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Fungi and Circular Economy: *Pleurotus ostreatus* Grown on a Substrate with Agricultural Waste of Lavender, and Its Promising Biochemical Profile

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Abstract: The increasing production of essential oils has generated a significant amount of vegetal waste that must be discarded, increasing costs for farmers. In this context, fungi, due to their ability to recycle lignocellulosic matter, may be used to turn this waste into new products, thus generating additional income for essential oil producers. The objectives of our work, within the framework of the European ALCOTRA project FINNOVER, were two-fold. The first was to cultivate *Pleurotus ostreatus* on solid waste of lavender used for essential oil production. The second was to provide, at the same time, new products that can increase the income of small and medium farms in the Ligurian Italian Riviera. This paper presents two pilot tests in which *P. ostreatus* was grown on substrates with five different concentrations of lavender waste, ranging from 0 to 100% (*w/w*). Basidiomata grown on all the substrates and their biochemical profiles were characterized using high-performance liquid chromatography coupled to mass spectrometry. The biochemical analysis of mushrooms proved the presence of molecules with antioxidant and potential pharmacological properties, in particular in mushrooms grown on lavender-enriched substrates. The results open the possibility of producing mushrooms classified as a novel food. Furthermore, the results encourage further experiments aimed at investigating how different substrates positively affect the metabolomics of mushrooms.

Keywords: essential oil production; agro-waste recycling; mushroom cultivation; closing the loop; HPLC-MS analysis

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1. Introduction

Due to environmental changes and the high degree of competitiveness of national and international markets, the agri-food industry faces numerous economic challenges; hence, the industry is constantly looking for economically sustainable solutions. These difficulties are manifested in rural areas where small- and medium-sized farms face challenges to market their products and to make them competitive with multinational corporations or foreign products. These difficulties may be caused by the poor efficiency of the production cycles and/or by the lack of competitiveness of the products themselves. A circular economy could be a valid alternative to the habitual "take-make-waste" approach, providing a concrete solution to transform the system into a more efficient, sustainable, and eco-friendly approach. Many studies have highlighted that a circular approach, through closing the production loop, minimizes external inputs and the production of additional waste, making the processes both economically and environmentally sustainable [1]. A circular approach, if properly applied, could allow the survival of many local farm businesses that would

otherwise not be economically sustainable. In recent years, the scientific community has worked to optimize production processes from a circular economy perspective.

Concerning bioresources, research has focused on the isolation and selection of particular organisms that are exploitable in circular processes [2]. In this context, fungi—due to their natural roles in ecological cycles—are good candidates to be exploited to recycle and to transform vegetal by-products and waste from agriculture into more valuable products. To date, there are more than 100,000 known species of fungi, but it is estimated that there are over 3,000,000 species that are still unknown [3]. Due to their biological characteristics, fungi are able to colonize almost every environment on Earth, and thus they play a key role in nutrient cycling in trophic chains. For example, lignicolous fungi (that is, wood-decaying fungi) play a crucial role in recycling lignin and cellulose in forest ecosystems [4]. On this basis, it is clear that several species of lignicolous fungi can also be applied fruitfully from a circular economy perspective and can be exploited to turn woody by-products and waste from agriculture into food or other valuable products. Mushrooms, in particular, are a group of fungi characterized as having sporomata (fruiting bodies) visible to the naked eye; they have been collected and cultivated by humans for thousands of years both for food and for medicinal purposes [5]. Almost all traded mushroom species today are saprotrophic or lignicolous fungi cultivated on substrates based on different decaying organic matter, most frequently wood. Recently, the increasing use of mushroom species such as *Pleurotus ostreatus* (Jacq.) P. Kumm., *Lentinula edodes* (Berk.) Pegler, *Ganoderma lucidum* (Curtis) P. Karst. and *Grifola frondosa* (Dicks.) Gray for food [6,7], nutraceutical products, and cosmetics [8,9], has motivated scientists to undertake new research regarding the applications of mushroom-forming fungi. Some examples are enzyme production through solid or liquid fermentation [10,11], bioremediation of polluted environments [12], new composite biomaterial for green building [13], and medical and nutraceutical applications [5,9]. In a recent paper, an international group of researchers [14] further underlined how, due to the research carried out in recent years, fungi can play a central role in our lives and, if properly exploited, they can help address many of the challenges humans are likely to face in the future.

P. ostreatus, also known as the oyster mushroom, is one of the most used and affordable species of cultivated mushrooms globally [6]. Due to its economic, ecological, and medicinal values, *P. ostreatus* is widely cultivated. Several authors have highlighted its usefulness in bioremediation of petroleum and aromatic polycyclic hydrocarbons [15–17]. Furthermore, from a biochemical perspective, *P. ostreatus* is an excellent supplier of different nutrients and well-known molecules with beneficial effects, such as vitamins, amino acids, and essential fatty acids. The nutritional value of these compounds, and their numerous effects, such as anti-inflammatory and antioxidant properties, are well known [18–20]. Within the extensive literature on *P. ostreatus*, research about cultivation techniques and the effects of different substrates on the production of sporomata has shown how different agricultural waste can be used for sporomata production [21–26]. These findings confirmed the versatility of this mushroom and suggest the possibility of cultivating it on other types of agricultural waste from a circular perspective.

True lavender (*Lavandula angustifolia* Miller) and its cultivars are widely utilized in the essential oil industry. The natural range of *L. angustifolia* lies in the south of France, the Pyrenees (Spain) and, partially, the north-western Italian Riviera [27]. Today, in addition to France and Spain, Bulgaria, the United Kingdom, China, Ukraine, and Morocco are among the biggest global producers. Bulgaria, in particular, has recently become the world's leading producer, producing 100 tons of lavender oil per year. During the extraction process, the relatively low quantity of essential oil in fresh lavender (0.8–1.3%) results in enormous quantities of solid residues (tens of thousands of tons globally) that still have a high content of useful substances. These wastes are usually discarded directly in nearby locations or disposed of as special waste, leading to potential environmental issues.

In this work, within the framework of the European project ALCOTRA 1198 FINNOVER (http://www.interreg-finnover.com accessed on 3 May 2021), we hypothesized that *P. os-*

treatus can be cultivated on substrates enriched with waste derived from the extraction of lavender essential oils. The pilot tests were established at the Stalla Company, a small agricultural business in Liguria (north-western Italy). The company was founded in 1900 and in the past 20 years has cultivated and hybridized several species of flowers, including the *L. angustifolia* cultivar *imperia* used in this work. The activities were carried out in two pilot tests with the main goal of confirming the feasibility of cultivating *P. ostreatus* mushrooms on waste of the *L. angustifolia* cultivar *imperia* in a small local and rural business. The biochemical profile of sporomata grown on lavender-based substrates was analyzed through high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) to evaluate the variation due to the lavender enrichment. The biochemical data showed that the mushrooms grown on the enriched substrates have a high content of useful substances that can add value to the final product. The results of this work highlight the possibility of using lavender residues according to the circular economy principle for the production of mushrooms.

2. Materials and Methods

2.1. Pilot Tests

P. ostreatus was used in the tests because it has several interesting characteristics: it grows quickly, is relatively easy to manage, has good organoleptic characteristics, and has interesting, well-known medicinal properties. Moreover, it also grows spontaneously in many areas of Liguria and northern Italy.

In 2017, two strains of *P. ostreatus* called POA (autochthonous wild strain, isolated from a Ligurian locality in the province of Savona) and POC (domesticated strain) were isolated and tested for the pilot plant cultivation. The two strains were isolated under a laminar flow on Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) at 24 °C for 15 days. Once purified, the two strains were preserved on PDA slants at 6 °C in the ColD collection of the Laboratory of Mycology DiSTAV (University of Genoa). The two tests described started in December (2018 and 2019, respectively) with spawn production and incubation, and the cultivation phases were carried out in spring at the Stalla Company.

Substrates were prepared according to Yang et al. [23] and modified as follows. In December, plant residues of the *L. angustifolia* cultivar *imperia* derived from the extraction of lavender essential oil were properly shredded and mixed with barley straw for the preparation of growth substrates with different lavender/straw ratios (Table 1). The water content of the final mixed substrates was adjusted to 70%.

Table 1. Details of the different percentages of lavender present in the substrates.

	% Barley Straw	% Lavender
1	100	0
2	0	100
3	50	50
4	60	40
5	70	30

The prepared substrates were placed in 25 × 45 cm thermoresistant polypropylene bags (600–700 g per bag), sealed, and autoclaved at 125 °C for 30 min. Once cooled, the substrates were inoculated under a laminar flow hood using 30 g of 7 day old cultures of the POA and POC strains grown on PDA. The spawn was incubated in the dark at 24 °C and 65–70% relative humidity (RH) for 50–60 days to allow the mycelium to colonize the entire substrate.

The pilot cultivation test was carried out in April at the Stalla Company. The pilot plant was set up in a company greenhouse adapted for cultivation. The 25 m^2 pilot area was set up using special sterilized plastic tarps. Cultivation benches (90 cm high) were set up in the pilot area, adjacent to a fog irrigation system to manage the RH during the cultivation phase. In addition, to prevent contamination, a small anteroom was set up to

provide double protection against contaminants to the pilot area. Before starting cultivation, all surfaces were sanitized using a 20% hypochlorite solution. The spawn was placed in the pilot area: all bags were placed on the benches, spaced 20 cm apart from each other. To enable the formation of primordia, six holes of 2 cm in diameter were made using a sterile scalpel on each spawn bag. During the cultivation period, the fog irrigation system was used to keep the RH at about 80%; moreover, the irrigation contributed to control the temperature at around 22 ± 3 °C during the entire period of cultivation.

The basidiomata were harvested when the pileus margins were flat to slightly rolled upwards. They were counted, weighed, and preserved at −20 °C for the subsequent biochemical analysis.

The weight data of the collected basidiomata were processed using Microsoft Excel to calculate the mean, perform one-way analysis of variance (ANOVA), and create charts of production on the different substrates. In addition, the percentage yield on the different substrates (Y) was calculated using the following equation:

$$Y = \frac{\text{basidiomata fresh weight}}{\text{weight of substrate}} \times 100$$

2.2. Sample Preparation and Biochemical Analysis

The basidiomata were divided into aliquots of 2 g fresh weight each, taking care to eliminate growth substrate usually attached to hyphae. Each aliquot was placed within an Eppendorf tube. The samples obtained were dried using a Speed-Vac freeze dryer slightly heated to 35 °C to facilitate the evaporation of the water in the vegetation. The samples were finely pulverized in a mortar to obtain a greater contact surface with the solvent, placed inside heat-resistant glass vials with 2 mL of anhydrous ethanol, methanol, or acetonitrile next to a magnetic anchor, and the vials were hermetically closed. Water was not used as an extraction solvent because in samples rich in polymeric sugars such as those examined, there is a rehydration of the sample itself, which is not desirable because it prevents effective filtration and centrifugation. The sample was left for 2 h on a heating plate at 55 °C and then filtered through filter paper and transferred to settle in a test tube in a refrigerator for 30 min. This allowed agglutination of the residual excess carbohydrates, causing it to precipitate. A final centrifugation at 13,000 rpm for 10 min was used to obtain clear extract without solid residue.

The HPLC-ESI-MS analysis was performed using an Agilent 1100 chromatograph directly coupled with an MSD ion trap mass spectrometer. Chromatographic separation was conducted using a C-18 Symmetry column (Waters Corporations). The column was chosen considering the complexity of the matrix to be analyzed and the reproducibility of the method.

For the mobile phase, HPLC-grade acetonitrile (Merck, Darmstadt, Germany) and Milli-Q water (Millipore Corp., Bedford, Italy) were used, and were both filtered, degassed, and added, respectively, to 0.5 and 1% formic acid (Carlo Erba, Sabadell, Spain) to facilitate and improve ionization. SIAD (Bergamo, Italy) supplied the research-grade nitrogen (>99.995%). Absolute ethanol (Carlo Erba) was used for the extraction. The characterization of a given signal can be undertaken by analyzing the "full scan" and tandem spectra with Massbank EU, an exhaustive "open access" tool available on the Internet. The m/z ratio of the parent ion was entered and, to focus the search and exclude some substances, the m/z ratios of one or more fragment ions were also entered. For each search, a relative intensity normalized to 100 and an adequate tolerance regarding the accuracy and resolution of the instrument used was set. In our case, although the instrument used has an accuracy of 0.05 UMA when considering the m/z ratios, the tolerance was set at 0.3 UMA.

The main issue with this kind of sample is the dominant presence of long-chained polysaccharides, such as chitin and related polymers, whose behavior as "chemical sponges" can interfere with the optimal separation in HPLC and ionization in the mass spectrometer. To overcome these problems, great care was applied in the filtration and centrifugation

processes; these steps are essential to ensure the quality of the samples that are injected in the HPLC-MS system.

3. Results

3.1. Pilot Tests

The entire mushroom production process on substrates enriched with lavender waste was established to be cost effective and well integrated, with the lowest impact on the usual activities of the company. As shown in Figure 1, the mushroom production process began with the waste derived from essential oil extraction (center of Figure 1). After the mushroom production process, the exhausted substrate consisting of vegetable material partially degraded by the biological activity of the mushrooms was used within the company as a soil conditioner or to produce biocompost.

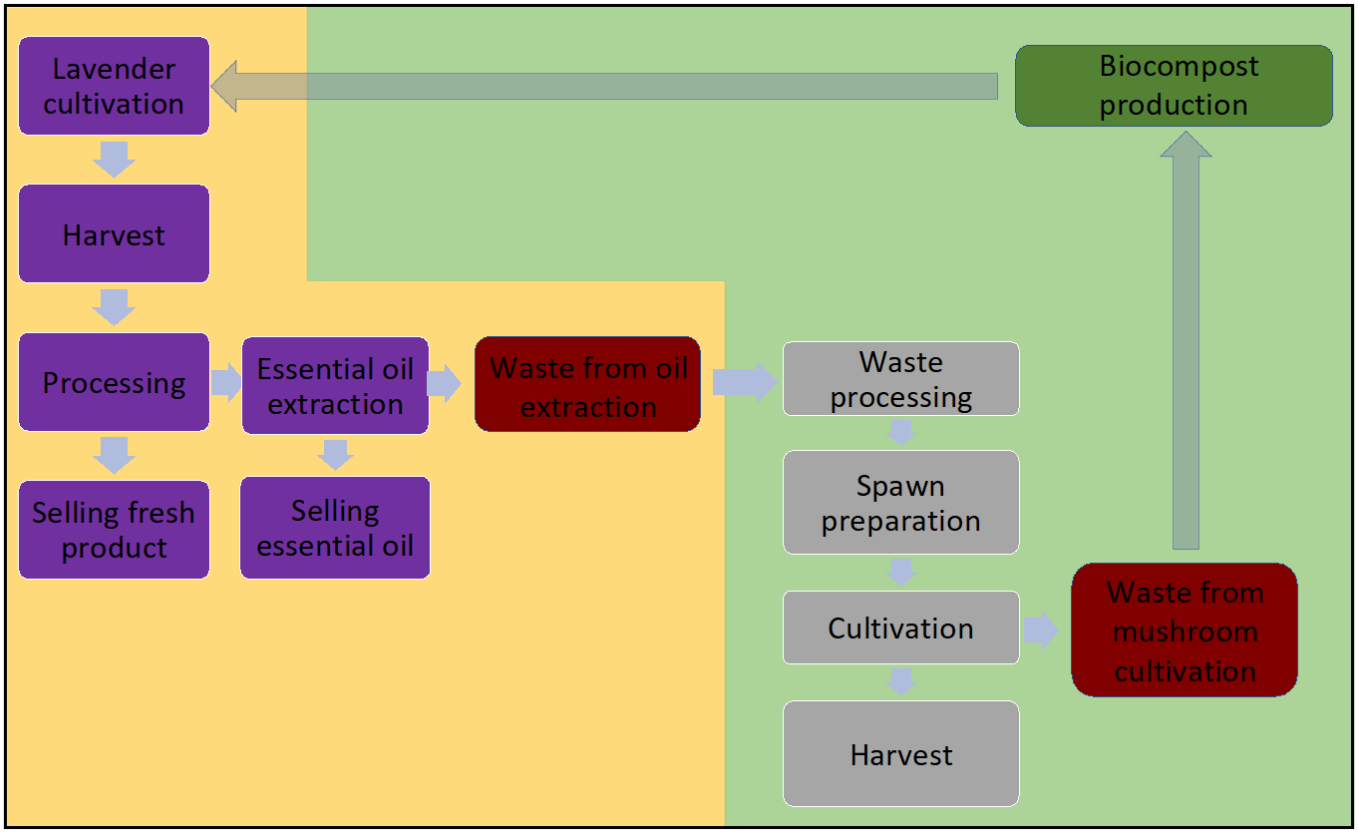

Figure 1. The entire circular process for mushroom cultivation. The yellow area concerns the cultivation of lavender and the essential oil production process. The green area represents the mushroom production process and the valorization of the spent substrates for biocompost production.

In 2018 (the first pilot test), during the incubation phase, 97% of the inoculated bags completed the incubation phase, whereas 3% were contaminated by a common microfungus of the genus *Trichoderma*. In 2019 (the second pilot test), all of the incubated bags reached the optimal colonization. The incubation time required to achieve complete colonization of the substrates was 40 ± 5 days in 2018 and 47 ± 7 in 2019. The substrates consisting of 100% lavender residues needed 12 ± 8 days longer in 2018 and 15 ± 7 days longer in 2019 to complete colonization compared with the control substrate. There were no significant differences between the two strains used regarding growth. During the cultivation phase, 90 and 95% of the bags in 2018 and 2019, respectively, reached the production of primordia and developed fruit bodies.

The weight of the basidiomata grown on the different substrates in 2018 and 2019 is shown in Table 2. The one-way ANOVA confirmed that the substrate composition

affected the weight ($p < 0.01$), but there was no difference in the growth between the two strains tested.

Table 2. Mushroom production (expressed in grams) on the different substrates tested.

Substrate	2018		2019	
	POA	POC	POA	POC
1	493	537	851	826
2	287	294	456	490
3	370	407	623	538
4	424	435	594	545
5	452	439	793	753

As shown in Figure 2, the percentage yield (Y) for both pilot tests showed a lower production efficiency for the substrate containing high lavender waste concentrations compared with the control (100% straw), which had a Y between 27.4 and 29.8. The substrate with 100% lavender had a Y between 15.2 and 16.9, the substrate with 50% lavender had a Y between 17.9 and 22.6, and the substrate with 40% lavender had a Y between 18.2 and 24.2. The Y for the substrate containing 30% lavender—between 24.4 and 26.4—was closest to the control substrate.

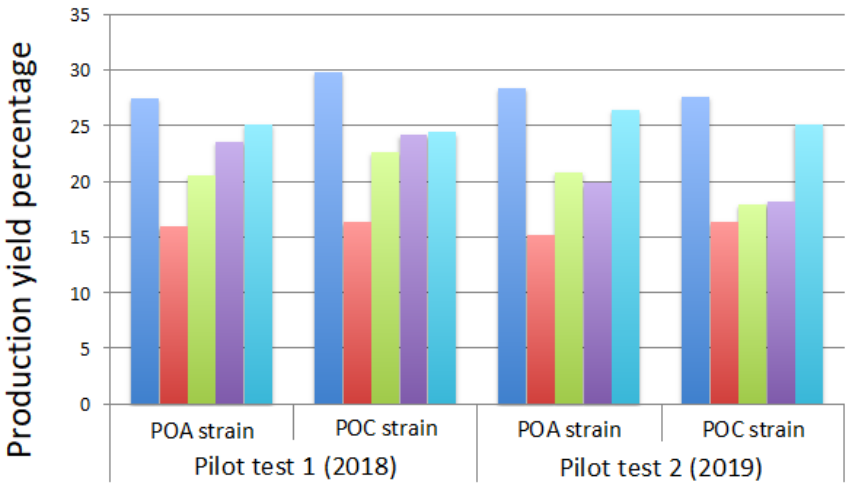

Figure 2. Production yield expressed as the percentage of the two strains grown on different substrates (each color refers to different straw/lavender ratios: blue = 100/0; red = 0/100; green = 50/50; violet = 60/40; light blue = 70/30) containing different concentrations of lavender waste in 2018 and 2019.

3.2. Biochemical Analysis

The analysis of sporomata extracts grown on the substrates with lavender showed that an average mass of extract between 1.6- and 2.1-fold higher in weight was produced compared with the sporomata grown on the control substrate. In addition, domesticated sporomata grown on the control substrate tended to produce fewer metabolites than the wild strain. The analysis was performed using multiple samples of mushrooms grown under the same conditions. This examination demonstrated the repeatability of the extraction and analytical methods. As shown in Figures 3 and 4, the chromatograms of the extracted samples overlap perfectly.

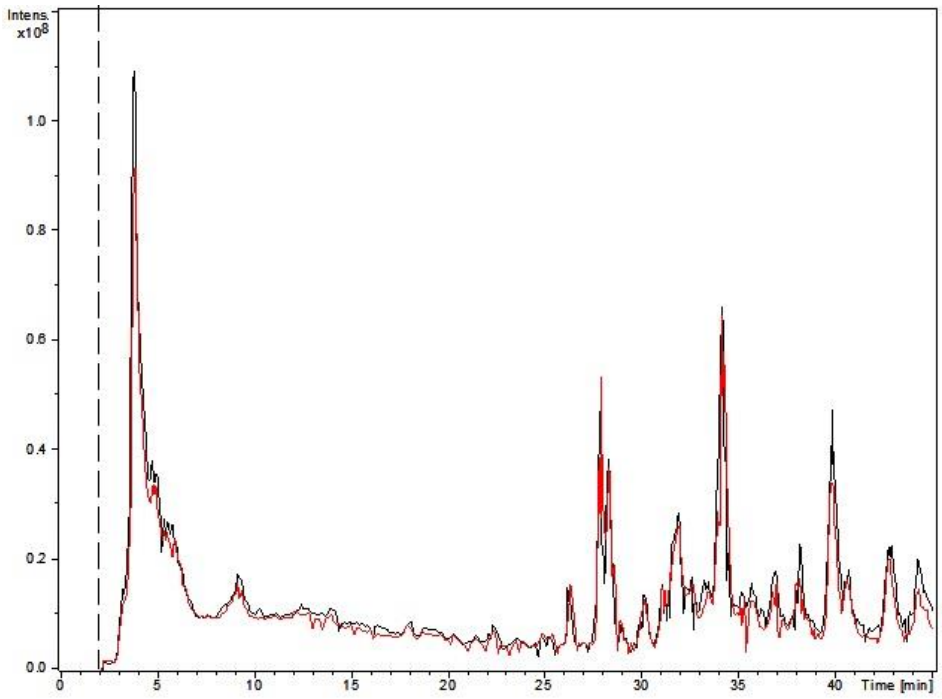

Figure 3. A comparison between samples of the same strain grown under the same conditions and extracted in ethanol. The perfect overlap of the different extracts confirms the reliability and reproducibility of the extraction and analytical methods.

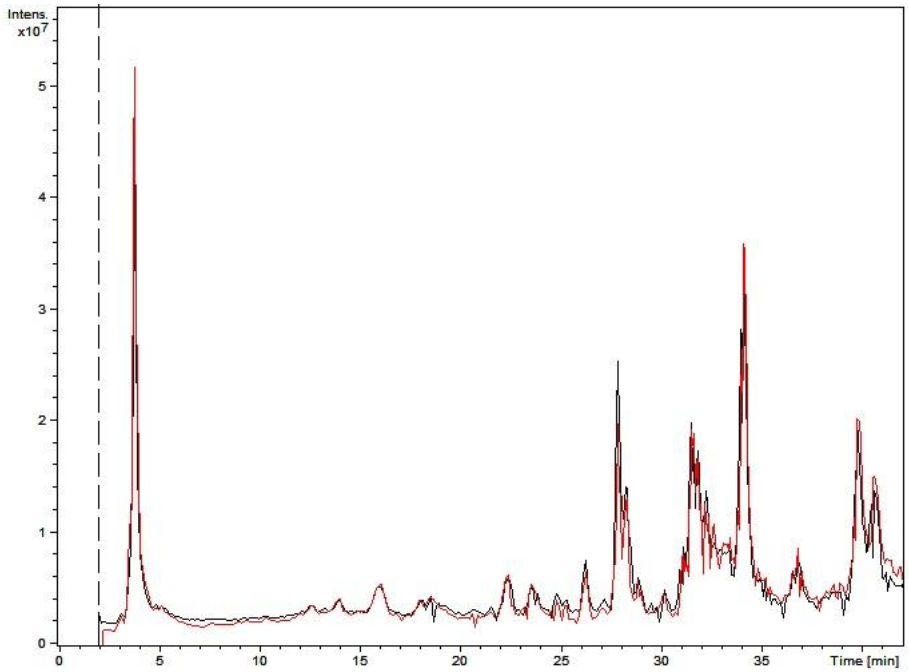

Figure 4. A comparison between samples of the same strain grown under the same conditions and extracted in acetonitrile.

This method isolated and identified >50 molecules belonging to numerous chemical families, such as di-tripeptides, fatty acids, and their epoxides. There were no major differences between the domesticated and wild strains regarding production of biomass and metabolites, although there were differences in the metabolite profile of *P. ostreatus* harvested on only straw compared with the enriched substrates. As shown in Table 3, the

HPLC-MS method allowed dividing the various classes of compounds into five groups relative to their retention time within the chromatographic analysis. The obtained separation could be very useful because it is predictive of the biological activities that each fraction, or specific compounds, from *P. ostreatus* extracts may have for different applications, including for human health.

Table 3. Classification of the isolated and identified compounds relative to the percentage of eluent B and their retention time in high-performance liquid chromatography.

Group	% of Acetonitrile	Retention Time (min)
Group 1 carboxylic acids, alcohols, and nucleosides	0–11	3–5
Group 2 carbohydrates, amino acids, dipeptides, tripeptides, and their derivatives (Amadori products)	15–32	7–15
Group 3 nucleotides, polyphenols, and lactones	35–48	16.5–20
Group 4 fatty acids and their derivatives	50–69	22.5–30
Group 5 cholanic acid and apolar compounds	70–100	35–40

4. Discussion

The two pilot tests carried out demonstrated the application of mushroom cultivation on a lavender farm following the circular economy principle. This process, as already proposed by other authors, enables farmers to reuse the agricultural waste produced during the main production cycle to obtain a new product [11,20,26]. The particular substrate leads to distinct metabolomic profiles, a phenomenon that improves the commercial value compared with mushrooms grown on traditional substrates. This difference compensates for the lower yield of mushrooms. These data are consistent with the feasibility study carried out as part of the FINNOVER project (www.interreg-finnover.com accessed on 3 May 2021). The vegetal waste partially inhibited the mycelial development, increasing the incubation time when grown on the substate with lavender, and, later, the development of basidiomata (lowering the production yield compared with the control). Based on the average yields reported in Table 1, there were significant differences in the weight of basidiomata produced on the substrates tested ($p < 0.001$), but there were no differences in the biomass of the produced sporomata between the two strains tested. This result was further confirmed by calculating the production efficiency index Y, as shown in Figure 2. These calculations revealed that the yields on a substrate composed of 100% of lavender residue were lower than those on the control substrate composed exclusively of straw. These differences in biomass production suggest the use of lavender residues to grow mushrooms may be unsuitable because, as shown in Figure 2, the Y value is inversely proportional to the lavender waste content in the substrate. However, as discussed below, the metabolomic characteristics and added nutraceutical value provided by the lavender to the cultivated basidiomata could compensate for a small drop in production. Because the substrate containing 30% lavender residue had slightly lower yields (ranging from 1 to −5.4%) than those observed in samples without lavender residue, this substrate may provide an appropriate balance between biomass production and improved metabolomic profile.

P. ostreatus is a well-known food that is rich in essential dietary elements [28,29]. Specifically, we found that the wild strain is a slightly better producer of metabolites in response to the presence of an environmental substrate than the domesticated strain, if dried extracts and just secondary metabolites are considered. This variable production probably depends on the fact that the domesticated strain, which has been cultivated for

generations in a protected and controlled environment through cloning of the mycelium—a type of asexual reproduction that does not allow genetic mixing—depresses or silences in successive generations various secondary metabolic pathways that are no longer useful for maintaining physiological activity. These metabolic pathways are not silenced in the wild strain, because in nature it is in direct contact with environmental stressors, and direct competitors and pathogens. The substrate enriched with lavender, a plant particularly rich in terpenes and other molecules it produces as antibacterial and antifungal agents, stimulates the fungus to activate its secondary metabolism, as evidenced by the presence of compounds that are not part of the fungus's usual primary metabolomic pattern. Although weight data from the different growth phases of the fungus showed that the fungus grown on straw generally produced greater biomass, HPLC-MS and tandem mass analysis showed that the levels of *n*-acetylglucosamine and its precursors remained constant in each sample, independently of the type of substrate used for growth.

Of the many molecules extracted and characterized, significant interest exists in the amino acids, glucosides, and small dipeptides, that are particularly known in the fungi kingdom. There are myriad roles for these peptides and their derivatives; it has been shown that these peptides have antifungal, antimicrobial, immunostimulant, and growth-promoting properties [30]. The evolution of these peptides can be seen in the large cyclized peptide molecules produced by some fungi, such as amatoxins of the *Amanita* genus, a clear evolutionary trend that leads from simple molecules to complex cycled peptides with defensive functions. Amanitin toxins, in general, are among the most effective toxins produced by superior fungi, with a clear defensive role. Therefore, the condition by which peptides and dipeptides are produced in *P. ostreatus* appears to be archaic or, at least, less evolved [30]. Of significant interest is the notable amount of glutathione in the samples. Its action is highly relevant regarding free radicals and peroxide ions, and largely justifies the powerful antioxidant action of *P. ostreatus* extracts analyzed on cell cultures treated with oxidizing agents (personal data).

Of note is the presence of fatty acid epoxides, particularly those of myristoleic, linoleic, and linolenic acids produced by the fungus grown on lavender-enriched substrates. Fungi are an excellent source of polyunsaturated fatty acids, whose role has long been recognized in the prevention of inflammatory heart and other diseases [31]. Moreover, it is evident that a balanced intake of polyunsaturated fatty acids is fundamental for human health. We also found a notable share of palmitic acid, a saturated fatty acid. The samples of *P. ostreatus* grown on substrate enriched with lavender also showed the presence of numerous molecules derived from fatty acids, in particular a series of fatty acid epoxides. These molecules are produced by the fungus in response to environmental stresses through the activation of the monooxygenase domain of cytochrome P450, which is common to all fungi and has been highly preserved during evolution [32]. In particular for linoleic and linolenic acid, in the samples cultivated on substrates enriched with lavender, we noticed a partial decrease in the content of polyunsaturated fatty acids in favor of the presence of fatty acid epoxides. This trend was also confirmed by the presence of cholanic acid in the samples of fungi cultivated on substrates enriched with lavender. These molecules of steroid origin are similar to those found in human bile salts and mammals in general. These molecules are probably involved in the fatty acid mobilization inside fungal cells, consistent with the presence of lipid drops in the fungal cells. The presence of fatty acid epoxides in fungi is interesting. It is known that fatty acids often have cytotoxic activity or otherwise are harmful to health [33]. It should be noted that epoxides of fatty acids were only present in fungi cultivated on enriched substrates, and were absent or present in trace amounts in fungi grown on only straw, confirming that lavender acts as a stressor, and suggesting the role of fatty acids epoxides as biomarkers of stress in Basidiomycota. In contrast to plants, in which the role of peroxygenases in the production of fatty acid epoxides is known, in fungi it appears that only the monooxygenase domain of cytochrome P450 is involved in their synthesis [34]. Nevertheless, the role of fatty acid epoxides in the regulation and suppression of inflammatory processes has been recognized [35]. Several

toxic and tumor growth-promoting activities are known [36], so further investigation to verify the activity of fatty acid epoxides produced by *P. ostreatus* would be useful.

5. Practical Implications of This Study

The pilot tests conducted allowed us to evaluate the technical feasibility of exploiting the cultivation of mushrooms to recycle agricultural residues in small rural farms. The results confirmed the feasibility of this approach, but showed a lower production yield compared with the standard substrate in optimal conditions. The reasons for this reduced efficiency are due to the different substrate and to the fact that the Stalla Company, which produces lavender essential oil, is not specialized in the production of mushrooms. Although the yields were low, a positive aspect that emerged from these tests is the presence of interesting substances within the basidiomata. This positive effect, due to the different substrates, should be investigated because it could add value to the product and make it extremely profitable for the farmers. Future tests will contribute to optimizing and improving the efficiency of the process, and to better understand how different residues can influence the biochemical composition of the fungi produced.

6. Conclusions

The pilot tests carried out in this work showed that mushrooms can be fruitfully exploited in the agricultural circular economy. In particular, we found that a lavender-based waste product could be recycled, resulting in interesting characteristics from a food that is currently commercially available, making it a niche product and a potential novel food. The same model could be tested using different residues and fungi. By evaluating different combinations, other interesting products could emerge that may be exploited in different rural contexts. The results obtained in this work should spur further research on this topic.

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