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Transcriptomic and Metabolomic Profiling of *Pleurotus eryngii* Cultivated on Olive Mill Solid Waste-Enriched Substrates

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Abstract

Olive Mill Solid Waste (OMSW) presents an environmental challenge due to its toxicity and difficulties in its recycling. Prior studies suggest its potential as a substrate ingredient for cultivating edible mushrooms. Here, we investigate how varying OMSW concentrations in the substrate affect the synthesis pathways of α-glucan and β-glucan polysaccharides, alongside transcriptional and metabolic changes in *Pleurotus eryngii*. We also assessed the mushroom's protein and nitrogen content. Our results highlight the critical role of substrate composition, demonstrating that the OMSW concentration significantly influences mushroom growth, yield, protein content, gene expression, and metabolite profiles. These findings establish OMSW not only as a viable recycling resource but also as a modulator of health-promoting compound synthesis in *P. eryngii*.

Keywords: *Pleurotus eryngii*; Olive Mill Solid Waste (OMSW); functional metabolites; differential gene expression; mushroom cultivation

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1. Introduction

The olive oil industry generates substantial byproducts, with Olive Mill Solid Waste (OMSW) presenting a major ecological concern. Concentrated mainly in Mediterranean countries, this industry produces over two million tons of OMSW each harvest season (November–February), constituting approximately 95% of global OMSW output. OMSW is rich in lignocellulosic material and polyphenolic compounds, which impede biodegradation, causing recycling challenges and potential water and soil pollution [1,2].

Current OMSW management strategies include composting, anaerobic digestion, and thermal combustion [3–5]. Fungal bioconversion has emerged as a promising alternative, utilizing lignocellulose-degrading white-rot fungi equipped with enzymes such as laccases, manganese peroxidases, and cellulases to efficiently break down complex plant polymers [6–8]. Among these, *Pleurotus* spp., including *Pleurotus eryngii*, are noted for rapid growth on diverse lignocellulosic substrates, and have been extensively studied for agricultural and industrial waste valorization [9–11].

P. eryngii is valued for its health-promoting properties, especially its immunomodulatory α-glucans and β-glucans, key structural polysaccharides of the fungal cell wall [12–15]. Previous studies showed that incorporating OMSW into cultivation substrates elevates glucan content, with *P. eryngii* exhibiting the highest glucan levels among seven *Pleurotus* species tested [16]. Notably, α-glucan concentrations positively correlate with OMSW levels, suggesting OMSW influences fungal metabolism and may mediate trade-offs between growth, enzyme activity, and secondary metabolite production.

We hypothesized that increasing OMSW concentration modulates *P. eryngii* metabolism, enhancing glucan synthesis by affecting development, gene transcription, and secondary metabolite pathways. This study evaluates the impact of OMSW content on *P. eryngii* growth and biochemical composition by examining fruiting body development, transcriptome and metabolite profiles, and protein content. Understanding these interactions will aid in optimizing *P. eryngii* cultivation for yield and nutritional value while addressing OMSW recycling.

2. Materials and Methods

2.1. Pleurotus eryngii Cultivation

Substrates were prepared with varying proportions of Olive Mill Solid Waste (OMSW: pH = 4.57; N = 1.21%; Ash = 18.08%), beer brewery grains, and eucalyptus wood shavings, as outlined in Table 1. A total of 0.8 kg substrate was packed into 2.0 L filtered autoclavable bags and sterilized at 121 °C for 1 h. After cooling to room temperature, bags were inoculated aseptically with 3% (*w*/*w*) *Pleurotus eryngii* Sylvan strain 3069 spawn and homogenized.

Table 1. Substrate composition for *P. eryngii* cultivation. Water content as percentage of fresh substrate. Contents of substrate components as percentage of dry matter.

Treatment	1	2	3	4
OMSW (%)	0	33	60	80
Beer brewery grains (%)	50	33	20	10
Eucalyptus shavings (%)	50	33	20	10
Water content (%)	60	58	54	52

Four substrate treatments were tested: a control (0% OMSW) and three with increasing OMSW concentrations (33%, 60%, and 80%). All bags were incubated at 23−24 °C and 50−60% relative humidity until full mycelial colonization. Fruiting was initiated by opening the bags and transferring them to a fruiting room at 18 °C and 80–90% relative humidity. Mature fruiting bodies were harvested, weighed, freeze-dried, and pulverized. The resulting mushroom powder was stored at −20 °C for further analysis. Three consecutive cultivation cycles in this methodology were examined.

2.2. RNA Extraction and Sequencing

Total RNA was extracted from 30 mg mushroom powder using the SV Total RNA Isolation System (Promega), following the manufacturer's protocol. RNA was stored at −80 °C prior to sequencing. Libraries were prepared using the PerkinElmer NEXTflex kit and sequenced on a NovaSeq 6000 platform (PE150), generating approximately 20 million paired-end reads (~6 GB) per sample.

2.3. Metabolite Profiling

Metabolites were extracted from 100 mg mushroom powder (four technical replicates per treatment) in 1 mL methanol with shaking for 2 h. After centrifugation at 14,000 rpm for 1 min, supernatants were filtered (0.2 μm) and analyzed using a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific).

2.4. Real-Time qPCR Analysis of Gene Expression

Total RNA was extracted from mushroom powder using SV-TOTAL RNA Isolation System (Promega). RT-qPCR analysis was performed with the ΔΔCt method of relative quantification with a Bio-Rad CFX, using SYBR Green (total of 20 μL reaction volume: 4 μL cDNA, 2 μL primers, 4 μL DDW, 10 μL SYBER Green mix) to monitor dsDNA synthesis,

and further analysis was performed with the CFX Maestro. GAPDH gene was used to normalize the values of relative expression. The specific primers used are as follows: β-glucan synthase Fw, 5′-CAA CAA TGC TTT TCT GGC TTC-3′. β-glucan synthase Rv, 5′-AAG AGT GCA GGC AAA ACG AT-3′. GAPDH Fw, 5′-ACC TCG AGA CTT ACG ACC CG -3′. GAPDH Rv, 5′-GTT AAC ACT ACG ACC TCC ACG-3′.

2.5. Nitrogen Analysis

Nitrogen content was determined using the Kjeldahl method. Mushroom powder samples (0.5 g) were digested with 150 mg Kjeldahl catalyst, 10 mL 98% sulfuric acid and 10 mL 32% hydrogen peroxide at 400 °C for 90 min (Büchi K-435). After cooling, the digest was distilled in 50 mL water and 60 mL 32% NaOH using a Büchi B-324 distiller. Titration was conducted with 0.1 N HCl in the presence of 65 mL 2% boric acid using a Metrohm 719 S autotitrator.

2.6. Protein Quantification

Total protein content was measured using Bradford assay. Mushroom powder samples (20 mg) were suspended in 400 µL distilled water, vortexed, and centrifuged (14,000 rpm, 15 min, 4 °C). Then, 5 µL of the supernatant was diluted and mixed with 200 µL Bradford reagent. Absorbance at 595 nm was measured using an Infinite 200 PRO spectrophotometer (Tecan). Protein concentrations were calculated from a BSA standard curve (0.125–2 mg/mL).

2.7. α- and β-Glucan Determination

Glucan levels were determined using the Megazyme Mushroom and Yeast β-glucan Assay Kit according to the manufacturer's instructions (2019 protocol). In short, total glucans were extracted from 100 mg mushroom powder samples using 12 M sulfuric acid followed by enzymatic hydrolysis with exo-1,3-β-glucanase and β-glucosidase. α-glucans were solubilized in NaOH and hydrolyzed using amyloglucosidase, invertase, and trehalase. Glucose concentration was measured using glucose oxidase/peroxidase (GOPOD) assay with absorbance at 510 nm, and glucan content was calculated accordingly.

2.8. Statistical Analysis

P. eryngii phenotypes, glucans, protein and nitrogen content: Statistical analyses were performed using JMP software v8.0 (SAS Institute). Differences between treatments were assessed using pairwise Student's *t*-tests, with significance set at $p < 0.05$.

RNA-seq: Gene expression differences were calculated using DESeq2, with significance determined at FDR-adjusted *p*-value < 0.05 (Benjamini—Hochberg correction).

Metabolite profiling: Differences between treatments were assessed using Student's *t*-tests, with significance set at $p < 1 \times 10^{-6}$.

3. Results

To evaluate the effect of Olive Mill Solid Waste (OMSW) on mushroom development, *P. eryngii* was cultivated on substrates containing varying concentrations of OMSW. Fruiting body yields were measured across two independent cultivation cycles.

Mushroom biomass production was significantly influenced by substrate composition. The highest yield was observed in the 33% OMSW treatment, indicating a potential growth-promoting effect at this concentration. Conversely, yield declined markedly at the highest tested concentration (80% OMSW), suggesting a detrimental impact for excessive OMSW on *P. eryngii* development (Figure 1).

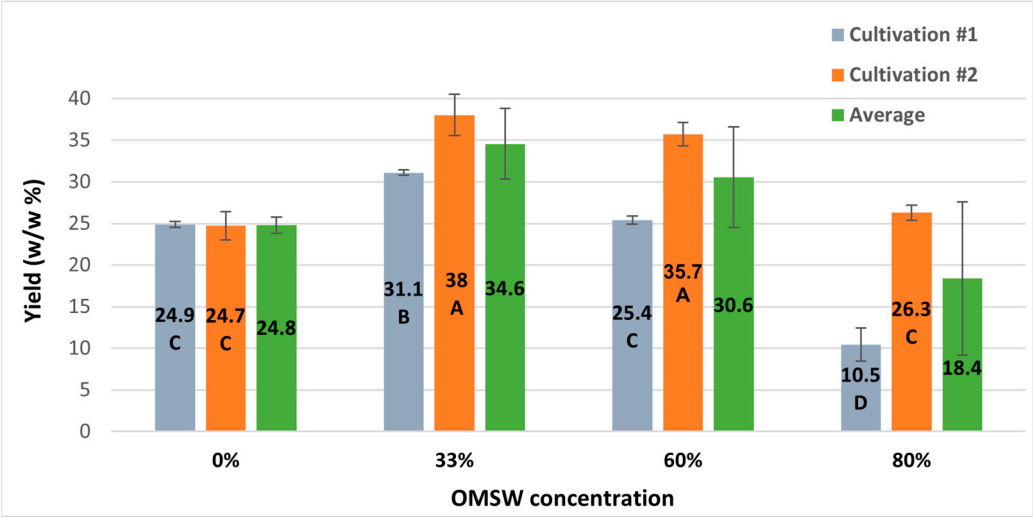

Figure 1. Mushrooms cultivated on various OMSW substrate concentrations in two cultivation cycle yields. Yields are expressed as the percentage ratio of the mushroom's fresh weight to the dry weight of the substrate. Results are expressed as mean ± SD. Statistical significance was determined using the "each-pair Student's *t*-test" ($p < 0.05$).

Morphological characteristics of the fruiting bodies, including stipe (stalk) diameter, pileus (cap) diameter, and overall height, were assessed across the different substrate treatments of the first cultivation cycle. A reduction in all measured dimensions was observed in mushrooms cultivated on substrates containing 80% OMSW. This reduction was most pronounced in fruiting body height, which was significantly lower compared to mushrooms grown on substrates containing 33% and 60% OMSW (Figure 2).

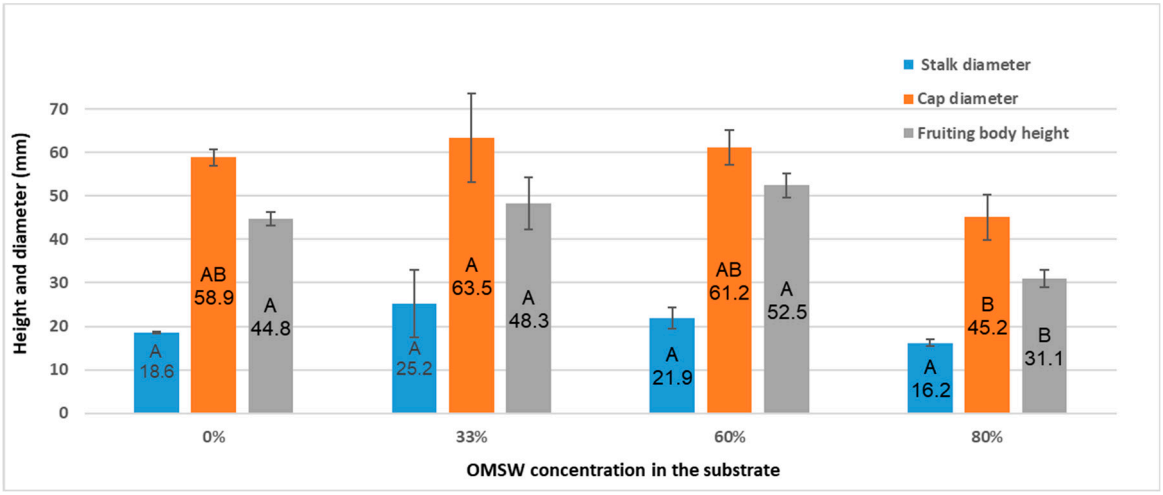

Figure 2. Height and diameter of *P. eryngii* fruiting bodies cultivated on various OMSW substrate concentrations. Results are expressed as mean ± SD. Statistical significance was determined using the "each-pair Student's *t*-test" ($p < 0.05$).

3.1. Transcriptional Profiling of P. eryngii Grown on OMSW-Enriched Substrates

To investigate the molecular basis underlying the observed phenotypic differences, transcriptome analysis was performed on *P. eryngii* fruiting bodies cultivated on substrates with varying OMSW concentrations. RNA-seq analysis revealed that OMSW content had a significant effect on global gene expression profiles.

The most prominent changes were observed in genes associated with ribosome biogenesis, secondary metabolite production, and polysaccharide biosynthesis. Gene expression

within these pathways varied significantly, showing strong upregulation or downregulation in response to different concentrations of OMSW. Additional metabolic pathways, including alanine, aspartate, and glutamate metabolism, as well as fatty acid biosynthesis, were also differentially expressed. Moreover, genes that are related to the peroxisome were upregulated, though to a lesser extent (Figure 3).

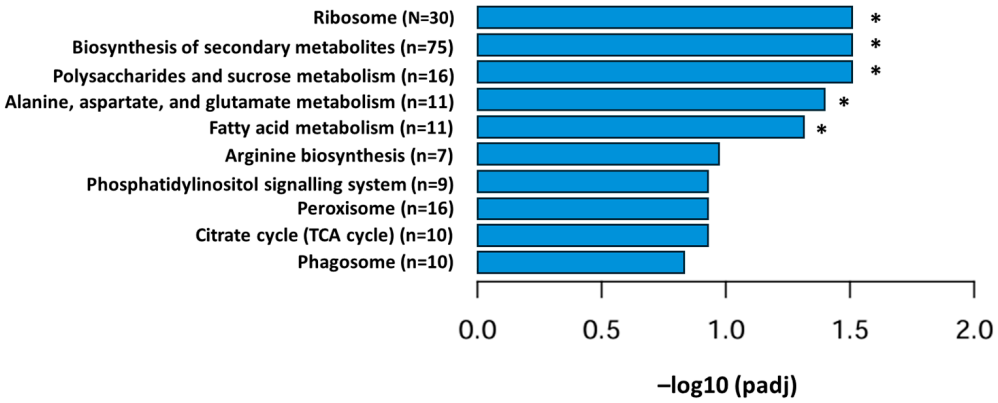

Figure 3. KEGG enrichment analysis of key metabolic pathways in *P. eryngii* fruiting bodies cultivated on 80% and 0% OMSW substrates. Gene expression was analyzed via RNA-seq, and functional annotation was performed based on KEGG pathway databases. "n" indicates the number of differentially expressed genes in the KEGG pathway. Asterisk (*) indicates FDR-adjusted p-value < 0.05 (Benjamini–Hochberg correction).

3.2. Expression of β-glucan Synthase Is Elevated in High-OMSW Substrates

The transcriptomic analysis has indicated upregulation of genes involved in polysaccharide metabolism and glycosyltransferase activity. This observation suggests an increased flux toward the synthesis and remodeling of structural polysaccharides including β-glucan, which is a key component of the fungal cell wall. (Supplementary Figure S1.) To further validate this observation, the expression of the β-glucan synthase gene was analyzed using real-time qPCR, revealing its upregulation in response to OMSW addition (Figure 4).

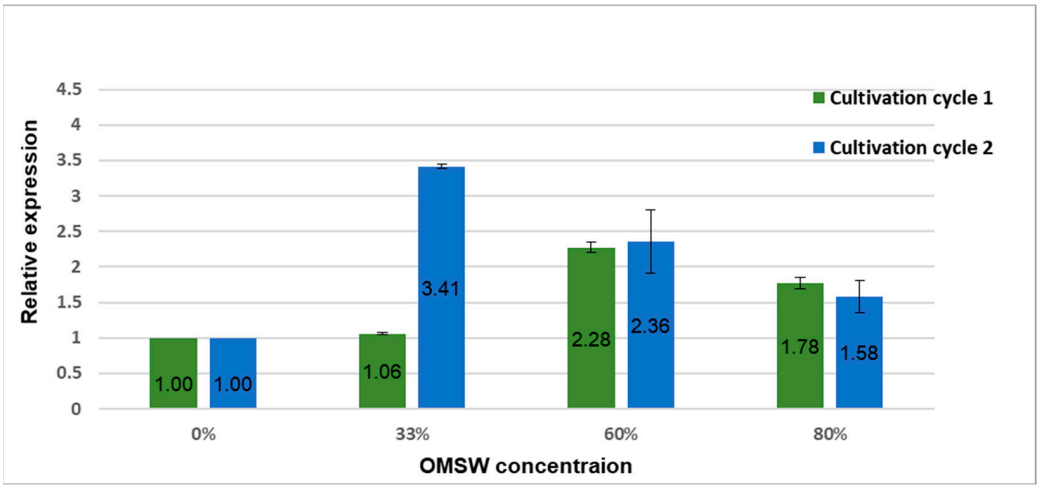

Figure 4. β-glucan synthase relative expression levels. Each column represents β-glucan synthase relative expression levels in one cultivation cycle (two technical repeats average), as tested using real-time qPCR. GAPDH expression in the 0% treatment is used as a normalizing gene. Results are expressed as mean ± SD.

3.3. Metabolomic Profiling of Fruiting Bodies Cultivated on High-OMSW Substrates

To further explore the biochemical impact of OMSW content, untargeted metabolomic profiling was conducted on fruiting bodies cultivated on substrates with 0% and 80% OMSW. LC-MS analysis identified distinct metabolic signatures between the two groups. The analysis revealed a total of 932 secondary metabolites that were differentially synthesized between the two cultivation conditions. Among these, 584 metabolites were upregulated and 348 were downregulated (80% OMSW vs. 0% OMSW). This indicates a substantial shift in the secondary metabolite profile in response to the addition of OMSW in the cultivation substrate (Figure 5).

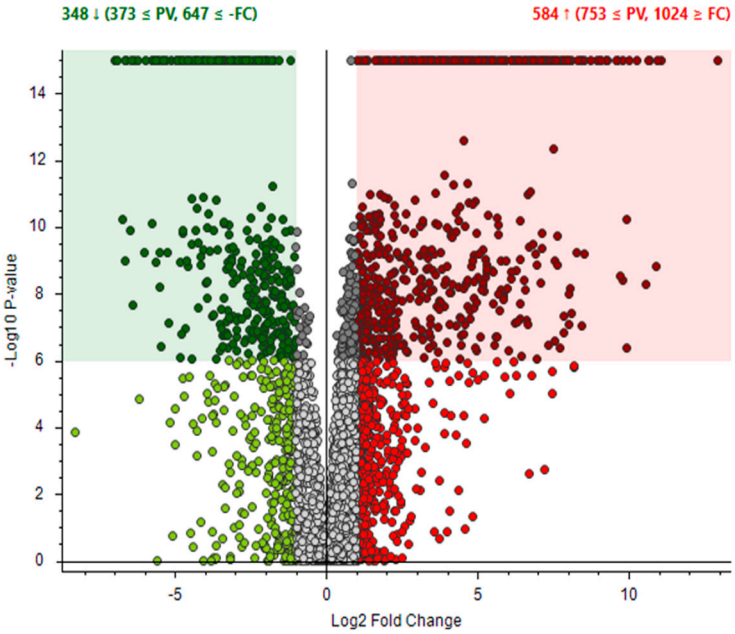

Figure 5. Relative concentration of metabolites in *P. eryngii* fruiting bodies cultivated on 80% or 0% OMSW substrate contents. The total metabolite profile was compared between mushrooms cultivated in two extremities of OMSW concentrations (obtained from a single cultivation cycle—referred to as cycle "A"). Red dots represent 584 upregulated metabolites, and green dots represent 348 downregulated metabolites. Shaded areas (green/red) indicate p-value $< 1 \times 10^{-6}$.

Among the most significantly upregulated metabolites in the 80% OMSW treatment were compounds that may aid the mushroom in mitigating oxidative stress, including veronal ($C_8H_{12}N_2O_3$), vitamin B_6 ($C_8H_9NO_3$), and mesalamine ($C_7H_7NO_3$). Mesalamine, in particular, is known as an anti-inflammatory agent widely used in the treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, where it reduces inflammation in the intestinal lining.

In addition, comprehensive Principal Component Analysis (PCA) using the metabolite profile composition in *P. eryngii* showed a clear separation between the metabolite profile of fruiting body extracts following cultivation on the four different OMSW substrate concentrations. According to the PC1 score (responsible for 44.1% of the overall variance), the metabolite profile of mushrooms cultivated on 80% OMSW substrates exhibited the most distinct metabolic profile (Figure 6).

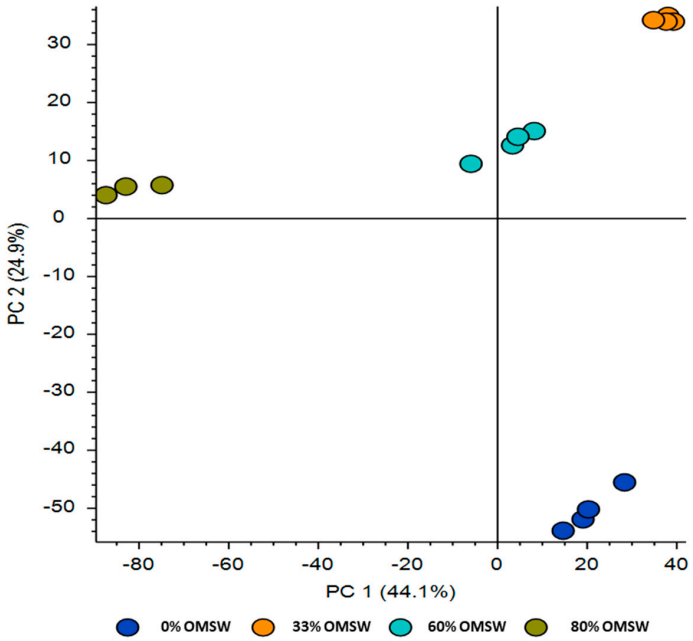

Figure 6. Relative variation in metabolite profile composition of *P. eryngii* fruiting bodies cultivated on 0%, 33%, 60%, and 80% OMSW substrate contents. The first two principal components of the PCA score plot were responsible for 69% (44.1% for PC1 and 24.9% for PC2) of the overall variance in the metabolomics profile.

3.4. Glucan Content Increases with Higher OMSW Substrate Concentrations

Given the well-documented health-promoting properties of fungal glucans, and prior evidence that the addition of olive mill waste to the cultivation substrate can influence glucan content in Basidiomycetes [17,18], we quantified α- and β-glucan concentrations in *P. eryngii* fruiting bodies in all four OMSW treatments. Our analysis revealed significantly elevated glucan levels in mushrooms cultivated on 60% and 80% OMSW substrates, compared to those grown at 0% and 33% OMSW (Figure 7).

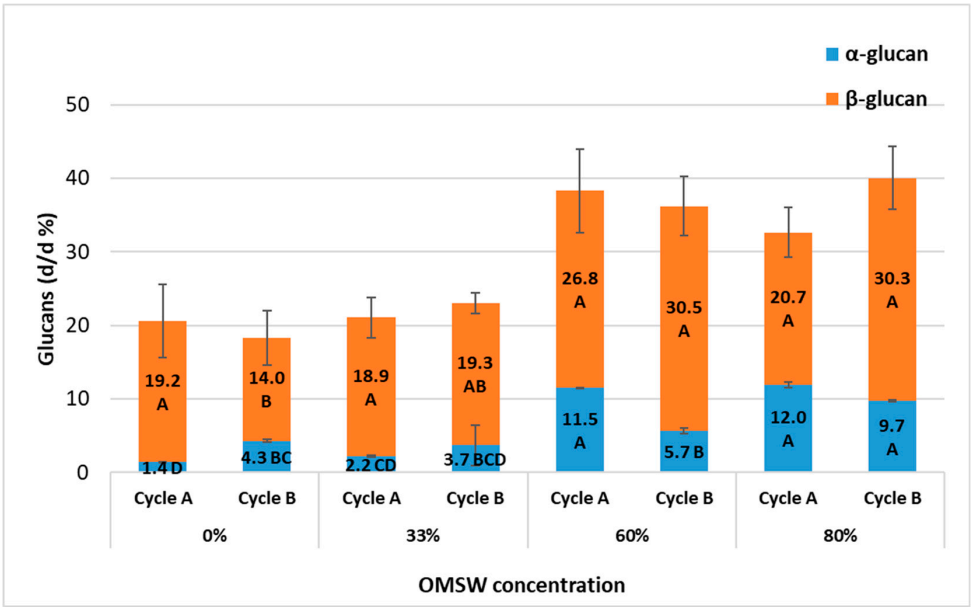

Figure 7. The α- and β-glucan concentrations (%) in *P. eryngii* dried fruiting bodies cultivated at various OMSW substrate concentrations, from two cultivation cycles. Results are expressed as mean ± SD. Statistical significance was determined using the "each-pair Student's *t*-test" ($p < 0.05$). Percent glucan values are relative to dry weight.

3.5. Protein and Nitrogen Content Decline with Increasing OMSW Concentrations

Total nitrogen and protein content in *P. eryngii* fruiting bodies, indicators of developmental status and nutritional quality [19], were quantified across the treatments with varying OMSW levels. Both nitrogen and protein concentrations exhibited a clear negative correlation with increasing OMSW content in the substrate, decreasing significantly in mushrooms grown with higher OMSW concentrations (Figure 8).

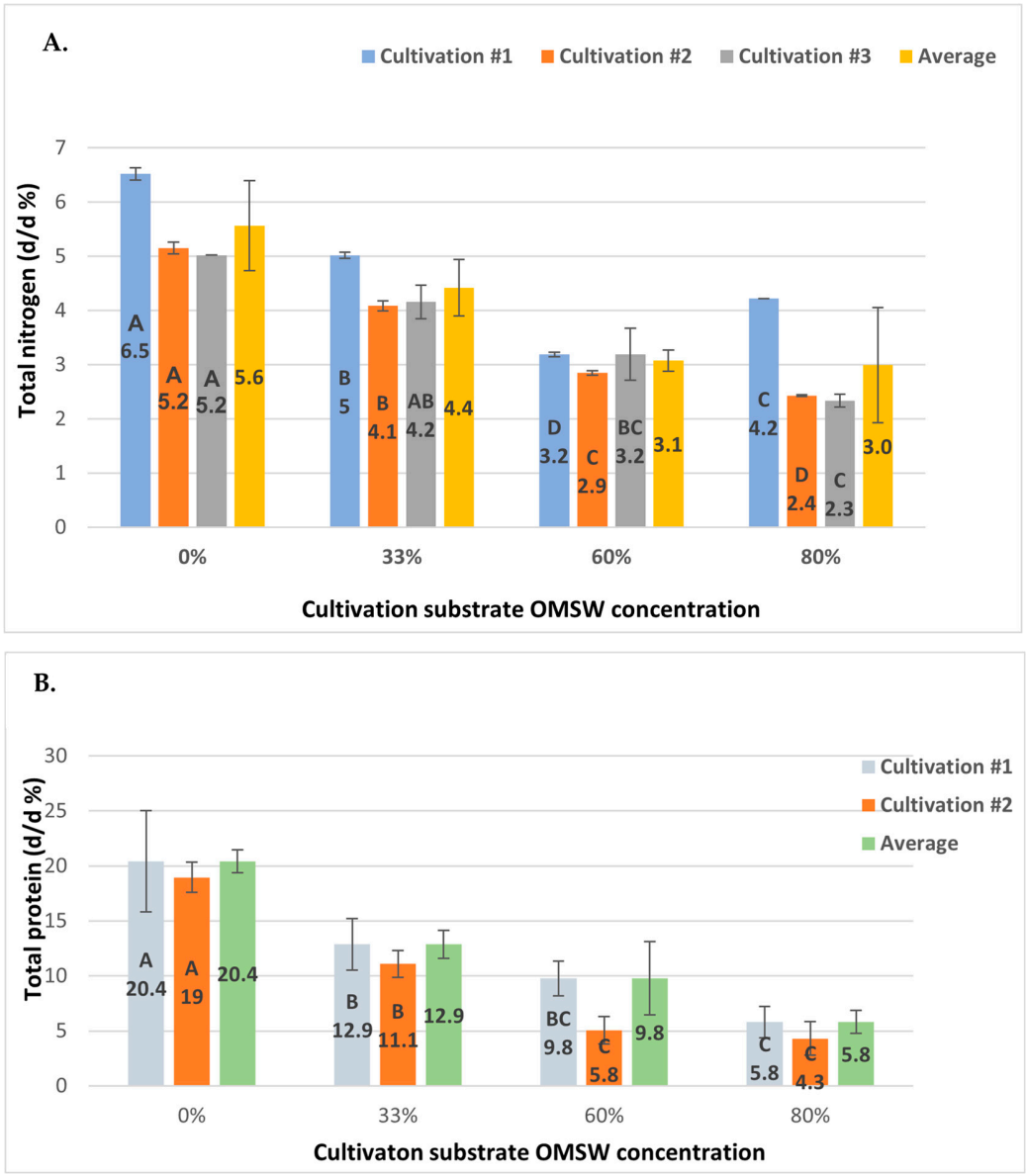

Figure 8. Nitrogen and protein concentrations in the dried fruiting bodies: (**A**) nitrogen concentration in dried fruiting bodies as measured in three independent cultivation cycles; (**B**) protein concentration in dried fruiting bodies as measured in two independent cultivation cycles. Results are expressed as mean ± SD. Statistical significance was determined using the "each-pair Student's *t*-test" ($p < 0.05$).

4. Discussion

Fungi play a pivotal role in ecological and environmental processes, particularly in the decomposition and recycling of organic waste. This activity leads to the synthesis of diverse compounds with nutritional and health-promoting properties, including vitamins, polysaccharides, proteins, and bioactive metabolites with medicinal potential [20,21].

In this study, we examined the relationship between cultivation substrate composition and *P. eryngii* development by integrating phenotypic characterization, transcriptomic analysis, metabolite profiling, and biochemical assays of glucan, nitrogen, and protein content.

Phenotypic measurements revealed that mushroom yield and fruiting body size were highest on substrates containing 33% OMSW and lowest on those with 80% OMSW. Developmental parameters followed a similar pattern, with the most robust fruiting bodies produced at 33% OMSW. These results suggest an optimal cultivation window between 33% and 60% OMSW substrate content, consistent with previous findings that identified OMSW as a viable substrate component for mushroom cultivation [22].

Considering the phenotypic differences originating in cultivation on substrates with varying OMSW percentages, we further employed a combined transcriptomic-metabolomic approach—an effective strategy widely used in studies aiming to optimize cultivation conditions and enhance natural metabolite production [23,24]. To this end, we compared transcriptomic profiles between 0% and 80% OMSW, representing the two extremes of the substrate compositions tested in this study. Key affected pathways included ribosome biogenesis, secondary metabolite biosynthesis, and polysaccharide metabolism. These transcriptional shifts likely reflect a metabolic adaptation to the substrate environment, potentially driven by nitrogen limitation and elevated polyphenol content characteristic of high OMSW substrates [25]. Consistent with this, biochemical analysis revealed a decline in both nitrogen and protein content with increasing OMSW concentrations. This reduction may reflect a metabolic reallocation of cellular energy toward enhancing cell wall rigidity, potentially at the expense of de novo protein synthesis, as an adaptive response to osmotic stress or carbon/nitrogen imbalance. Notably, the protein-to-nitrogen (P/N) ratio decreased progressively from 3.5 in the 0% OMSW treatment to 1.7 at 80% OMSW. This reduction in the P/N ratio at OMSW levels reflects changes in the cell's overall composition. Exposure to phenolic compounds present in OMSW is known to induce osmotic and oxidative stress in fungi, often triggering adaptive cell wall remodeling. The transcriptomic analysis has indicated upregulation of genes involved in chitin metabolism—including UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.9), glucosamine-1-phosphate N-acetyltransferase (EC 2.7.7.23), and chitinase (EC 3.2.1.14) (Supplementary Figure S2). This observation suggests enhanced chitin turnover as part of a possible stress response. Together, these findings point to a reallocation of nitrogen resources toward cell wall maintenance at the expense of cytoplasmic protein accumulation. This hypothesis is supported by concurrent changes in α- and β-glucan levels.

Metabolomic profiling revealed pronounced differences in the overall metabolite composition between mushrooms grown on varying OMSW substrates (Figures 5 and 6), in agreement with the detected transcriptional regulation of secondary metabolism genes (Figure 3). Similar upregulation of secondary metabolite pathways under oxidative stress has been reported recently [26], suggesting that high OMSW concentrations induce stress responses that impact growth and metabolic output. Furthermore, the observed upregulation of peroxisome-related genes (Figure 3) suggests a stress response aimed at detoxifying reactive oxygen species (ROS), likely induced by the high phenolic content in OMSW. The elevated expression of polysaccharide biosynthesis genes in mushrooms cultivated on 80% OMSW substrates corresponded with increased α-glucan content, peaking at 80% OMSW, and β-glucan content, which peaked at 60% OMSW. These results reinforce prior observations linking OMSW substrate enrichment to enhanced glucan accumulation in *P. eryngii* fruiting bodies. Given the immunomodulatory and pathogen-resistance properties attributed to mushroom glucans [27–29], these findings highlight the dual benefits of OMSW valorization: environmental waste recycling and production of mushrooms

enriched in health-promoting compounds. While OMSW has demonstrated beneficial effects on mushroom cultivation and the production of valuable metabolites, it is important to note that the chemical composition of OMSW can vary depending on its specific origin. These compositional differences are expected to influence fungal metabolism and growth, potentially leading to variability in mushroom yield and biochemical profiles.

5. Conclusions

Our findings demonstrate that optimizing mushroom yield—measured by weight and size—does not necessarily correlate with maximized levels of nutritionally and therapeutically valuable components such as glucans and bioactive secondary metabolites. These results align with and expand upon previous studies, highlighting the critical influence of substrate composition on the metabolic profile of cultivated mushrooms [30]. Consequently, the selection of cultivation substrates and conditions should balance both biomass production and the targeted enhancement of specific bioactive compounds with a unique functionality in the mushroom's fruiting body. Protein is a significant component of mushrooms, although it may also be derived from non-fungal sources. However, sources of glucans and metabolites of medicinal value are less common, and some are unique to mushrooms. Therefore, incorporating OMSW into *P. eryngii* cultivation substrates offers a dual benefit: providing an environmentally sustainable strategy for recycling problematic agricultural waste and promoting the synthesis of mushrooms enriched in health-promoting constituents.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15081811/s1, Figure S1: Differences in the expression of genes involved in the synthesis and remodeling of structural polysaccharides between mushrooms grown in 80% OMSW and 0% OMSW substrate contents. Overexpressed genes are in red; underexpressed genes are in green; Figure S2: Differential gene expression, in the amino sugar and nucleotide sugar metabolic pathway between 80% OMSW and 0% OMSW substrate content. In red, overexpressed gene expression; in green, underexpressed genes.

Author Contributions: Conceptualization, O.D. and I.P.; methodology, N.E. and I.P.; validation, I.P., S.K. and N.E.; investigation, N.E., A.A., S.K. and I.P.; resources, N.E.; data curation, N.E., D.L. and I.P.; writing—original draft prep—D.L. and I.P.; writing—review and editing, N.E., S.K., D.L. and I.P.; visualization, N.E. and I.P.; supervision, S.K. and I.P.; project administration, N.E.; funding acquisition, I.P. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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