

Article

From Waste to Value: Investigating Mushroom Stems from *Pleurotus ostreatus* Grown on Mealworm Frass as a Nutritional Source for Aquaculture Feed

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Abstract

This study investigated mealworm frass as a sustainable substrate for *Pleurotus ostreatus* cultivation while valorizing mushroom stems as aquaculture feed. Mushrooms were grown on substrates containing 0–15% frass, and nutritional analyses were conducted on both fruiting bodies (for human consumption) and stems (for fish feed). Increasing frass levels significantly enhanced protein content, rising from 7.78% to 22.31% in stems and 24.74% to 30.99% in fruiting bodies. Lipid concentrations showed minor fluctuations while, in contrast, β -glucan content declined with high frass inclusion percentages. Essential amino acid levels peaked at 7.37% in stems (15% frass) and 8.08% in fruiting bodies (12.5% frass). Polyunsaturated fatty acids dominated the fatty acid profile, increasing with high frass levels. Mushroom bodies and stems were additionally investigated for their antimicrobial activity to determine whether they could offer protection against common fish and human pathogens. Antimicrobial assays revealed that dichloromethane extracts from stems grown on 12.5% and 15% frass exhibited inhibitory activity (inhibition zones of 10–11 mm) against *Tenacibaculum maritimum*, a microorganism that poses a significant threat to aquaculture. These findings highlight mealworm frass as a promising substrate for enhancing mushroom nutritional value while providing a sustainable, protein-rich feed ingredient for aquaculture.

Keywords: oyster mushroom; insect frass; antimicrobial properties; amino acid profile; fatty acid profile



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1. Introduction

The growth of aquaculture underscores the urgent need for sustainable practices to address environmental challenges, while ensuring food security. In aquaculture, conventional feed ingredients such as fishmeal and soybean meal are associated with significant ecological impacts, including overfishing, habitat destruction, and deforestation [1,2]. As industry

strives to meet the growing demand for fish protein, there is a pressing need to develop sustainable feed alternatives that minimize these environmental burdens while supporting fish health, namely by adding functionalities such as antibacterial properties that contribute to disease prevention and reduce reliance on antibiotics, ultimately enhancing aquaculture sustainability [3].

The *Pleurotus* genus has shown significant promise as a functional feed additive and nutraceutical in both human health and aquaculture. Rich in bioactive compounds like β -glucans and polysaccharides [4,5], *Pleurotus* mushrooms possess cholesterol- and glucose-lowering effects, immune-modulatory properties, and antiproliferative activity against cancer cells [6]. In aquaculture, dietary supplementation with *Pleurotus* extracts has positive effects on the growth rate (SGR), feed conversion ratio (FCR), lysozyme activity, phagocytosis, and disease resistance in species like rainbow trout, Amur catfish, Nile tilapia, and *Pangasius catfish* [7–10].

Insect frass, particularly produced by mealworms, has gained attention as an eco-friendly agricultural input due to its high content of organic matter and essential nutrients. Mealworm frass, a waste product obtained from mealworm farming, consists of the excrement generated by the mealworm larvae throughout their entire life cycle, which makes the total output at an industrial scale substantial. This, together with its nutrient-rich composition and a very low production cost, positions mealworm frass as an efficient and sustainable resource within circular farming systems [11].

Studies have shown that insect frass, particularly from species like *Tenebrio molitor* and *Hermetia illucens*, significantly improves plant growth, soil health, and nutrient uptake when used as a soil amendment. Its richness in essential macro and micronutrients, combined with beneficial microbial communities, promotes biomass production and induces plant defenses against pathogens [12,13]. Using it as a substrate for cultivating edible mushrooms represents a promising innovation, enhancing the valorization of insect farming by-products and opening new pathways for sustainable agricultural practices.

According to Putri et al. [14], white oyster mushroom (*Pleurotus ostreatus*) cultivation with mealworm frass significantly enhances the nutritional profile of the harvested mushrooms. The addition of frass, alone or in combination with molasses, led to notable increases in ash, crude protein, and crude fat content, while reducing crude fiber. The highest protein content was observed in the frass-only treatment (23.4%), underscoring frass's potential as a sustainable, nutrient-rich growth substrate. These findings position mealworm frass as a promising alternative substrate for optimizing the nutritional value of oyster mushrooms in commercial settings.

Edible mushrooms themselves generate significant agricultural residues, such as stems, which are often discarded despite their valuable nutritional properties [15]. Transforming these stems into aquaculture feed ingredients offers a dual solution: reducing agricultural waste and providing a sustainable alternative to conventional feed sources.

This study investigated the potential uses of mushroom stems from *Pleurotus ostreatus*, the second most widely cultivated and distributed edible mushroom worldwide, after the champignon (*Agaricus bisporus*), thanks to its remarkable adaptability [16]. The mushrooms used in this study were cultivated using mealworm frass, as an emerging ingredient for fish feed. The nutritional composition, safety, as well as the functional properties, namely in terms of antimicrobial potential, of these stems and fruiting body were assessed to ensure their viability for both human consumption and aquaculture applications. This research examined the effect of different inclusion levels of insect frass in mushroom substrates, on mushroom yield, quality, and aquaculture feed potential. The aim is to demonstrate how waste valorization can contribute to a more sustainable and resilient aquacultural feed production system.

2. Materials and Methods

2.1. Insect Frass

The mealworm frass, commercially known as :oFrass[®], is a natural product, suitable for organic farming. It consists exclusively of the excrement of the yellow mealworm (*Tenebrio molitor*). The frass is separated from the insects and their rearing substrate by mechanical means using proprietary technology. The product has a sandy texture, brownish color, is odorless, and feels like dry granules to the touch.

2.2. Mushroom Cultivation

The mealworm insect frass provided by TEBRIO (Salamanca, Spain), was used as an ingredient for mushroom production at different percentages (CONTROL = 0%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15%) mixed with commercial mushroom substrate, supplied by the compost yard Micelios Fungisem S.A. (Calahorra, Spain). To prepare the commercial mushroom substrate, the wheat and corn straw are first chopped. The chopped straw is then moistened with water containing urea to ensure the appropriate nitrogen content. The chopped and moistened straw is left to drain for a few hours to eliminate excess moisture and initiate fermentation. After this conditioning, the mixture is placed in a fermentation tunnel, where it will remain for approximately 7 days. After fermentation, the mixture is transferred to another tunnel for pasteurization. This process eliminates potential competing microorganisms from the compost and leaves it ready for mycelium inoculation.

Trials were in 2023 performed at CTICH facilities. CTICH has had experience in mushroom research since 2003. Ten replicates per percentage of inclusion were prepared. The different substrates were mixed, watered, placed in polypropylene bags, and sterilized in an autoclave at 121 °C for 180 min. The already sterilized substrate was spawned with *Pleurotus ostreatus* mycelium (strain H9) at 1% rate. The bags were sealed with a heat sealer and taken to an incubation room where they remained for 12 days at 25 °C. Once the mycelium had colonized the entire substrate, the bags were moved to a growing room at 16 °C, with 90% humidity, and 800 ppm of CO₂ to force pinning. Mushroom were collected individually from each bag, weighed, and the stems were separated from the caps manually with a knife. Each fraction was chopped with a chopper (SAMMIC CK 38V; Gipuzkoa, Spain) and dried at 35 °C in a dryer (Klarstein Master Jerky 550; Chal-Tec GmbH, Berlin, Germany). The dry material was ground in a centrifugal mill ULTRA ZM 200 (Retsch; GmbH, Haan, Germany). The mushroom powder obtained was then analyzed following the next protocols (Section 2.3). Samples of the commercial mushroom substrate, insect frass, and the different mixes were collected and the following elements and parameters were measured following AOAC guidelines [17]: total nitrogen, ashes, humidity, fibers, pH, conductivity, and organic content (Table 1).

Table 1. Parameters of the commercial substrate and mealworm frass used in the trial.

Sampe	Humidity (%)	Ashes (%s.m.s.)	Nitrogen (%)	pH	Conductivity (mS/cm)	Organic Matter (%)	Lignin (%)	Cellulose (%)	Hemicellulose (%)
Substrate	72.5 ± 2.1	17.0 ± 4.0	0.9 ± 1.1	8.4 ± 0.5	1.8 ± 0.6	83.0 ± 4.0	9.9 ± 2.4	42.2 ± 3.6	24.5 ± 4.4
Insect frass	6.9 ± 2.5	7.1 ± 0.1	3.4 ± 0.2	5.8 ± 0.2	6.2 ± 0.9	92.9 ± 0.1	7.4 ± 2.6	16.0 ± 3.1	41.6 ± 4.6

The parameters of the commercial substrate and mealworm frass used in the trial are presented in Table 1.

2.3. Nutritional Analysis

2.3.1. Protein Quantification

The protein content was quantified by nitrogen determination using the Kjeldhal method. The sample is mineralized with concentrated sulfuric acid in the presence of a catalyst, converting organic nitrogen into ammonium sulphate. Ammonia is released by adding sodium hydroxide to the cooled mineralization. This ammonia is then distilled into an excess boric acid solution. The whole is titrated with a titrated solution of hydrochloric acid. The quantity of ammonia produced is used to determine the nitrogen content of the sample.

The protein content is finally calculated by multiplying the nitrogen value by a conversion factor of 4.38, appropriate for mushrooms [18].

2.3.2. Lipid Content

The lipid content of the samples was determined using Soxhlet extraction. Approximately 5 g of dried and ground sample was placed in a Soxhlet cartridge and extracted with hexane as the solvent for 4 h under reflux. After extraction, the hexane solvent was evaporated using a rotary evaporator, and the remaining lipid residue was dried to a constant weight in a desiccator.

2.3.3. Glucan Determination

The β -glucan content of mushroom samples was determined using the Megazyme β -Glucan Assay Kit (K-YBGL), following the manufacturer's protocol (Megazyme®; IDA Business Park, Bray, Wicklow, Ireland). Briefly, approximately 200 mg of each sample was subjected to two separate hydrolysis procedures to quantify total glucans and α -glucans (100g for each hydrolysis).

For α -glucan determination, 100 mg of the sample was hydrolyzed with 1.7 M sodium hydroxide to solubilize α -glucans and free glucose. The extract was then neutralized and treated with amyloglucosidase and invertase to enzymatically hydrolyze starch and sucrose into glucose. The released glucose was quantified using a glucose oxidase/peroxidase (GOPOD) assay.

For total glucan determination, the remaining 100 mg of the sample was hydrolyzed using sulfuric acid, breaking down all glucan types (α - and β -) into glucose, which was again quantified using the GOPOD assay.

The β -glucan content was calculated by subtracting α -glucan and free glucose contributions, based on corresponding control assays, and expressed as a percentage of the dry weight of the sample.

2.3.4. Chitin Quantification

For the chitin quantification, 1 g of samples and standards (chitin and glucosamine) was hydrolyzed using 25 mL of concentrated hydrochloric acid and 15mL of Milli-Q water to partially depolymerize chitin into glucosamine. Hydrolysis was conducted in a water bath at 90 °C with continuous agitation (140 rpm) for 4 h. The hydrolysates were diluted with 50 mL Milli-Q water (ELGA LabWater, Veolia Water Technologies, High Wycombe, UK) and analyzed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry UPLC-MS/MS (Waters Corporation, Milford, MA, USA) using an Acquity UPLC-DAD-MS/MS system (including TQD Acquity) equipped with a Waters Acquity UPLC HILIC column (1.7 μ m, 2.1 $\times$ 100 mm). Glucosamine was quantified by monitoring specific mass transitions and comparing peak areas to calibration curves generated from standard solutions.

The concentration of chitin was expressed as a percentage using the following equation:

$$\text{Chitin\%} = \frac{FD \times C_m}{10000000}$$

where FD represents the dilution factor (final volume in mL divided expressed in g), and C_m is the concentration of glucosamine obtained from the UPLC-MS/MS analysis (in ppb).

2.4. Fatty Acid Profiling and Quantification by GC-FID

First, an acid hydrolysis breaks the ester bonds of fatty acids in glycerides, gluco- and phospholipids, and sterol esters. Fatty acids are extracted with n-hexane, then methylated with methanolic solution of BF_3 to form fatty acid methyl esters (FAMES). FAMES are measured by gas chromatography with flame ionization detection (GC-FID). The fatty acid profile was analyzed using a Shimadzu chromatograph, equipped with a split-splitless injector, a FI detector, and a Shimadzu autosampler. Both the injector and detector temperatures were set at 225 °C. Separation was performed on a 100 m $\times$ 0.25 mm i.d. $\times$ 0.20 μm HP-88 column. Helium served as the carrier gas at an internal pressure of 250 kPa. The column temperature was initially held at 120 °C for 5 min, then increased to 240 °C at a rate of 3 °C/min and held for 10 min. The split ratio was 1:30, and the injection volume was 1 μL . Fatty acids were identified by comparing the relative retention times of FAME peaks from samples with standards. A Supelco mixture of 37 FAMES (standard CRM47885) purchased from Sigma-Aldrich (St. Louis, MO, USA) was used.

2.5. Amino Acid Profiling and Quantification by UPLC-DAD-MS

The amino acids (AA) profile was determined according to Kairos amino acid kit (Waters, Milford, MA, USA). The samples were hydrolyzed prior to the UPLC analysis with 6N HCl. Reverse-phase chromatography was performed using a 2 μL injection volume and a flow rate of 0.4 mL/min, in a high-performance liquid chromatography system (Waters, Milford, MA, USA). Separation was achieved using a Cortecs UPLC C18 column with dimensions of 2.1 mm $\times$ 150 mm, 1.6 μm (Waters, Milford, MA, USA) at 55 °C.

2.6. Heavy Metal Analysis

Heavy metal concentrations in the samples were quantified using inductively coupled plasma mass spectrometry (ICP-MS) Thermo ICAP RQ. Prior to analysis, approximately 0.5 g of each dried and ground sample was subjected to acid digestion using a mixture of concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a microwave digestion system to ensure complete mineralization using Ultrawave ECR. The digested samples were diluted with deionized water to a final volume, filtered, and analyzed by ICP-MS. Calibration curves were prepared using certified standard solutions and the results were expressed as milligrams of heavy metal per kilogram of dry sample.

2.7. Preparation of Crude Extracts for Antimicrobial Analysis

Three crude extracts from each mushroom biomass sample (14 samples: stems and fruiting bodies cultivated with 0%, 2.5%, 5%, 7.4%, 10%, 12.5%, 15% of mealworm frass) were prepared to evaluate its antimicrobial properties, following the process described by Girão et al., 2019 [19], with small adjustments to the protocol. All samples were freeze-dried, ground to a fine powder, and stored at -20 °C until further use. For each sample, 5 g of freeze-dried biomass was subjected to independent extractions with each of the following solvents, used separately and in order of decreasing polarity: deionized water, acetone/methanol (1:1, v/v), and dichloromethane. The biomass was placed in 100 mL Erlenmeyer flasks, followed by the addition of 30 mL of the appropriate solvents, and the flasks incubated for 30 min, at 200 rpm and room temperature. The organic or aqueous

layer was recovered and concentrated using a rotary evaporator, with the extraction process being repeated twice for enhanced yield. The recovered crude extract was weighed and dissolved in dimethyl sulfoxide (>99.9%, DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 1 mg mL^{-1} to be used for the antimicrobial assay.

2.8. Antimicrobial ASSAY

The antimicrobial properties of the crude extracts were tested using the agar-based disk diffusion method, following a previously described method [20], against a panel of reference pathogenic strains relevant for aquaculture—*Edwardsiella tarda* DSM30052, *Aeromonas hydrophila* DSM3018, *Pseudomonas anguilliseptica* DSM1211, *Yersinia ruckeri* ATCC29473, *Listonella (Vibrio) anguillarum* ATCC19264, *Tenacibaculum maritimum* ATCC43397, and *Lactococcus garvieae* DSM 20684—and human health—*Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC29213, *Salmonella enterica* ATCC25241, and *Candida albicans* ATCC10231. Each reference microorganism was grown in the appropriate culture medium and incubation temperature (Table A1). For the bioassay, each strain was suspended in the respective liquid medium, D and its turbidity adjusted to 0.5 McFarland standard ($\text{OD}_{625} = 0.08\text{--}0.13$) [21]. The suspensions were used to inoculate agar plates, and blank paper disks (6 mm in diameter) loaded with 15 μL of each crude extract (1 mg mL^{-1}) were placed on the surface of the inoculated medium. Positive control disks were inoculated with 15 μL of enrofloxacin (1 mg mL^{-1} ; Sigma-Aldrich, MO, USA) for the bacterial strains, and 15 μL of nystatin (1 mg mL^{-1} ; Sigma-Aldrich, MO, USA) for the yeast *C. albicans*, while negative control disks were inoculated with 15 μL of DMSO. Each extract was tested in two independent experiments. Antimicrobial activity was determined by measuring the diameter of the inhibition halo formed around each disk, while negative control disks were inoculated with 15 μL of DMSO. Each extract was tested in two independent experiments. Antimicrobial activity was determined by measuring the diameter of the inhibition halo formed around each disk.

2.9. Statistical Analysis

All statistical analyses were performed using R software (version 4.4.0; R Core Team, 2024). A one-way ANOVA was used to assess the effect of inclusion level. When significant differences were detected ($p < 0.05$), Tukey's HSD test was applied for multiple comparisons. Values in the same column followed by the same superscript letter are not significantly different according to Tukey's multiple comparison test ($p < 0.05$). Different letters indicate statistically significant differences between groups.

3. Results

3.1. Mushroom Yield Due to Substrate Variation

The average mushroom yield (expressed as kg of mushrooms/kg of substrate) for each treatment can be found in Figure 1. The yield from the control group was approximately 0.3 kg mushrooms per kg of substrate. The highest yield was achieved with the addition of 2.5% insect frass, averaging around 0.35 kg/kg substrate. However, at 5% insect frass, the yield notably dropped to 0.25 kg/kg substrate. As the inclusion level increased to 7.5%, the yield was not significantly different to the control level, averaging 0.3 kg/kg substrate. Similarly, the 10% frass group showed a yield of around 0.3 kg/kg substrate. A slight decrease in yield was observed at 12.5% frass, averaging just below 0.3 kg/kg substrate. The lowest yield occurred at 15% insect frass, where the yield dropped to 0.22 kg/kg substrate.

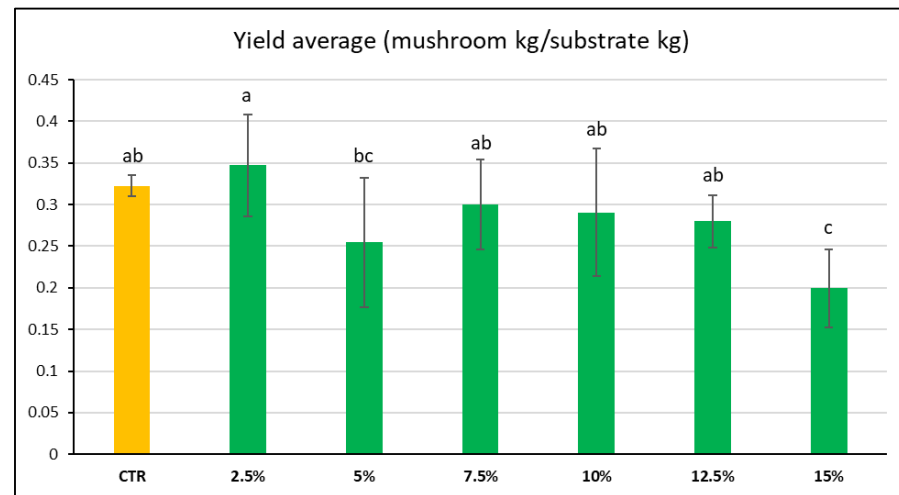


Figure 1. Mushroom yield (kg mushrooms/kg substrate) with different insect frass inclusion percentage (p -value ≤ 0.001). The a, b, c indicate statistically significant differences between groups based on Tukey's HSD test.

3.2. Nutritional Composition of Mushroom Stems and Fruiting Body

The nutritional composition of the mushroom fruiting bodies and stems are presented in Table 2. Protein concentrations increased significantly with higher frass levels in both the stems. It was observed to be the lowest at 0% frass inclusion (7.8%) and peaks at 15% frass inclusion (22.3%). For the fruiting body, protein levels start at 24.7% and steadily rise to 31.0% at 15% frass. However, according to the p -value obtained via the ANOVA to test the variation, is not statistically significant (p -value = 0.332).

Table 2. Nutritional composition of PO fruiting body (PO.FB) and PO stems (PO.St) at different frass inclusion percentage.

Inclusion Percentage	Protein		β -Glucan (%)		Chitin (%)	
	PO. St *	PO. FB	PO. St *	PO. FB *	PO. St	PO. FB
0%	7.8 $\pm$ 0.6 ^d	24.7 $\pm$ 0.5 ^a	37.9 $\pm$ 0.8 ^a	26.4 $\pm$ 0.4 ^a	5.5 $\pm$ 1.2 ^a	6.1 $\pm$ 0.5 ^a
2.5%	10.5 $\pm$ 0.8 ^{cd}	26.5 $\pm$ 0.7 ^a	36.6 $\pm$ 0.8 ^{ab}	19.2 $\pm$ 0.3 ^b	6.2 $\pm$ 0.8 ^a	6.5 $\pm$ 0.7 ^a
5%	15.0 $\pm$ 0.7 ^{bc}	27.8 $\pm$ 1.7 ^a	26.7 $\pm$ 0.1 ^{cd}	14.9 $\pm$ 0.6 ^c	6.6 $\pm$ 0.6 ^a	6.9 $\pm$ 0.5 ^a
7.5%	14.5 $\pm$ 0.1 ^{bc}	30.5 $\pm$ 1.0 ^a	25.3 $\pm$ 3.1 ^{cd}	16.1 $\pm$ 0.4 ^c	6.6 $\pm$ 0.7 ^a	6.8 $\pm$ 0.3 ^a
10%	15.2 $\pm$ 1.2 ^b	30.6 $\pm$ 2.2 ^a	29.8 $\pm$ 0.8 ^{bc}	15.1 $\pm$ 0.4 ^c	7.3 $\pm$ 0.6 ^a	7.3 $\pm$ 0.6 ^a
12.5%	14.8 $\pm$ 1.0 ^{bc}	29.1 $\pm$ 3.8 ^a	24.4 $\pm$ 0.4 ^{cd}	14.2 $\pm$ 0.2 ^c	6.7 $\pm$ 0.5 ^a	7.1 $\pm$ 0.5 ^a
15%	22.3 $\pm$ 0.7 ^a	31.0 $\pm$ 2.1 ^a	20.3 $\pm$ 0.9 ^d	14.3 $\pm$ 0.8 ^c	6.4 $\pm$ 0.6 ^a	6.4 $\pm$ 0.5 ^a

* p -value ≤ 0.001 .

In contrast to this, for protein, the β -glucan decreases in both stems and fruiting bodies as frass percentage increases. In the stems, beta-glucan concentration dropped from 37.9% at 0% frass to 20.3% at 15% frass. A similar decline occurred in the fruiting bodies, with beta-glucans going from 26.4% to 14.3% at the highest frass concentration.

As for the chitin concentration, it remained relatively stable with different frass concentrations in the fruiting bodies. In the stems, chitin content increased slightly, from 5.5% at 0% frass inclusion to 7.3% at 10% frass inclusion, and then decreased to 6.4% at 15% frass inclusion. This variation is statistically significant with a p -value of 0.009.

In both stems and fruiting bodies, lipid concentrations were also measured and the values slightly fluctuated with increasing frass levels. In the stems, lipid content starts at 1.6% with 0% frass inclusion and increases slightly to 2.1% at 15% frass inclusion. Similarly,

the fruiting body showed a lower increase in lipid content, from 3.4% at 0% to 3.9% at 5%, before reaching slightly higher frass levels (3.3% at 15%).

3.3. Amino Acid Composition

Total amino acids, including essential and non-essential contents in the stems and fruiting bodies, are presented in Figure 2. *P. ostreatus* fruiting bodies and stems have a higher non-essential amino acid content than essential amino acids content. For the stems, total essential amino acids content is 2.44% in the 0% frass treatment and was highest at 7.37% in the 15% frass treatment. For the fruiting body, amino acids levels were at their lowest at 6.13% with 2.5% frass and highest at 8.08% in the 12.5% frass treatment. The amino acid profile is presented in more detail in Tables A2 and A3.

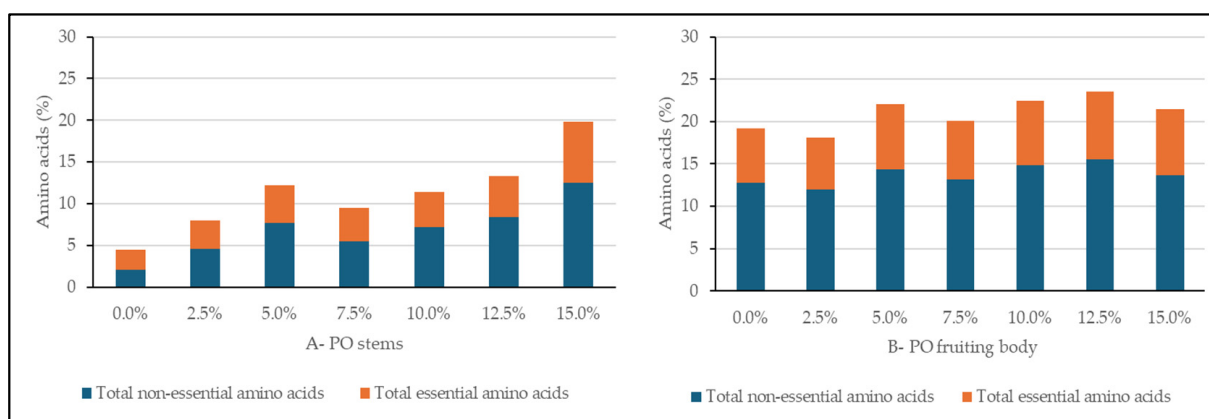


Figure 2. Effect of the insect frass from mealworm on total amino acid contents (% DM) of *P. ostreatus* stems (A) and fruiting body (B).

3.4. Fatty Acid Composition

Figure 3 presents the effect of the mealworm frass on fatty acid contents of *P. ostreatus* stems and fruiting body (Figure 3B), specifically the concentration of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). The data indicates that PUFAs are the dominant fatty acid class in both PO stems and fruiting bodies, significantly increasing with frass inclusion at 7.5% and then again at 15% for PO stems and slightly increasing at 10% frass concentrations for the fruiting body. SFAs in PO stems are consistently higher than MUFAs but lower than PUFAs, suggesting a balanced lipid profile. MUFA levels remain the lowest across all concentration with the lowest concentration around 7.5% inclusion for PO stems. The detailed fatty acid profile is presented in Tables A4 and A5.

3.5. Heavy Metal Content Analysis

The data in Figure 4 indicates varying concentrations of heavy metals in stems cultivated with mealworm frass at different levels (0–15%).

For the fruit body cultivated with mealworm frass at different levels, the key toxic metals such as Cd, Pb, As, and Hg were also below detection levels. Essential minerals like Zn, Cu, Fe, and Mn increase with high frass levels and are present in beneficial amounts.

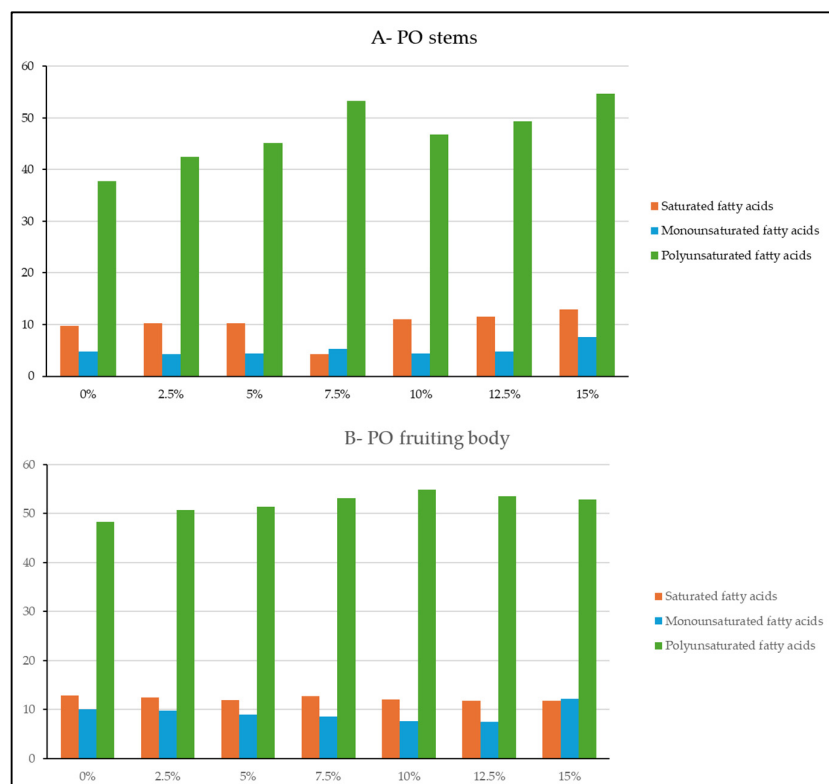


Figure 3. Effect of the mealworm frass on fatty acid contents of *P. ostreatus* stems (A) and fruiting body (B) expressed in percentages (%).

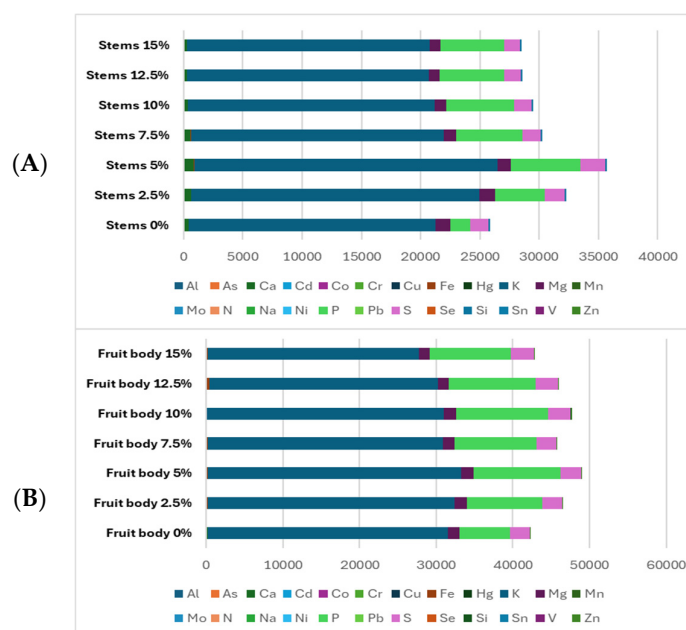


Figure 4. Traces elements and potentially toxic metals concentration in the fruiting bodies (A) and stems (B) of *P. ostreatus*, expressed in mg/kg of DM.

3.6. Analysis of Antimicrobial Activity

The 14 mushroom biomass samples analyzed in this study, comprising both stems and fruiting bodies, and cultivated using different percentages of insect frass, were extracted with three independent solvents: water, an acetone/methanol mixture (1:1, *v/v*), and dichloromethane. This process yielded 14 extracts per solvent, totaling 42 crude extracts, with extraction yields ranging from 1503.8 mg for the aqueous extract of the fruiting

body cultivated with 10% of insect frass to 22.2 mg for the dichloromethane extract of the stems cultivated with 0% of insect frass. For all biomass samples, higher yields were obtained using water as solvent, followed by the acetone/methanol mixture, and finally dichloromethane (Figure 5A).

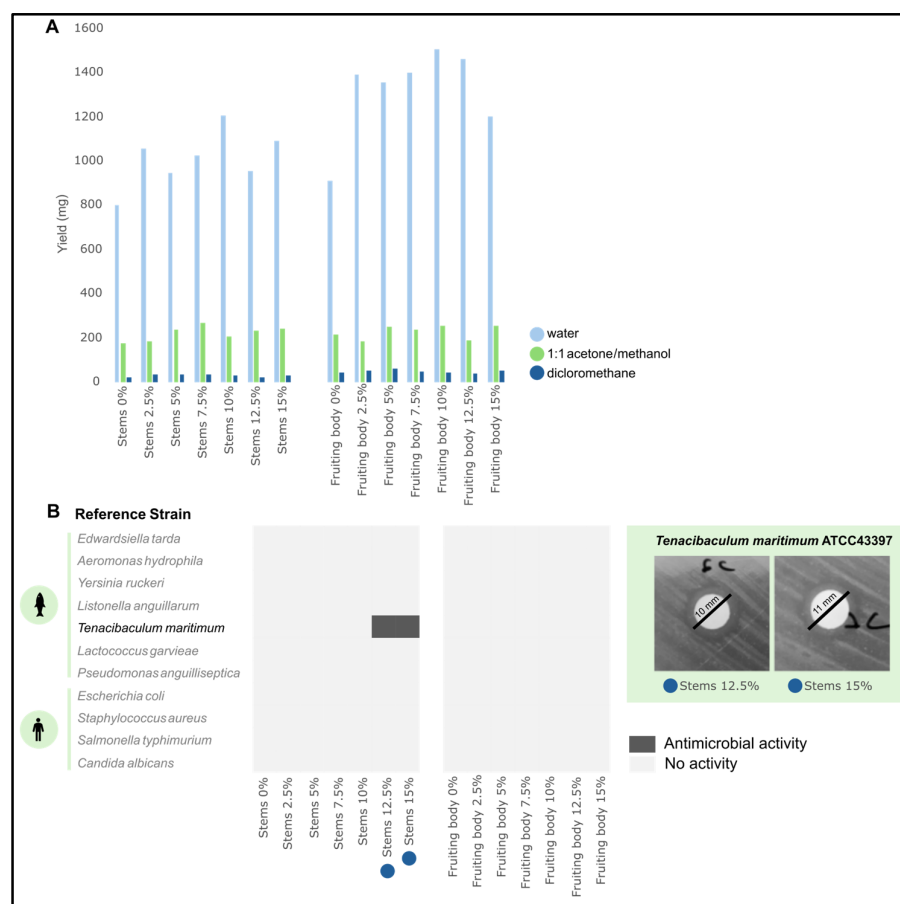


Figure 5. (A) Extraction yield of mushrooms stems and fruiting bodies samples using the different solvents. (B) Antimicrobial activity of crude extracts from mushrooms samples. The inhibition halos for the active extracts are presented, as well as the solvent used for their obtention.

Based on solvent affinity, these results indicate that the compounds in these samples are predominantly polar. All 42 crude extracts were tested for antimicrobial properties using the agar-based disk diffusion method. Interestingly, only two of these extracts, both obtained using the non-polar solvent dichloromethane, and derived from stem samples (cultivated with 12.5 and 15% of insect frass, respectively), were revealed to have inhibitory activity against the aquaculture-relevant strain *Tenacibaculum maritimum* ATCC43397, exhibiting inhibition halos with 10 and 11 mm of diameter, respectively (Figure 5B).

4. Discussion

4.1. Yield, Nutritional and Microbiological Benefits, and Potential of Mushroom Stems

4.1.1. Yield Analysis

The data indicates that low levels of insect frass (2.5%) enhance mushroom yield, likely by supplying additional nutrients to the substrate or due to microbial benefits. Insect frass specifically from *Tenebrio molitor* is rich in nitrogen, and micronutrients such as phosphorus and potassium, which can promote fungal growth at these lower inclusion levels [22–24]. However, as the concentration of insect frass increases, mushroom yield begins to decline. This reduction in yield at higher levels (10%, 12.5%, and 15%) could be attributed to several

factors. First, excessive insect frass may disrupt the carbon-to-nitrogen ratio in the substrate, creating a nutrient imbalance that inhibits fungal development. Lastly, the lower porosity and more compact structure of the substrate with high percentages could not allow the growth and aeration of the mycelium, which could face higher difficulty in its development.

4.1.2. Nutritional Potential

The current study indicates that incorporating insect frass into the mushroom substrate significantly enhances the nutritional profile of *Pleurotus ostreatus* (PO), affecting both the fruiting bodies and stems. Notably, frass inclusion boosts protein concentration in both parts of the mushroom, a result attributed to the additional nitrogen provided by the frass, which was used to synthesize proteins. Protein content in fruiting bodies increased notably with higher frass levels, making them a more valuable source of dietary protein for human consumption [25]. Similarly, in the stems, protein concentration rose from 7.8% at 0% frass to 22.3% at 15% frass.

The amino acid profiles of both fruiting bodies and stems were also improved with frass inclusion. Total amino acid content in fruiting bodies increased from 6.1% with 2.5% frass to 8.1% at 12.5% frass, showing that substrate composition directly influences mushroom quality. In the stems, essential amino acids (EAAs) increased from 2.4% to 7.4% from 0% and 15% frass inclusion, with notable improvements in lysine (0.4% to 1.2%), leucine (0.4% to 1.5%), and methionine (0.1% to 0.3%). These EAAs are vital for both human and aquatic nutrition due to their roles in growth, muscle development, and metabolic functions. They are also crucial for fish growth, immune function, and overall health, since they cannot be synthesized by the organism and must be obtained from their diet [26].

It was also found that the PO can serve as a valuable dietary protein source, particularly due to its higher content of total non-essential amino acids (NEAAs) compared to total essential amino acids (EAAs). While essential amino acids are crucial for human health, as they must be obtained from the diet, non-essential amino acids play significant roles in various physiological functions and can contribute to overall protein nutrition [27].

Given its amino acid profile, *P. ostreatus* can be considered an excellent dietary supplement for various groups with dietary restrictions, including vegetarians and vegans, and populations with limited access to animal protein sources. Some additional benefits can be linked to the presence of essential amino acids such as leucine and valine (both slightly increased with frass inclusion), which modulate the resistance to fatigue and muscle and liver glycogen degradation [28].

In terms of lipid content, frass inclusion had a minimal impact on both fruiting bodies and stems. Lipid concentrations in the fruiting body increased from 3.4% at 0% to 3.9% at 5%, before reaching slightly higher frass levels (3.3% at 15%). As for the stems, the lipid increased only slightly, from 1.6% with 0% frass to 2.1% with 15% frass, while lipid levels in fruiting bodies remained largely stable. Despite the modest change in quantity, the fatty acid profile of PO remained favorable. In fruiting bodies, polyunsaturated fatty acids (PUFAs) were the dominant class, peaking at around 10% frass inclusion. Saturated fatty acids (SFAs) were higher than monounsaturated fatty acids (MUFAs), but lower than PUFAs, while MUFAs remained the lowest across all concentrations, indicating a balanced lipid composition.

The stems similarly showed beneficial fatty acid profiles. PUFA and SFA contents are particularly relevant for aquaculture applications. PUFAs support fish growth, immunity, and reproduction, while SFAs contribute to energy storage, lipid metabolism, and feed conversion efficiency. A balanced ratio of PUFAs to SFAs is crucial in aquafeeds, as high PUFA levels without sufficient SFAs can lead to oxidative stress [29].

Conversely, β -glucan levels decrease in both fruiting body and stems. For the fruiting body, the value decreased from 26.4% to 14.2 with 12.5% inclusion level. The highest level obtained with the frass inclusion was 19.2% with 2.5% inclusion. As for the stems, β -glucan levels decreased from 37.9% at 0% frass inclusion to 20.3% at 15% frass inclusion. Since β -glucans contribute to immune modulation in fish and other aquatic organisms, this decline might reduce the potential immunostimulatory benefits of the stems as feed [30].

Chitin variation was not statistically significant and remained relatively unaffected by frass inclusion. For the PO stems, the chitin levels fluctuated from 5.5% at 0% inclusion to a peak of 7.3% at 10% frass inclusion before slightly declining to 6.4% at 15% frass inclusion. Chitin can support gut health and act as a prebiotic for aquatic organisms, but since its variation is minimal, frass supplementation does not appear to have a significant impact on the concentration of this component in the stems.

Thus, the use of frass as a substrate for mushroom stem production appears to be a promising strategy for improving their suitability as aquaculture feed. Although the reduction in β -glucans might slightly affect immune-boosting properties, the enhanced protein content likely outweighs this drawback.

4.1.3. Safety Analysis

Fish feed quality is considered one of the most important factors impacting the success of aquaculture practices. In this study, the results shown in Figure 4 (Tables A6 and A7) suggest that the levels of heavy metals of different mushroom stems cultivated using mealworm frass ensure the safety of fish for human consumption. Even if there are levels slightly above the standard, these values are only found at low level in fish and are non-toxic [31]. In addition, trace elements, such as copper, are essential for hemoglobin synthesis and act as a cofactor for various enzymes involved in energy metabolism and antioxidant defense. It supports the immune system and overall growth [32]. As for the stems, the trace metal concentrations in the fruiting body are generally within safe limits and can offer beneficial contributions to human health. Based on the results, the best percentage of stems for having a non-toxic and beneficial ingredient for fish (stems) and also human (fruiting body) appears to be between 10% and 12.5%. At these percentages, the trace metal concentrations are generally within safe limits and can offer beneficial contributions to fish or human health.

4.1.4. Antimicrobial Potential

The results obtained in this study reveal two mushroom extracts (from stems grown on 12.5% and 15% frass) exhibiting antimicrobial activity against *T. maritimum*. This ulcerative bacterial disease represents a major challenge in marine aquaculture, particularly among economically important fish species, due to its rapid transmission, high mortality rates, and limited treatment options. The resulting losses underscore the need for novel and sustainable strategies to sustain the industry and protect fish populations [20]. Although mushrooms are widely recognized as sources of bioactive compounds with antimicrobial, antifungal, and antiviral properties [33], evidence of their effectiveness against aquaculture-relevant pathogens remains scarce. Nevertheless, recent research has highlighted that fungi and their secondary metabolites are being explored for use in aquaculture, where they show promising roles in enhancing fish immunity, improving water quality, and controlling pathogenic bacteria [34]. The antimicrobial response observed in our study suggests the potential of mushroom-derived biomasses—particularly those cultivated on frass—as promising agents in aquaculture disease control. The activity against *T. maritimum* is especially interesting, given this pathogen's resistance to conventional antibiotics, which contributes to severe disease outbreaks and rising antimicrobial resistance. Incorporating

porating these biomasses into aquafeeds could offer a practical route for enhancing fish health through dietary means, potentially strengthening preventative health strategies in farming systems.

In fact, the inclusion of fungal biomasses in animal feeds has been found to function as an immunostimulant, enhancing fish resistance to diseases [35]. This effect is attributed to the presence of various immunostimulatory compounds such as oligosaccharides, polysaccharides, amino acids, triterpenoids, alkaloids, phenols, vitamins, and/or minerals such as zinc, copper, iodine, selenium, and iron [36]. Fungal polysaccharides, in particular, are recognized for their ability to regulate growth and immune response. They are potent immunological stimulators, capable of directly activating defense mechanisms like leukocytes and boosting their phagocytic, cytotoxic, and antimicrobial activities. Additionally, fungal polysaccharides promote gut microbiota growth, acting as prebiotic substances [37]. These polysaccharides are usually present in the form of glucans, which can account for 50–60% of the dry weight of the fungal cell wall [38]. Glucans can be α -glucans or β -glucans, the latter being associated with a stronger immunostimulatory effect [39]. β -Glucans are directly recognized by immune cells via specific receptors on plasma membranes, triggering downstream signaling processes that enhance non-specific immune factors [40] across various fish species [41–44]. Indeed, several studies have reported on the potential of the dietary inclusion of fungal biomasses or extracts derived from them. For example, the dietary inclusion (1 and 2% DM) of extracts derived from chaga mushroom (*Inonotus obliquus*) and *Phellinus linteus* increased immune, cellular, and humoral response and overall resistance of longtooth grouper (*Epinephelus bruneus*) against *Vibrio harveyi* infection [45,46]. The dietary inclusion of king trumpet mushroom (*Pleurotus eryngii*) (3% DM) stimulated lysozyme activity, phagocytosis, and respiratory burst activity in Pangasius catfish (*Pangasius bo-courti*), increasing its survivability against *Aeromonas hydrophila* challenge [10]. The dietary inclusion of oyster mushroom (*Pleurotus ostreatus*) extracts (1 and 2% DM) increased respiratory burst, phagocytic and lysozyme activity, and reduced mortality in rainbow trout (*Oncorhynchus mykiss*) subjected to infection by *Aeromonas hydrophila* and *Lactococcus garvieae* [7,37].

Thus, the use of glucan-rich mushroom stems with a balanced composition of amino acids and polyunsaturated fatty acids (PUFAs) presents a multifaceted opportunity in aquaculture feed formulation. Not only does this approach provide a sustainable avenue for valorizing mushroom byproducts, but it also significantly enhances fish health and resilience.

However, further research is needed to identify and characterize the specific bioactive compounds responsible for these effects and to assess their efficacy and safety under real farming conditions. Overall, our findings support the continued exploration of mushroom-based bioproducts as part of integrated, sustainable approaches to managing disease in marine aquaculture.

4.2. SWOT Analysis: Valorization of Mushroom Stems Cultivated Using Insect Frass for Aquaculture

To support the growing demand for sustainable aquaculture, innovative feed solutions are essential, especially those aligned with circular economic principles. This strengths, weaknesses, opportunities, and threats (SWOT) analysis explores the potential of mushroom stems, a byproduct often considered waste, as a nutritional ingredient for aquaculture feed. Grown on insect frass, the stems demonstrate an improved protein profile and, in some cases, show microbacterial activity against the growth of the aquaculture-relevant strain *Tenacibaculum maritimum*, a major pathogen in affecting fish species of high economic value.

Figure 6 outlines the key strengths, weaknesses, opportunities, and threats associated with the use of mushroom stems cultivated with insect frass as a sustainable feed resource in aquaculture systems.



Figure 6. SWOT analysis of the mushroom stems grown using mealworm frass for aquaculture feed.

4.2.1. Strengths

- Enhance nutritional values:

Increase the protein and lipid content compared to the stems grown using commercial substrates, both key nutrients for aquaculture species.

- Inhibitory activity and immunostimulant potential:

Mushroom stems with 12.5 and 15% frass inclusion have shown inhibitory effects against *Tenacibaculum maritimum*, a major pathogen causing ulcerative disease in high-value marine fish. While mushrooms are known for their bioactive antimicrobial properties, research targeting *T. maritimum* is limited. These findings suggest mushroom stems could offer a promising natural antimicrobial option for aquaculture feed, leading to substantial mortality and financial losses within the aquaculture industry.

Although research on combating *T. maritimum* remains limited, their known antimicrobial properties and promising results from mushroom stems suggest potential for use in aquaculture feed. Additionally, mushroom stems may act as immunostimulant, enhancing the immune system, thereby boosting the host's ability to resist and defend against pathogens [47].

- Use of waste products (frass and mushroom stems):

Using agricultural byproducts like mushroom stems and insect farming residues aligns with global efforts to reduce waste and promote resource efficiency. This approach directly contributes to Development Goal SDG 12 on responsible consumption and production, reducing food waste and lowering the environmental impacts of production [48].

Repurposing byproducts like mushroom stems and insect frass for aquaculture reduces reliance on unsustainable raw materials and lowers the agriculture ecological footprint. This circular approach supports multiple SDGs, beyond SDG 12, including SDG 13 (by cutting methane emissions), SDG 14 (by reducing fishmeal use), and SDG 15 (by promoting sustainable land use). Studies also show that such practices offer environmental and economic benefits by turning waste into valuable resources [49].

- Cost-effectiveness:

Mealworm frass, a nutrient-rich byproduct of insect farming, shows strong economic and agronomic potential as a substrate supplement for mushroom cultivation. Rich in nitrogen, chitin, and organic matter, it can improve the yields when added at optimal levels. Compared to traditional supplements like wheat bran or soybean meal, frass is often cheaper or even free from local insect farms seeking waste disposal solutions. In mushroom production, the stems are typically considered a byproduct due to their tough, fibrous texture and limited culinary use [50]. As such, they are often discarded during mushroom processing. The stems' lower protein content limits their use as primary protein sources in aquafeeds. Instead, their applicability lies in replacing lower-protein filler ingredients like plant-based meals, such as wheat meal or rapeseed meal, which are also rich in fiber in the form of non-starch polysaccharides, such as cellulose, hemicellulose, and lignin [51]. These conventional filler feedstuffs are subject to price fluctuations, being influenced by market demand and disturbances, such as supply chains disruptions [52]. The cost effectiveness perspective of incorporating a by-product with little intrinsic value is especially relevant in the aquaculture sector, where feed constitutes the largest input costs [53].

- Food vs. feed competition:

The food vs. feed concept is especially relevant in a world increasingly challenged by a rising population, resource constraints, and food insecurity [54]. In this context, the practice of allocating edible, high-value crops such as cereals, legumes, and oilseeds for livestock feed rather than human diet is facing increasing scrutiny for its implications on sustainable development and resource use efficiency [55].

Considering these challenges, using mushroom stems, typically discarded during edible mushroom processing, as a component of aquaculture feed presents a significant advantage, aligning with the principle of resource decoupling by utilizing not-in-demand staple food crops as inputs in animal feeds. This approach also addresses the “sustainability trilemma” of producing sufficient food, using resources efficiently, and minimizing environmental impacts [56,57], since incorporating agricultural by-products into feed redirects an existing biomass stream that would otherwise contribute to organic waste. Furthermore, from a policy and global food systems perspective, the inclusion of agro-industrial by-products in animal diets is increasingly encouraged. It supports the principles of the FAO's sustainable livestock agenda [58], which emphasizes valorization of non-human-edible biomass to reduce pressure on natural resources and promote food security.

4.2.2. Weaknesses

- Nutritional variability due to substrate differences

Despite its strengths, this approach has certain weaknesses. A major concern is the variability in the nutritional composition of mushroom stems, which depends on the type of mealworm frass and cultivation conditions. Such inconsistencies could pose challenges for aquaculture feed producers striving for standardized nutritional profiles.

- Market hesitation about unconventional ingredients

Market perception presents a concerning difficulty. Aquaculture producers may hesitate to adopt unconventional feed ingredients due to concerns about their efficacy and consumer acceptance. Furthermore, the shelf life of mushroom stems and frass-based products may be shorter than traditional feed ingredients, necessitating efficient preservation techniques to maintain their nutritional value.

4.2.3. Opportunities

- Growing demand for sustainable aquaculture solutions

The global expansion of aquaculture creates a pressing demand for sustainable feed solutions, presenting a significant opportunity for this approach. Governments and international organizations are increasingly promoting sustainable practices in aquaculture through policies, subsidies, and regulatory frameworks [59]. Such support can facilitate the adoption of novel feed.

- Consumers preference for sustainability in aquaculture and support from governments and eco-friendly policies

Consumer demand for sustainability is another promising opportunity. With growing awareness of environmental issues, consumers are more inclined to support products derived from eco-friendly practices. This trend could incentivize aquaculture producers to integrate mushroom-stem-based feed grown using mealworm frass into their operations.

4.2.4. Threats

- Regulatory barriers and strict feed safety standards

Several threats could hinder the implementation of this solution. Regulatory barriers remain a key concern, as the use of novel feed specifically by-products still must meet strict safety and quality standards [2].

- Strong competition from established feed ingredients

The dominance of traditional feed ingredients and established supply chains. These established products benefit from robust supply chains and economies of scale, making it difficult for alternative feed solutions to compete without significant cost advantages or subsidies [60].

- Environmental risks and economic viability

Environmental risks also deserve attention. While mealworm frass and mushroom stems are sustainable resources, improper handling or processing could lead to unintended ecological impacts, undermining the credibility of the approach.

Economic viability is another potential obstacle, as fluctuations in the costs of insect farming, mushroom cultivation, and feed processing could affect profitability.

5. Conclusions

The mushroom stems, typically considered a low-value byproduct, were shown to be nutritionally suitable for aquaculture feed, contributing to resource efficiency and reducing waste in the production process. The inclusion of mealworm frass in the mushroom cultivation substrate presents significant benefits and trade-offs in terms of yield, nutritional value, and potential aquaculture applications.

Nutritionally, frass supplementation enhanced the protein content of both *P. ostreatus* fruiting bodies and stems, making them valuable protein sources for human and aquaculture consumption. The amino acid profile of the fruiting body improved with frass inclusion, particularly in non-essential amino acids, while the stems demonstrate a substantial increase in essential amino acids critical for fish growth.

Beyond its nutritional potential, *P. ostreatus* grown using mealworm frass exhibited promising antimicrobial properties, with potential applications against fish pathogens such as *T. maritimum*. Further studies are required to characterize its bioactive compounds and explore its role in disease prevention within aquaculture.

Overall, integrating frass into mushroom cultivation enhanced yield at a concentration of 7.5% to 12.5%, improving nutritional quality, and offering a sustainable approach for developing functional feed ingredients, supporting both human nutrition and aquaculture sustainability.

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Appendix A.

Table A1. Culture conditions for the reference strains used in the antimicrobial assay.

Strain	Culture Medium	Incubation Time (h)	Incubation Temperature (°C)
<i>Edwardsiella tarda</i> DSM 30052	Tryptic soy	24	28
<i>Aeromonas hydrophila</i> DSM 3018	Tryptic soy	24	28
<i>Pseudomonas anguilliseptica</i> DSM 12111	Tryptic soy	48–72	25
<i>Yersinia ruckeri</i> ATCC 29473	Tryptic soy	24–48	28
<i>Listonella (Vibrio) anguillarum</i> ATCC 19264	Tryptic soy	48	28
<i>Tenacibaculum maritimum</i> ATCC 43397	Marine agar	24–48	25
<i>Lactococcus garvieae</i> DSM 20684	Tryptic soy yeast	24–48	28
<i>Escherichia coli</i> ATCC 25922	Mueller–Hinton	24	37
<i>Staphylococcus aureus</i> ATCC 29213	Mueller–Hinton	24	37
<i>Salmonella enterica</i> ATCC 25241	Mueller–Hinton	24	37
<i>Candida albicans</i> ATCC 10231	Sabouraud dextrose	24	37

Appendix B. Amino Acid Content in *P. ostreatus* Stems and Fruiting Body

Table A2. Amino acids content in the PO stems.

(a) PO Stems	0%	2.5%	5%	7.5%	10%	12.5%	15%
Alanine	0.46	0.68	0.93	0.82	0.84	1.03	1.52
Arginine	0.34	0.54	0.79	0.72	0.73	0.88	1.57
Aspartic acid	LQ	LQ	1.37	LQ	1.33	1.51	2.22
Cystine	LQ	0.11	0.16	0.12	0.11	0.11	0.21
Glutamic acid	LQ	1.51	2.04	1.89	1.98	2.22	3.10
Glycine	0.41	0.56	0.72	0.63	0.65	0.80	1.15
Histidine	0.16	0.21	0.29	0.27	0.30	0.34	0.49
Hydroxyproline	LQ	LQ	LQ	LQ	LQ	LQ	LQ
IsoLeucine	0.29	0.43	0.55	0.47	0.50	0.61	0.92
Leucine	0.44	0.66	0.88	0.75	0.78	0.98	1.48
Lysine	0.37	0.51	0.68	0.63	0.67	0.82	1.20
Methionine	0.08	0.11	0.17	0.13	0.14	0.17	0.31
Ornithine	0	0	0.08	LQ	0.16	0.15	0.17
Phenylalanine	0.36	0.48	0.60	0.53	0.56	0.65	0.92
Proline	0.36	0.49	0.60	0.54	0.56	0.66	0.94
Serine	0.31	0.45	0.63	0.53	0.57	0.68	1.00
Threonine	0.36	0.50	0.62	0.55	0.58	0.66	0.97
Tryptophan	LQ	LQ	LQ	LQ	LQ	LQ	LQ
Tyrosine	0.19	0.26	0.36	0.29	0.30	0.32	0.60
Valine	0.39	0.54	0.68	0.61	0.63	0.76	1.08
Total non-essential amino acids	2.07	4.59	7.68	5.53	7.24	8.36	12.49
Total essential amino acids	2.44	3.44	4.47	3.94	4.14	4.98	7.37
Total	4.51	8.04	12.16	9.48	11.38	13.34	19.86

Table A3. Amino acids content in the PO fruiting body.

(b) PO Fruiting Body	0%	2.5%	5%	7.5%	10%	12.5%	15%
Alanine	1.23	1.19	1.40	1.20	1.36	1.46	1.43
Arginine	1.20	1.49	1.81	1.75	1.94	2.14	1.71
Aspartic acid	1.93	1.85	2.19	1.84	2.17	2.39	2.22
Cystine	0.21	0.21	0.27	0.25	0.25	0.24	0.24
Glutamic acid	4.90	4.06	4.58	4.35	4.99	4.71	3.84
Glycine	1.01	0.97	1.08	0.97	1.07	1.20	1.13
Histidine	0.46	0.44	0.57	0.53	0.64	0.64	0.59
Hydroxyproline	LQ	LQ	LQ	LQ	LQ	LQ	LQ
IsoLeucine	0.77	0.74	0.95	0.80	0.87	0.91	0.90
Leucine	1.27	1.19	1.37	1.15	1.34	1.45	1.42
Lysine	1.03	1.03	1.32	1.21	1.32	1.45	1.34
Methionine	0.27	0.26	0.36	0.34	0.35	0.38	0.37
Ornithine	0.00	0.00	0.23	0.23	0.28	0.31	0.22
Phenylalanine	0.80	0.77	0.97	0.92	0.95	0.99	0.99
Proline	0.78	0.76	0.98	0.88	0.96	1.03	1.01
Serine	0.94	0.89	1.16	1.04	1.14	1.20	1.11
Threonine	0.88	0.82	1.03	0.96	1.04	1.09	1.02
Tryptophan	LQ	LQ	LQ	LQ	LQ	LQ	LQ
Tyrosine	0.55	0.52	0.66	0.65	0.70	0.81	0.77
Valine	0.91	0.88	1.11	1.03	1.10	1.17	1.16
Total non-essential amino acids	12.74	11.95	14.36	13.16	14.87	15.50	13.69
Total essential amino acids	6.41	6.13	7.67	6.93	7.63	8.08	7.78
Total	19.14	18.08	22.02	20.10	22.50	23.58	21.47

Appendix C. Fatty Acid Profile in PO Stems and Fruiting Body

Table A4. Fatty acid profile in the PO stems.

(a) PO Stems	0.0%	2.5%	5.0%	7.5%	10.0%	12.5%	15.0%
Myristic acid	0.00	0.00	0.00	0.12	0.11	0.11	0.14
Caproic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pentadecanoic acid	1.47	1.53	1.45	1.93	1.55	1.44	1.47
Palmitic acid	7.16	7.72	7.82	0.93	8.19	8.83	10.16
Palmitoleic acid	0.00	0.00	0.00	0.11	0.07	0.09	0.25
Heptadecanoic acid	0.00	0.11	0.10	0.15	0.13	0.13	0.12
10-heptadecenoic acid	0.35	0.30	0.19	0.28	0.25	0.20	0.13
Stearic acid	0.94	0.72	0.70	0.84	0.69	0.71	0.83
Elaidic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oleic acid	4.29	3.76	3.96	4.66	3.89	4.30	6.76
Linolelaidic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.48
Linoleic acid	37.81	42.45	45.10	53.22	46.17	49.32	54.06
Arachidic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
g-Linolenic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.06
Alpha-linolenic acid (ALA)	0.00	0.00	0.00	0.00	0.50	0.00	0.07
11-Eicosenic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.05
Eicosadienoic acid	0.00	0.00	0.00	0.00	0.10	0.00	0.06
Behenic acid	0.00	0.00	0.08	0.09	0.09	0.08	0.09
Gamma-Eicosatrienoic acid (DGLA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Erucic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tricosanoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lignoceric acid	0.12	0.13	0.14	0.21	0.16	0.16	0.20
Nervonic acid	0.12	0.16	0.20	0.23	0.18	0.20	0.38
Docosahexaenoic acid (DHA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table A5. Fatty acid profile in the PO fruiting body.

(b) PO Fruiting Body	0.0%	2.5%	5.0%	7.5%	10.0%	12.5%	15.0%
Myristic acid	0.08	0.09	0.10	0.10	0.09	0.10	0.10
Caproic acid	0.06	0.00	0.00	0.00	0.00	0.00	0.00
Pentadecanoic acid	1.93	1.73	1.63	1.81	1.83	1.58	1.26
Palmitic acid	8.57	8.80	8.71	9.14	8.65	8.82	8.96
Palmitoleic acid	0.11	0.15	0.17	0.17	0.12	0.15	0.16
Heptadecanoic acid	0.10	0.09	0.08	0.10	0.11	0.10	0.09
10-heptadecenoic acid	0.16	0.15	0.11	0.10	0.10	0.10	0.09
Stearic acid	1.16	1.06	0.95	0.95	0.84	0.78	0.92
Elaidic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oleic acid	9.38	9.16	8.33	7.94	6.59	6.95	11.63
Linolelaidic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic acid	47.78	48.49	50.86	52.34	52.59	53.09	52.12
Arachidic acid	0.05	0.00	0.00	0.00	0.00	0.00	0.07
g-Linolenic acid	0.09	0.10	0.09	0.10	0.10	0.10	0.09
Alpha-linolenic acid (ALA)	0.26	0.04	0.23	0.48	0.06	0.10	0.55
11-Eicosenic acid	0.00	0.00	0.05	0.07	0.54	0.00	0.07
Eicosadienoic acid	0.00	0.04	0.19	0.00	0.06	0.12	0.08
Behenic acid	0.23	0.18	0.10	0.17	0.11	0.09	0.17
Gamma-Eicosatrienoic acid (DGLA)	0.17	0.14	0.09	0.23	0.33	0.16	0.00
Erucic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.03
Tricosanoic acid	0.06	0.00	0.00	0.04	0.03	0.03	0.00
Lignoceric acid	0.58	0.51	0.32	0.39	0.38	0.32	0.25
Nervonic acid	0.33	0.29	0.27	0.26	0.23	0.24	0.24
Docosahexaenoic acid (DHA)	0.00	1.94	0.00	0.00	1.80	0.00	0.00

Appendix D. Trace Elements Content in PO Stems and Fruiting Body

Table A6. Trace elements content in PO fruiting body.

Trace Metals (mg/kg DM%)	Stems 0%	Stems 2.5%	Stems 5%	Stems 7.5%	Stems 10%	Stems 12.5%	Stems 15%
Al **	21.3 ± 0	<20	<20	<20	<20	<20	<20
As **	<5	<5	<5	<5	<5	<5	<5
Ca **	159.5 ± 6.5	122 ± 9	128.5 ± 8.5	107.9 ± 25.1	81.65 ± 8.1	99.4 ± 16.6	107.8 ± 9.2
Cd **	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Co **	<1	<1	<1	<1	<1	<1	<1
Cr **	<2	<2	<2	<2	<2	<2	<2
Cu **	<10	<10	<10	<10	<10	12.2 ± 1.9	13.6 ± 0.15
Fe **	72.85 ± 4.15	67.5 ± 4.1	74.6 ± 0.8	66.65 ± 0.15	64.7 ± 00	62.85 ± 3.95	61.5 ± 1
Hg **	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
K *	31,250 ± 550	32,150 ± 450	33,050 ± 250	30,700 ± 200	30,850 ± 350	29,750 ± 50	27,600 ± 400
Mg *	1525 ± 5	1670 ± 20	1650 ± 10	1515 ± 15	1615 ± 5	1445 ± 25	1340 ± 10
Mn *	9.7 ± 0.1	11 ± 0.2	11.8 ± 0.1	11.1 ± 00	12.75 ± 0.15	12.8 ± 1.2	11.55 ± 0.05
Mo **	<1	<1	<1	<1	<1	<1	<1
Ni **	<1	<1	<1	<1	<1	<1	<1
P *	6580 ± 30	9845 ± 155	11,300 ± 200	10,650 ± 50	12,000 ± 100	11,300 ± 100	10,600 ± 200
Pb **	<10	<10	<10	<10	<10	<10	<10
S *	2550 ± 20	2535 ± 75	2685 ± 15	2620 ± 20	2910 ± 10	2850 ± 10	3025 ± 55
Se **	<10	<10	<10	<10	<10	<10	<10
Si *	63.15 ± 0.75	36.95 ± 14.15	25.6 ± 7.3	21.65 ± 2.25	38.35 ± 0.9	27.8 ± 6.3	44 ± 6.3
Sn **	<5	<5	<5	<5	<5	<5	<5
V **	<2	<2	<2	<2	<2	<2	<2
Zn **	84.2 ± 2.4	82.15 ± 2.35	78.7 ± 0.4	73.7 ± 1	82.65 ± 0.15	95.45 ± 11.55	74.3 ± 1.9

* p -value < 0.05; ** p -value ≥ 0.05.

Table A7. Trace element content in PO stems.

Trace Metals (mg/kg DM%)	Stems 0%	Stems 2.5%	Stems 5%	Stems 7.5%	Stems 10%	Stems 12.5%	Stems 15%
Al *	40.45 ± 7.05	43 ± 9.8	60.9 ± 4	45.75 ± 3.55	32.7 ± 1	26.8 ± 3.9	<20
As **	<5	<5	<5	<5	<5	<5	<5
Ca *	410.5 ± 21.5	616 ± 60	777.5 ± 55.5	536.5 ± 2.5	325 ± 9	253 ± 6	248.5 ± 48.5
Cd **	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Co **	<1	<1	<1	<1	<1	<1	<1
Cr **	<2	<2	<2	<2	<2	<2	<2
Cu **	<10	<10	14.9 ± 0.4	15.25 ± 0.65	16.1 ± 0.5	17.65 ± 1.75	15.75 ± 0.05
Fe **	<50	<50	70.8 ± 2.4	49.85 ± 2.45	<50	<50	71.5
Hg **	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
K *	20,800 ± 600	24,300 ± 0	25,550 ± 50	21,300 ± 300	20,800 ± 200	20,400 ± 100	23,400 ± 400
Mg *	1240 ± 60	1305 ± 15	1160 ± 10	1020 ± 10	965.5 ± 10.5	871.5 ± 9.5	1055 ± 25
Mn *	7.25 ± 0.32	9.83 ± 0.46	9.89 ± 0.41	7.59 ± 0.09	7.8 ± 0	6.92 ± 0.17	9.41 ± 0.35
Mo **	<1	<1	<1	<1	<1	<1	<1
Ni **	<1	<1	<1	<1	<1	<1	<1
P *	1715 ± 5	4215 ± 5	5860 ± 40	5610 ± 10	5715 ± 125	5475 ± 115	7815 ± 35
Pb **	<10	<10	<10	<10	<10	<10	<10
S *	1510 ± 30	1655 ± 30	2040 ± 70	1530 ± 10	1480 ± 20	1430 ± 50	2200 ± 30
Se **	<10	<10	<10	<10	<10	<10	<10
Si *	145 ± 19	157.5 ± 4.5	190 ± 9	155.5 ± 2.5	126 ± 5	123 ± 2	88.75 ± 2.95
Sn **	<5	<5	<5	<5	<5	<5	<5
V **	<2	<2	<2	<2	<2	<2	<2
Zn	<50	<50	51 ± 0	<50	<50	<50	62.8

* p -value ≥ 0.05; ** p -value < 0.05.

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