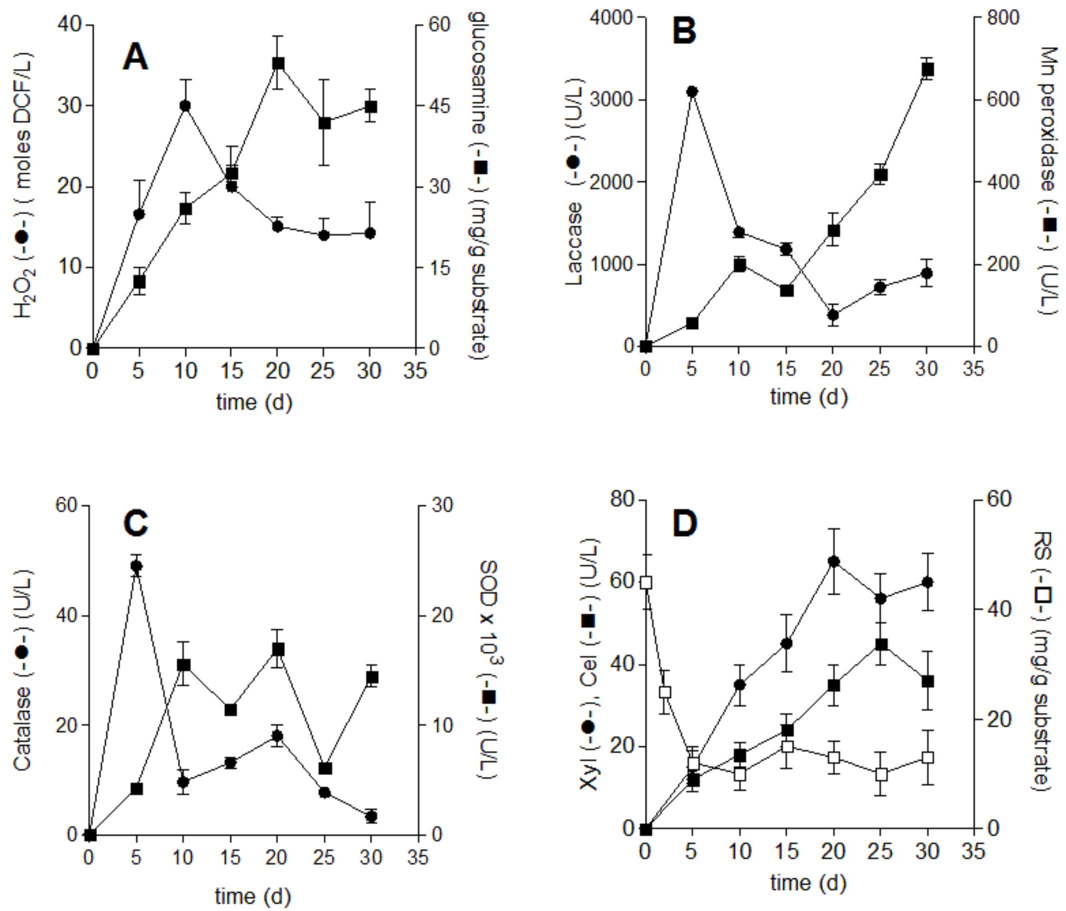

Figure 1

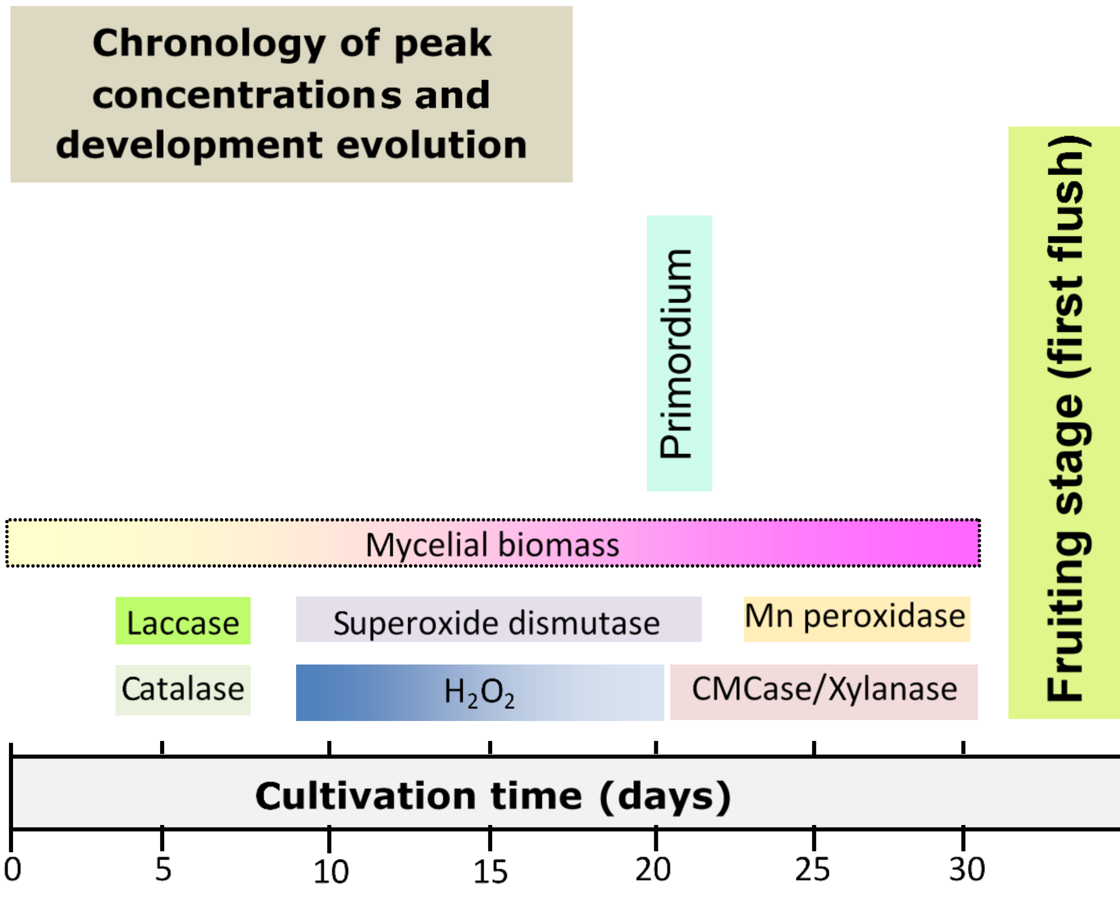

Figure 2.

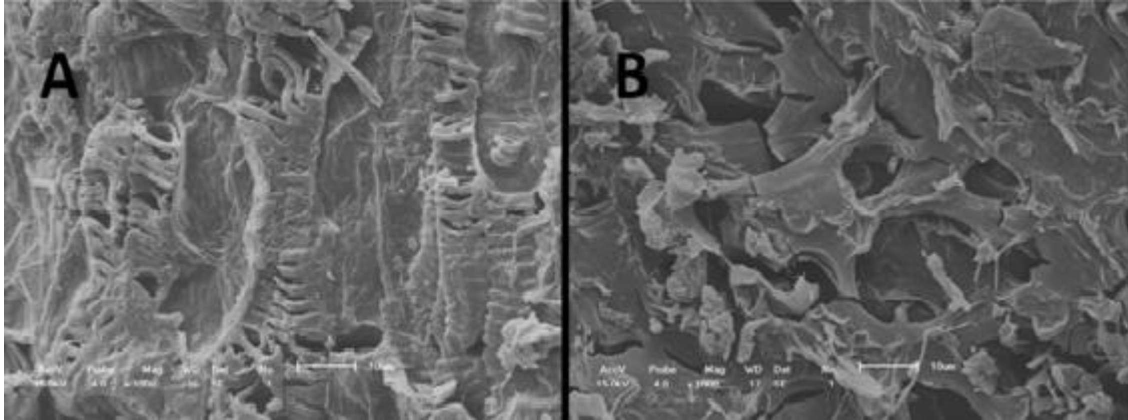

Figure 3.

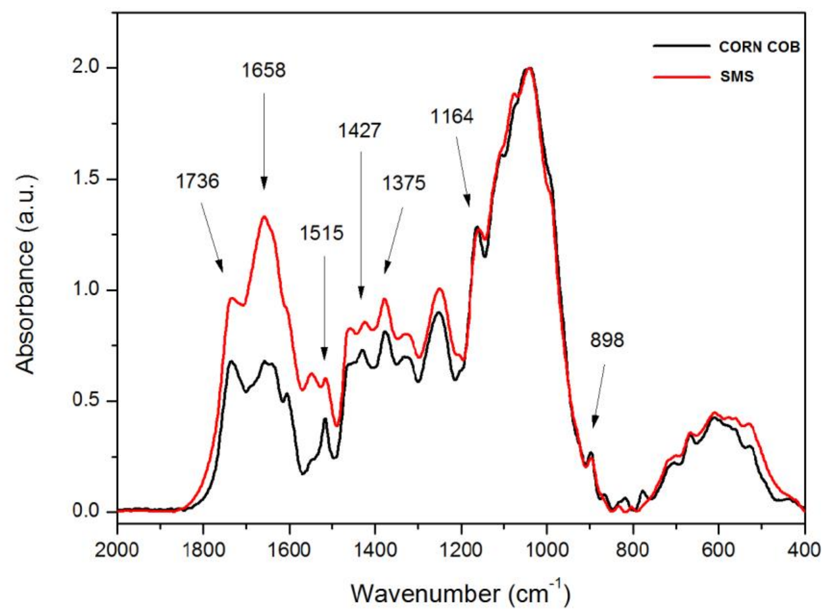

Figure 4.

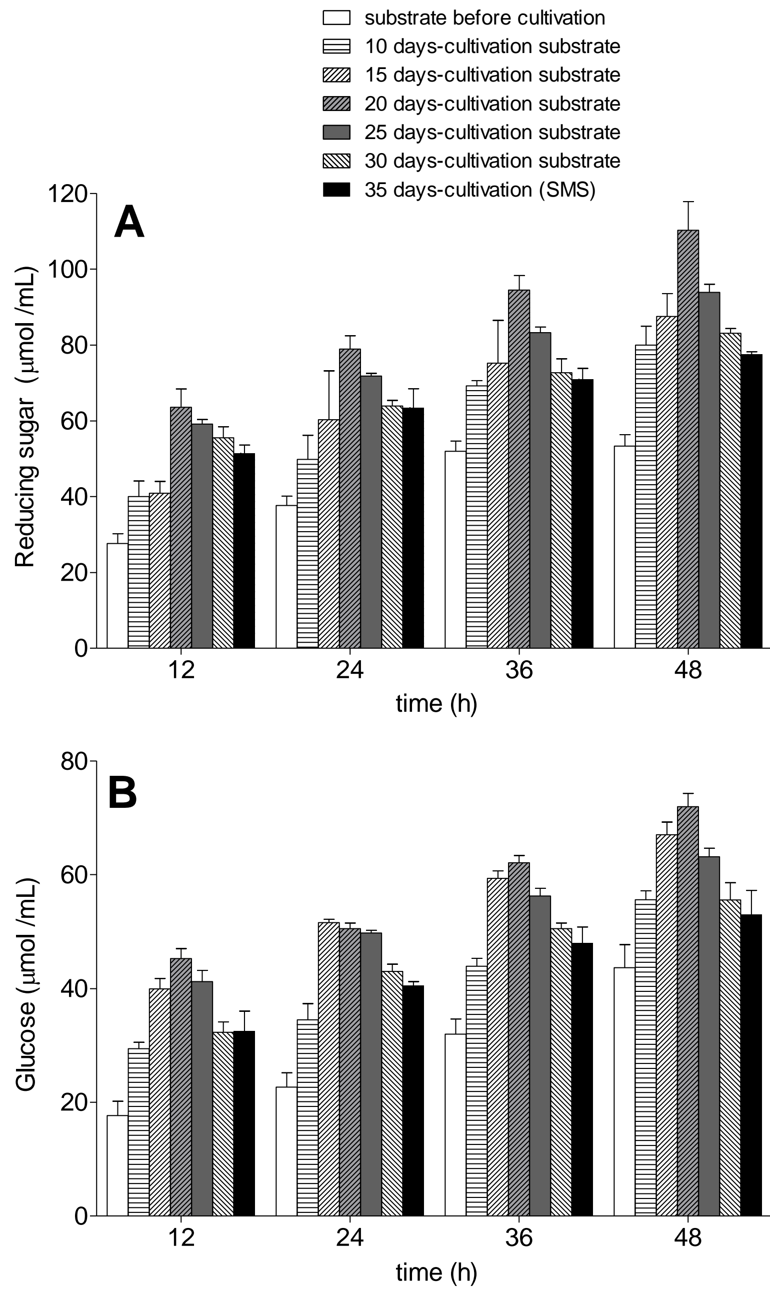

Figure 5

Table 1. FTIR bands assignments and diminutions in specific bands in the spectra of corn cob substrate caused by *P. pulmonarius* (after mushroom production, first flush).

Band (cm^{-1})	Assignments	Percent diminution
1736	C=O carbonyl and acetyl group vibration of hemicellulose and lignin	47.30 ± 4.50
1515	aromatic skeletal vibrations in lignin	39.00 ± 6.90
1427	syringyl and guaiacyl condensed nuclei	26.10 ± 2.80
1375	cellulose and hemicellulose	12.50 ± 3.30
1164	C–O –C asymmetric stretching of hemicellulose and lignin	13.70 ± 4.10
1098	crystalline cellulose	21.30 ± 2.00
898	amorphous cellulose	7.80 ± 1.20