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Comparative and integrated OMICS characterization of Italian *Hericium erinaceus* strains: Implications for food composition

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ABSTRACT

The transition toward plant-forward diets is driving interest in sustainable, nutrient-rich food sources, with edible mushrooms emerging as promising alternatives. *Hericium erinaceus* mycelium, traditionally valued in Chinese medicine, is gaining recognition for its potential in food applications. This study evaluates the proteomic and metabolomic profiles of four strains of *H. erinaceus* mycelia collected in Italy, namely He1, He2, He4, and He5. MS proteomics and NMR metabolomics allowed the identification of 2180 proteins and 31 metabolites, revealing a rich nutritional composition. Functional analyses highlighted key pathways involved in protein synthesis and energy metabolism. Notably, strain-specific differences in amino acids, organic acids, and sugars profiles suggest opportunities for targeted strain selection to optimize nutritional profiles. These findings support the development of *H. erinaceus* mycelium as a sustainable, functional ingredient for novel food products, aligning with consumer demand for healthier and more environmentally conscious dietary options.

1. Introduction

A significant dietary shift from meat-centric to plant-forward diets is occurring from both consumers and industry, motivated by concerns over environmental sustainability, public health, and animal welfare (Cheskin et al., 2008; Hartmann and Siegrist, 2017; Lang, 2020). This trend reflects a growing awareness of the ecological and physiological impacts of food choices and a commitment to more sustainable and health-conscious consumption.

Edible mushrooms represent a promising class of alternative functional foods, offering a rich source of essential nutrients and bioactive compounds, including amino acids, fibers, vitamins, minerals, and being naturally low in calories, sodium, fat, and cholesterol (Das et al., 2021). Their production is also associated with a substantially lower environmental footprint compared to animal-based protein sources (Poore and Nemecek, 2018). Moreover, mushrooms possess unique organoleptic properties, making them suitable for enhancing the nutritional and sensory profiles of muscle foods (Kashyap et al. 2025). They are particularly suitable for flexitarian diets, where the amount of consumed meat or fish is drastically reduced. Despite these advantages, many edible mushroom species remain underutilized in food applications, although they may offer distinct nutritional, sensory, and environmental benefits compared to commonly used varieties (Kumar et al., 2021). With the rising consumer demand for healthier and more sustainable

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food options, the incorporation of mushrooms into muscle foods is poised for continued growth. Therefore, further investigation into these lesser-known species is warranted.

Hericium erinaceus (Bull.) Pers., commonly known as Yamabushitake or Lion's Mane, is a culinary medicinal mushroom that grows predominantly on oak and holm oak (Thongbai et al. 2015). Across its three growth stages - mycelium, primordium, and sporophore - it produces a diverse array of bioactive metabolites. To date, over 80 small molecules have been isolated and characterized, including isoindolinones, isobenzofuranones, erinacines, alkaloids, sterols, and polyketides (Tan et al., 2024). These compounds exhibit a broad spectrum of biological activities, such as anticarcinogenic, antibiotic, anti-fatigue, cardioprotective, nephroprotective, anti-senescence, and antidepressant effects (I. C. Li et al., 2018; Trovato Salinaro et al., 2018).

In addition to its pharmacological potential, *H. erinaceus* is characterized by a rich nutritional profile. Since 2015, its dehydrated mycelium powder has been included in the European Union's official list of Novel Foods (European Commission, 2015). Up to now, the attention has been mainly focused on the main macro and micro nutrients that characterize this species. Anyway, it is well known that the features of a food matrix, namely nutritional properties, nutraceutical activities, and organoleptic characteristics are also defined by small metabolites (Łysakowska et al., 2023). For this reason, to further valorize the potential of *H. erinaceus* as a Novel Food ingredient, a deeper understanding of its metabolite profile is required, as well as a better understanding of the biochemical factors, such as protein expression, activated in the matrix.

In this context, OMICs approaches offer a powerful framework for comprehensive molecular characterization (Horgan and Kenny, 2011). Among them, proteomics enables the systematic analysis of protein expression, post-translational modifications, and functional interactions, providing insights into metabolic pathways and regulatory networks relevant to fungal development and environmental adaptation (Ball et al., 2020). In particular, proteomics provides valuable insights into strain-specific protein expression, which can support the selection of strains with desirable traits for nutritional, nutraceutical, and industrial applications (Xie et al., 2025). However, proteomics studies on edible and medicinal mushrooms primarily focused on differential expression across environmental conditions, growth stages, and tissue morphologies (Al-Obaidi, 2016; Cao et al., 2023), while investigations into intraspecific variation, particularly among *H. erinaceus* strains, remain limited. Indeed, proteomics studies on *H. erinaceus* regarded the identification of the expressed proteins in the sporophore (Horie et al., 2008) and in the mycelium, unravelling the differences between growth stages (Zeng et al., 2018).

Complementing proteomics, metabolomics captures the downstream biochemical phenotype, highly responsive to environmental factors (Guijas et al., 2018). An LC-MS/MS approach has been previously applied to define the metabolomic profile of an *H. erinaceus* sporophore Chinese strain (Yang et al., 2021). However, untargeted NMR-based metabolomics offers a robust and reproducible platform for comprehensive profiling of complex biological matrices and for assessing the impact of variables, such as strains and growing conditions, on the chemical profile (Sobolev et al., 2022).

In this study, we employed MS-based proteomics and NMR-based metabolomics complementary approaches to characterize and compare the mycelial proteome and metabolome of four *H. erinaceus* strains grown in Italy. Our aim was to obtain a comprehensive overview of the protein and metabolite profiles of the species, to better define its potential nutritional, nutraceutical, and organoleptic properties. Additionally, we considered strain variability as an important interspecies factor influencing the chemical profile and potential applications of *H. erinaceus*. These findings provide a molecular basis for selecting and optimizing strains for food and health-related applications, contributing to the valorization of this species in functional food development.

2. Materials and methods

2.1. Chemicals and reagents

Gradient-grade water, chloroform, methanol, p otassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4), and 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP) were purchased from Merck Life Science (Milano, Italy). Deuterium oxide 99.97% of deuterium, methanol-d_4 99.80% of deuterium, and chloroform-*d* 99.80% of deuterium were purchased from Euriso-Top (Saclay, France). Gradient-grade solvents for LC/MS experiments (solvent A: 100% milliQ water, 0.1% formic acid; solvent B: 20% milliQ water, 80% acetonitrile, 0.1% formic acid) were purchased from Thermo Fisher Scientific (Waltham, MA USA).

2.2. Fungal strains

Four strains namely He1, He2, He4, and He5, belonging to the Fungal Research Culture Collection (MicUNIPV) of the Mycology Laboratory at the Department of Earth and Environmental Sciences (DSTA) (University of Pavia, Italy) were analyzed. The strains analyzed in this study were chosen due to their preservation within our Research Culture Collection and their prior comprehensive characterization through chemical analyses and functional assays. Wild Type (WT) sporophores were collected in Italy and then mycelia isolated in pure culture. Sporophores of the strains He1, He2 and He4 were collected in Siena (SI), Italy, from *Quercus ilex* L. whereas sporophore of the strain He5 was collected in Bedonia (PR), Italy, from a live specimen of *Quercus cerris*. As previously reported (Cartabia et al., 2022), the sporophores were aseptically cut into small portions (about 1 mm^3) placed into Petri dishes with 2% malt extract agar as a culture medium (MEA, Biokar Diagnostics). Chloramphenicol at 50 ppm was added in this first step. Incubation was carried out at 24 °C in complete darkness. This allowed the strains to be isolated in pure culture. Then, all the strains were identified both by macro and micro-morphological cultural characteristics and by molecular analysis of the Internal Transcribed Spacer (ITS) regions (Cesaroni et al., 2019; Corana et al., 2019; Girometta et al., 2024). The assigned accession number is MN861073.1 for He1, MN861074.1 for He2, PZ040150.1 for He4, and PV037528.1 for He5. The strains were stored in different cultural media at − 80 °C.

2.3. Samples preparation

Fungal strains (He1, He2, He4 and He5) were initially cultivated for 15 days at 25 °C on Petri dishes containing 2% w/v Malt Extract Agar (MEA, Biokar) to standardize inoculum conditions. and ensure consistent growth reactivity. Liquid cultures were initiated by aseptically transferring five colonized MEA portions (approximately 0.125 cm^3 each) per 300 mL of medium into 1 L and 2 L flasks containing 2% *w/v* Malt Extract (Biokar, initial pH 5.0 ± 0.5). The flasks were plugged with raw cotton to allow passive gas exchange while maintaining sterility. To ensure uniform environmental parameters, incubation was conducted in a temperature-controlled incubator at 25 °C, under dark, and strictly static conditions. This standardized, long-term setup (30 days) was specifically designed to allow the mycelia to reach a mature and stable metabolic state. By strictly controlling initial parameters and environmental conditions, we ensured high reproducibility and minimized stochastic physiological variations that could otherwise emerge across the long-term cultivations. Finally, the resulting mycelia were gently rinsed with deionized water, lyophilized (24 h at −50 °C and 1 mbar), and subsequently stored at −20 °C.

2.4. Protein extraction procedures

Samples were prepared with a method previously developed by our group for the proteomic analysis of *Schizophyllum commune* (Desiderio

et al., 2025). Specifically, dehydrated samples (~400 mg) were plunged in liquid nitrogen (~ −200 °C) for 60 s and reduced in thin powder using pestle and mortar. Cell lysis and protein extraction were performed as follows: 100 mg of fungal powder was suspended in 1 mL of 10 mM Tris buffer pH 8 in the presence of glass beads (100 mg, diameter ~ 500 μm), homogenized by mechanical stress (FastPrep-24, 4 cycles of 30 s each), and centrifuged for 15 min at 14000 x g at room temperature. The pellet was then washed twice with acetone, centrifuged, suspended in 10 mM Tris buffer pH 8 and 8 M urea for 24 h, and finally centrifuged to remove glass beads.

The amount of extracted proteins in each sample was quantified by absorbance spectroscopy employing a Bradford assay (calibrated using bovine serum albumin as standard protein).

2.5. LC-MS/MS analysis

Samples were diluted to final 10 mM Tris buffer pH 8 and 4 M urea, and proteins reduced by dithiothreitol 10 mM, alkylated by iodacetamide 50 mM and digested by trypsin (16 h at 37 °C, urea diluted to final 2 M). Digested peptides have been desalted by Ziptip C-18 columns, lyophilized and suspended in 0.1% formic acid. Samples have been analyzed by LC/MS employing an Orbitrap Fusion instrument (Thermo Fisher Scientific) equipped with a HPLC system (EASY-nLC 1000). Peptides have been separated by a 50 cm C-18 column (Thermo Fisher Scientific) at 40 °C using a 120 min gradient with a flow rate of 250 nL/min (solvent A: 100% milliQ water, 0.1% formic acid; solvent B: 20% milliQ water, 80% acetonitrile, 0.1% formic acid), detected in the orbitrap analyzer and fragmented in the ion trap analyzer by high-energy collision dissociation (HCD) with helium gas.

2.6. Protein identification, functional and statistical analysis

The identification and quantification was performed with MaxQuant 2.1.4.0 (Tyanova et al., 2016) against the *H. alpestre* database (proteome ID: UP000298061) downloaded from Uniprot in April 2023. The selected proteome is the only one available for the *Hericium* genus, and was used as *H. erinaceus* and *H. alpestre* are part of the same monophyletic genus with a close evolutionary relationship within the genus (Cesaroni et al. 2019, Wang et al. 2024). The default parameters have been used with the following exceptions. Mass search tolerances were set to 10 ppm for MS and 0.6 Dalton for MS/MS. Aminoacid residues modifications were used as fixed for carbamidomethylation of cysteines and as variable for methionine oxidation. The possible missed cleavages for trypsin/P were set to 3. False discovery rate was set to 1% at peptide and protein levels based on a target/decoy search. Unique and razor peptides were used for the quantification of proteins by Peptide Spectrum Match (PSM). The extracted proteins were quantified taking into account the average spectral count among the two replicates. Quantification data for each protein in the different samples is reported in Supplementary Table S1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) with the dataset identifier PXD066941 and 10.6019/PXD066941.

Perseus 2.0.5.0 was used for post-processing data, statistical analysis, and data visualization. ImageGP 2 (https://www.bic.ac.cn/BIC/#/) was used to prepare the volcano plots picture (Chen et al. 2024). Gene ontology terms enrichment analysis were performed with DAVID, considering Gene Ontology Biological Process terms (Sherman et al., 2022). The enriched terms were then clustered according to semantic similarity using Revigo (Supek et al., 2011). ITS sequences were aligned and their pairwise distances were calculated using MEGA12.1 (https://www.megasoftware.net/), with the following parameters: Bootstrap method for variance estimation (5000 replicates) and Maximum Composite Likelihood as Substitution model. Mantel test was performed using the ecodist package in R (Goslee and Urban, 2007).

2.7. Extraction and analysis procedures for NMR metabolomics

Sample extraction for NMR metabolomics analysis was carried out by applying the Bligh-Dyer protocol previously reported for this matrix (Spano et al., 2024). In particular, 100 mg of sample were added with 3 mL of $CH_3OH/CHCl_3$ 2:1 *v/v* mixture and 0.8 mL of distilled H_2O, followed by sonication. Subsequently, a further 1 mL of $CHCl_3$ and 1 mL of distilled H_2O were added to the system. Finally, after centrifugation, the obtained hydroalcoholic and organic phases were separated. The procedure was repeated another two times on residual pellets to obtain a quantitative extraction of the metabolites. Both hydroalcoholic and organic phases were dried with a soft N_2 flux (1 bar, room temperature) and preserved at −20°C until analysis.

NMR analyses were carried out on a 600 MHz spectrometer (Jeol JNM-ECZ 600 R) equipped with a 5 mm FG/RO DIGITAL AUTOTUNE probe.

Dried hydroalcoholic extracts were dissolved in 700 μL of 100 mM phosphate buffer/D_2O, containing 0.5 mM TSP (3-(trimethylsilyl)propionic acid sodium salt) as internal standard. Organic extracts were dissolved in 700 μL of $CDCl_3/CD_3OD$ 2:1 *v/v* mixture. ^1H NMR spectra were acquired at 298 K using a 9000 Hz spectral width, 4 dummy scans, 128 scans, 7.723 s relaxation delay, 90° pulse of 8.8 μs, 64 K data points, and residual water signal suppression with a presaturation pulse. Raw data were processed (Fourier transformation, baseline and phase correction, signal area integration) using JEOL Delta software (v5.3.1).

Quantitative data from hydroalcoholic extracts were obtained referring to the internal standard TSP and expressed as mg/100 g sample DW (dry weight) on three replicates ($n = 3$). Quantitative data obtained from organic extracts were expressed as relative molar percentage (molar %) on three replicates ($n = 3$).

Metabolite identification was carried using both information obtained by ^1H spectral features (signal chemical shift, multiplicity, *J* coupling constants) and literature data regarding *H. erinaceus* and other pertaining fungal species analyzed under the same experimental conditions (Goppa et al., 2023; Spano et al., 2024).

For each quantified metabolite, one-way ANOVA, followed by Tukey's multiple comparisons test, was applied to observe significant differences ($p < 0.0001$) among the samples using GraphPad Prism 8.0.2 software.

3. Results and discussion

3.1. Proteins identification and quantification through a proteomic approach

Proteomic analysis of the four investigated *H. erinaceus* mycelia identified a total of 2180 proteins, Supplementary Table S1. Notably, approximately 45% of these proteins (923) were consistently detected across all samples, suggesting the presence of a conserved core proteome, Fig. 1. Among the pairwise comparisons, strains He1 and He2 shared the highest number of proteins (n = 1310), followed by the He4-He5 pair with 1251 shared proteins, indicating varying degrees of proteomic similarity among the strains.

3.2. Functional analysis of identified protein

A comprehensive functional analysis of proteins identified across all *H. erinaceus* mycelial samples was performed. This analysis revealed significant enrichment in 16 Gene Ontology Biological Process (GO-BP) terms, Table 1. The results suggest that the cultivation conditions were favorable for protein biosynthesis, as evidenced by the enrichment of processes such as translation, protein folding, mRNA splicing, intracellular protein transport, and cellular amino acid biosynthesis. Additionally, enrichment in pathways related to the tricarboxylic acid cycle (TCA) and glycolysis was observed, indicating elevated energy production consistent with active cellular growth. Notably, terms associated

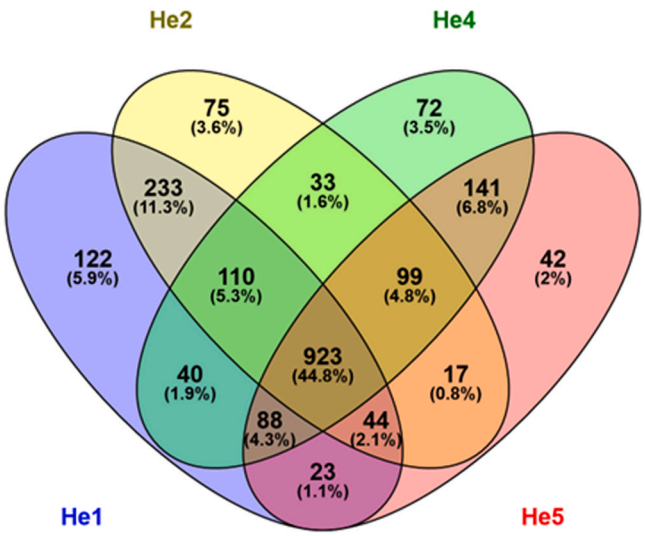

Fig. 1. Venn's diagram reporting the identified proteins present in all analyzed samples. He1 (blue), He2 (yellow), He4 (green), He5 (red).

Table 1
Gene Ontology Biological Process terms enriched considering all identified proteins.

Term number	Term description
GO:0006412	translation
GO:0006099	tricarboxylic acid cycle
GO:0006457	protein folding
GO:0000398	mRNA splicing, via spliceosome
GO:0016192	vesicle-mediated transport
GO:0001732	formation of cytoplasmic translation initiation complex
GO:0006511	ubiquitin-dependent protein catabolic process
GO:0006096	glycolytic process
GO:0006886	intracellular protein transport
GO:0008652	cellular amino acid biosynthetic process
GO:0007017	microtubule-based process
GO:0006913	nucleocytoplasmic transport
GO:0009097	isoleucine biosynthetic process
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process
GO:0006897	endocytosis
GO:0019752	carboxylic acid metabolic process

with metabolite transport and secretion, specifically GO:0016192 (vesicle-mediated transport) and GO:0006897 (endocytosis), were also enriched, suggesting active intracellular trafficking and secretion processes.

3.3. Differences in protein expression between different strains

Pairwise comparisons of protein expression profiles among the four mycelium strains revealed distinct patterns of differential expression. Strains He1 and He2 samples exhibited highly similar protein expression profiles, with only 16 proteins showing significant differences, Fig. 2A. Among them, 14 proteins were overexpressed in He1 and are associated with biological processes such as carboxylic acid metabolism, isoprenoid biosynthesis, lipid transport, dimethylallyl diphosphate biosynthesis, and endocytosis, Supplementary Table S2. The presence of enzymes involved in carboxylic acid metabolism is possibly related to higher amounts of threonine and asparagine found in He1, as reported in 3.4.1, Fig. 6. Furthermore, the upregulation of isoprenoid biosynthesis- and dimethylallyl diphosphate biosynthesis-related proteins in He1 suggests a potential for enhanced production of terpenoids, bioactive compounds known for their anti-cancer, anti-inflammatory, and antioxidant properties (Chen et al., 2023). Terpenoid compounds have been found abundant in *H. erinaceus* mycelium and sporophore (Zeng et al., 2018), but further metabolite profiling is warranted to confirm the presence and quantify the levels of these compounds in this strain, that could add functional value to *H. erinaceus* as a food ingredient. This observation underscores the complementary role of proteomics in enriching and extending the metabolic profiling of *H. erinaceus*, since some classes of compounds such as terpenoids are not detected with the here used metabolomics approach.

In contrast, He1 displayed markedly different expression profiles when compared to He4 and He5, with 327 and 402 differentially expressed proteins, respectively. The He1–He4 revealed 167 proteins upregulated in He1 and 160 in He4, Fig. 2B. Proteins overexpressed in He1 were enriched in GO-BP terms related to amino acid biosynthesis and metabolism, vesicle-mediated transport, glyoxylate cycle, protein folding and ubiquitination, endocytosis, and protein transport, Fig. 3 and Supplementary Table S3. The overexpression of proteins related to amino acid biosynthesis in He1 is consistent with the total amino acid (TAA) content measured by NMR. As mentioned in 3.4.1, strain He1 showed a similar TAA level to He2, but a marked difference compared to He4, which exhibited the lowest TAA content among the strains. Conversely, He4 showed increased expression of proteins involved in ergosterol biosynthesis, response to fatty-acid biosynthesis, lipid metabolism, oxidative stress, and melanin biosynthesis, Fig. 4 and Supplementary Table S4. Although He4 exhibited an increase in protein expression related to lipid biosynthesis, no significant differences in total fatty acid or ergosterol content were observed, Fig. 4. This is possibly due to the presence of different enzymes in different classes involved in the biosynthesis of these molecules. However, He4 upregulation of melanin biosynthesis enzymes, including tyrosinase, correlated with its visibly darker pigmentation. Fungal melanin is a multifunctional compound with anti-radiation, anti-oxidation, photoprotection, biosorbent, and antibacterial properties. It also has various biological activities, including blood lipids reduction, hepatoprotection and anti-tumor effects and blood sugar reduction. In the food industry melanin finds applications as a functional ingredient (e.g. for color enhancement, shelf-life extension, etc.) and the food-grade melanin is generally derived from edible and medicinal fungi (Liu et al., 2022).

The He1-He5 comparison identified 253 proteins overexpressed in He1, Fig. 2C. These proteins were associated with biological processes such as isocitrate and malate metabolism, amino acid biosynthesis, chorismate biosynthesis, arginine biosynthesis via ornithine, endocytosis, galactose metabolism, cellular response to oxidative stress, cellular nitrogen compound biosynthesis, organic cyclic biosynthesis, steroid biosynthesis, and isoprenoid biosynthesis. These findings support the hypothesis that He1 may produce a broader range of bioactive secondary metabolites, in particular isoprenoids. In contrast, 139 proteins were overexpressed in He5, linked to malonyl-CoA biosynthesis, glucose metabolism, fatty acid metabolism, GDP-mannose biosynthesis, arginine biosynthesis, leucine biosynthesis, transmembrane transport, autophagy, organonitrogen compound biosynthesis, response to oxidative stress, cell redox homeostasis, and cellular aromatic compound metabolism, Supplementary Table S5. Interestingly, He5 exhibited the highest glucose concentration, Fig. 7A, which aligns with the observed overexpression of proteins associated with glucose metabolism. In contrast, He1 showed a marked overexpression of proteins involved in galactose metabolism, indicating the activation of distinct sugar metabolic pathways across the strains.

The He2-He4 comparison revealed 23 differentially expressed proteins, with 4 upregulated in He2 and 19 in He4, Fig. 2D. The He2 strain exhibited an overexpression of proteins related to endocytosis and the metabolism of compounds containing nitrogen. In contrast, the proteins overexpressed in He4 were involved in the tricarboxylic acid cycle, aldehyde metabolic process, and melanin biosynthetic process. The overexpression of tyrosinase in He4 provides molecular evidence for its intensified melanin biosynthesis production, corroborating the observed pigmentation phenotype and highlighting a potentially upregulated

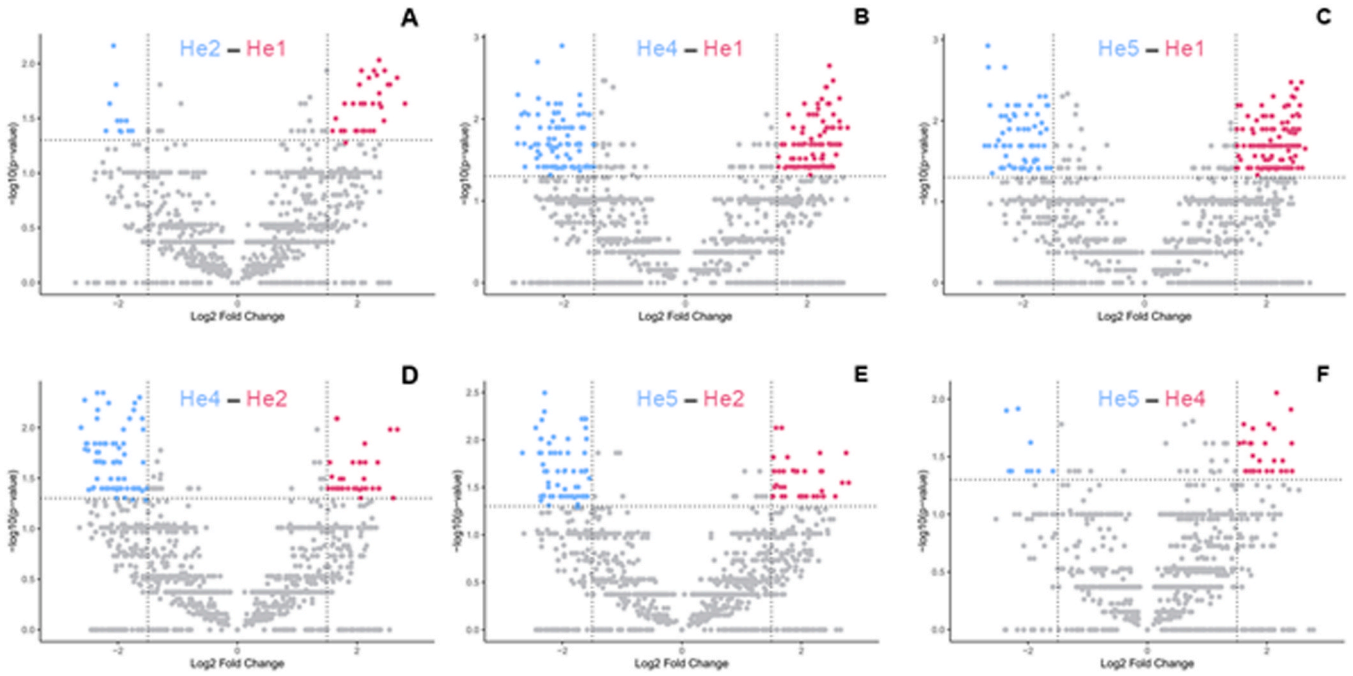

Fig. 2. Volcano plots illustrating the differential protein expressions between various strains. Each panel (A–F) compares protein expression between two strains, with the x-axis representing $\log_2$ fold change and the y-axis representing $-\log_{10}$(p-value). Red and blue dots indicate significantly upregulated and downregulated proteins, respectively. Proteins are considered significantly differentially expressed if they meet the criteria of p-value < 0.05 and fold change > 1.5. Comparisons are as follows: (A) He2 vs. He1, (B) He4 vs. He1, (C) He5 vs. He1, (D) He4 vs. He2, (E) He5 vs. He1, and (F) He5 vs. He4.

Fig. 3. GO Biological process terms associated with proteins overexpressed in He1 *vs* He4. The GO-BP terms are clustered by semantic similarity using Revigo.

Fig. 4. GO Biological process terms associated with proteins overexpressed in He4 *vs* He1. The GO-BP terms are clustered by semantic similarity using Revigo.

melanogenic pathway.

In the He2–He5 comparison, 82 proteins were differentially expressed, Fig. 2E. He2 showed upregulation of 32 proteins involved in prenylcysteine catabolic process, mitochondrial alanyl-tRNA aminoacylation, proteolysis, cellular nitrogen compound biosynthetic process, and catechol-containing compound metabolic process. He5 exhibited 50 upregulated proteins associated with response to oxidative stress, fatty acid biosynthetic process, cellular aldehyde metabolic process, glycine catabolic process, cysteine biosynthetic process, ergosterol biosynthetic process, carboxylic acid metabolic process, methionine biosynthetic process, and polysaccharide catabolic process. He5 exhibits significant activation of diverse biosynthetic routes, reinforcing the role of proteomic analysis as a complementary approach to deepen and extend the metabolic profiling of *H. erinaceus*.

Hierarchical clustering based on the protein expressions of the different strains, Fig. 5 and Supplementary Table S6, confirmed the close similarity between He1 and He2, which formed a distinct cluster separate from He4 and He5. We investigated whether genetic distances among strains, calculated from ITS sequences, Supplementary Table S7, were correlated with differences revealed by hierarchical clustering based on protein expression profiles. A Mantel test indicated no significant correlation between the two distance matrices ($r = -0.089$, $p = 0.7889$), suggesting that ITS-based genetic variation does not predict differences in protein expression. It should be noted, however, that genomic divergence extends beyond the ITS region and probably involves other genes that more directly influence proteomic patterns. However, it should be noted that, despite being isolated from the same host plant species and the same area as He1 and He2, He4 is genetically more similar to He5. This is in line on what has been observed for the proteomic profile which is more closely aligned with He5, Figure2F.

In fact, although He4 was sampled within the same macro-area as He1 and He2, the exact sampling site was not the same. For this reason, it exhibited a distinct profile, highlighting how subtle variations in the micro-environmental conditions can markedly influence the chemical signature of this type of biological sample.

He5, which was collected from a different area, displayed a unique and clearly differentiated profile.

This underscores the importance of extensive molecular characterization, as strains from similar environments can exhibit markedly different biochemical traits.

3.4. NMR metabolomics

NMR analysis of the examined samples enabled the identification of various metabolite classes in both hydroalcoholic and organic extracts. In the hydroalcoholic extracts, sixteen amino acids, five sugars and polyols, seven organic acids, and six nitrogen-containing compounds were identified. In contrast, the organic extracts revealed the presence of fatty acids, ergosterol, and phospholipid polar heads.

The following sections provide a detailed discussion of each metabolite class.

3.4.1. Amino acids

From a qualitative perspective, all samples exhibited a similar amino acid profile, with the exception of histidine, betaine, and tryptophan, Fig. 6. Histidine was detected exclusively in He1, whereas betaine was absent in this sample. Tryptophan, on the other hand, was not detected in He4. Quantitatively, He1 showed the highest concentrations of essential amino acids (EAA), asparagine, and tyrosine, as confirmed by one-way ANOVA ($p < 0.0001$, respect to He4 (a) and He5 (b)). The

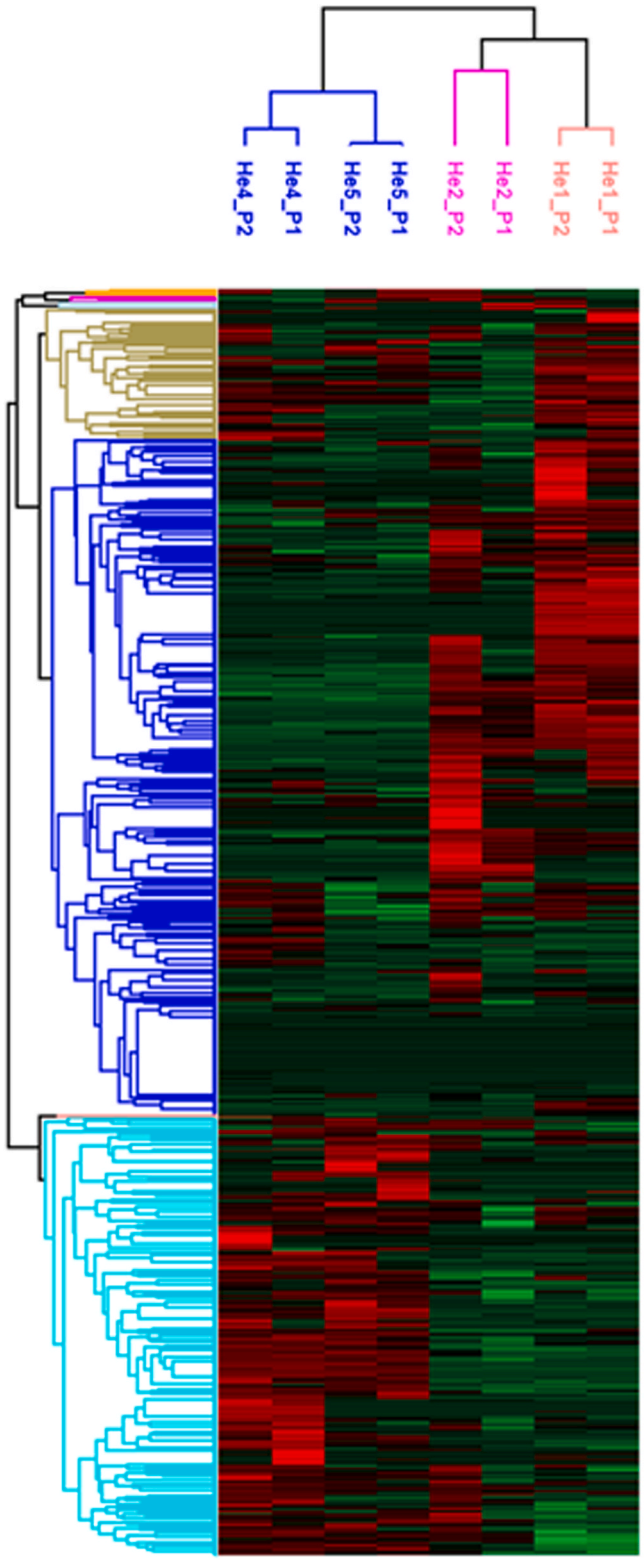

Fig. 5. Heatmap of protein expression in all the analyzed samples. The columns represent the samples with the technical replicates and are divided in 3 clusters with a distance threshold of 62.4. The rows represent the protein identified and are divided in 7 clusters with a distance threshold of 3.17. The intensity of the colors is proportional to protein expression after Z-score normalization. Upregulated proteins are coloured in red and downregulated in green, while proteins that were not identified in the samples are coloured in black. The matrix used to generate the heatmap is provided in Supplementary Table S6.

elevated asparagine levels observed in He1 are consistent with the overexpression of enzymes involved in carboxylic acid metabolism. These enzymes also contribute to threonine biosynthesis, which is indeed significantly higher in He1 than in the other strains. Notable findings were also observed for He4, which exhibited the highest glutamine content. The overexpression of proteins involved in glutamine metabolic process was observed in He4, supporting the higher amount measured in this strain. The amount of betaine, aspartate, and glutamate were the highest in He5, but we did not observe the overexpression of specific class of proteins related to the synthesis of these amino acids. This discrepancy is possibly due to the presence of distinct biosynthetic pathways that are not directly reflected by the protein classes analyzed, allowing metabolite accumulation to occur independently of detectable changes in enzyme abundance. The strain He5, was also the richest in arginine and we also observed an upregulation of enzymes responsible for the synthesis of arginine in He5 with respect to other strains.

The NMR analysis of He2 mycelial sample was previously conducted under the same experimental conditions (Spano et al., 2024). A comparison with the current results reveals that the amino acids profile of He2 is close to those of He1, particularly in terms of EAA content. These results are consistent with the protein expression profiles of the two strains. Indeed, He1 and He2 displayed a strong similarity in their proteomic patterns, and among the 16 differentially expressed proteins, only those involved in carboxylic acid metabolism were related to amino acid biosynthesis. This further underscores the close correspondence between the two profiles.

To further evaluate the potential of *H. erinaceus* as a food ingredient and to facilitate a comparison among samples, quantitative data on the main classes of amino acids, in terms of both nutritional and organoleptic properties, are presented in Table 2. Notably, He1 and He2 had similar TAA as well as the highest levels of EAA, with the same concentration range, underlining how these two strains can be used as a source of essential amino acids and/or proteins. However, He5 exhibited the highest overall TAA content, whereas He4 had the lowest one.

In terms of flavor-active amino acids (FAAs) and sweet amino acids (SAAs), He1 and He2 again showed comparable levels, whereas He5 had the highest concentrations in both categories, making this strain a potential candidate as food flavoring, mainly thanks to the high levels of glutamate.

Comparison with existing literature on *H. erinaceus* provides valuable insights. It is important to note that due to different experimental protocols, namely extraction methods, analysis methodology, and data reporting, direct quantitative comparison are limited. Nevertheless, some meaningful similarities and differences can be observed. Only a single study in literature has provided a metabolomics NMR characterization of *H. erinaceus*, focusing on fruit body analysis of a Chinese strain (D. Liu et al., 2019). Anyway, due to a not well-defined extraction protocol and a different quantification approach, only a qualitative comparison is possible, In particular, most of the here detected free amino acids have been also identified in the cited work, except for histidine, lysine, and tyrosine not measured in the analysis of D. Liu et al. (2019) that, on the contrary, identified serine, here not detected. Previous studies on *H. erinaceus* powder (J. Li et al., 2022) (growth stage and origin not indicated in the paper) have reported glutamate as the most abundant amino acid, underlining the importance of this metabolite in mushrooms and confirming its main role in umami taste (Woraharn et al., 2015, 2016), as also confirmed by the here obtained results. Anyway, it is noteworthy to underline that in the mycelial samples here analyzed, higher concentrations of all free amino acids were measured with respect to those reported by J. Li et al. (2022). This significative difference could be attributed to both different sample extraction protocols (boiling water in the paper of J. Li et al. (2022)) and strain origin. Moreover, the growth stage is not indicated by J. Li et al., thus introducing a potential variability factor. Another paper has analyzed the content of free tryptophan in both mycelium and fruit body

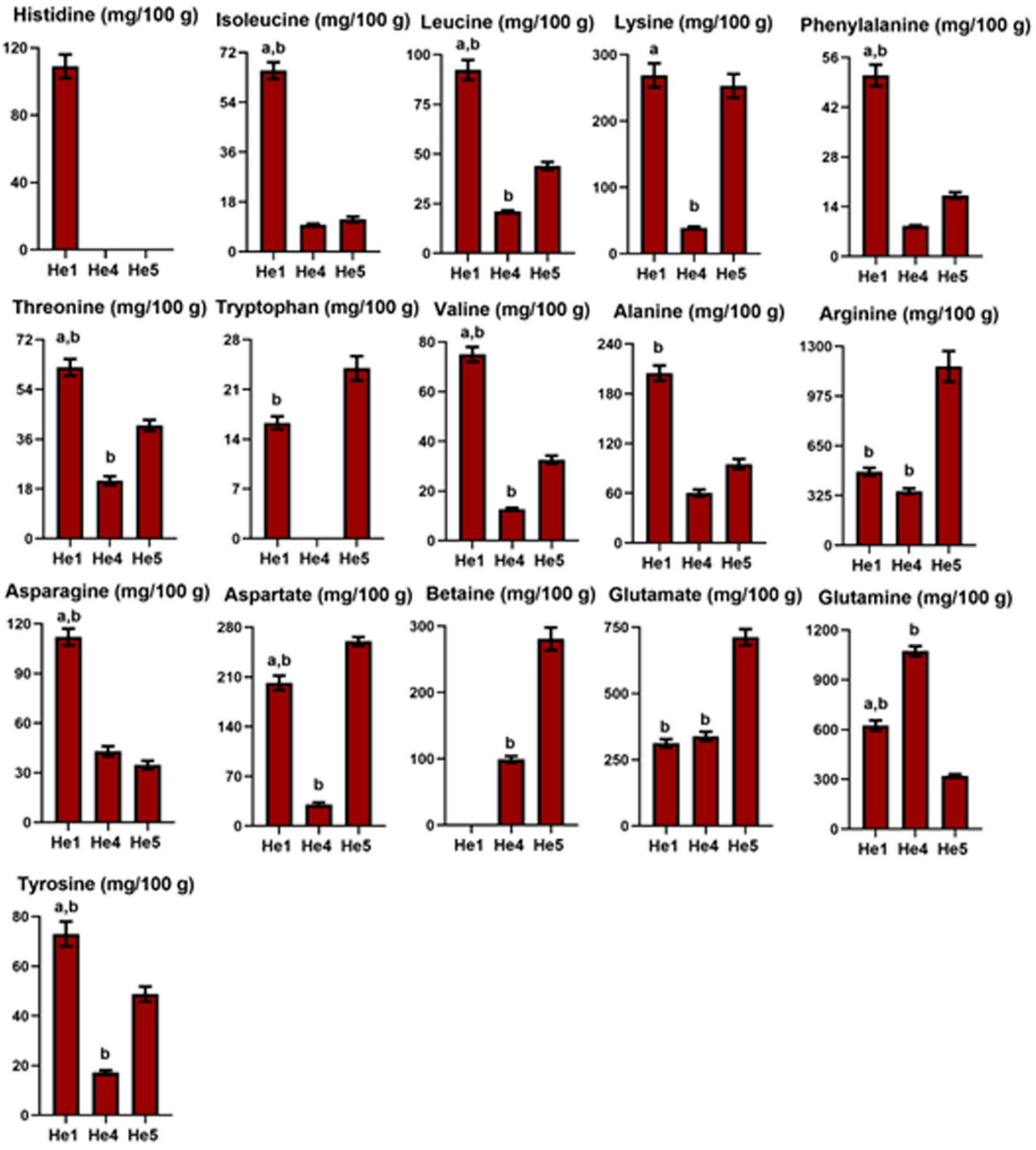

Fig. 6. Histograms relative to free amino acids quantification (mg/100 g of dried sample ± SD, $n = 3$) in the Bligh–Dyer hydroalcoholic extracts. One-way ANOVA, followed by Tukey's multiple comparisons test, was applied to underline significant differences ($p < 0.0001$): a) *vs* He4, b) *vs* He5.

hydroalcoholic extracts of *H. erincaeus* strain from US (Lazur et al., 2024). Interestingly, the detected concentration of tryptophan in *H. erinaceus* mycelium (Lazur et al., 2024) is similar to those here measured, whereas fruit bodies have been characterized by higher amounts respect to mycelium stage. Other analyses regarding the free amino acids content of *H. erinaceus* mycelia are not available, underlining a lack of knowledge in this field. Anyway, a recent study has focused the attention on essential free amino acids content of Korean strain of *H. erincceus* fruit body growth in liquid medium and solid one (brown rice) (Ryu et al., 2026). Interestingly, the here measured content of essential amino acids was higher with respect to those measured in liquid culture by Ryu et al., and similar to those growth with solid medium. These differences in terms of quantified AAs between mycelium and sporophore, as well as origin, growth condition, and other variables, that can be extended to all metabolites, are the result of a different gene expression, caused by the high mushroom adaptability required to

Table 2
Amounts (mg/100 g of dried sample ± SD, $n = 3$) of total free amino acids (TAA), essential amino acids (EAA), flavor amino acids (FAA) and sweet amino acids (SAA) measured in the analyzed mycelia.

Amino acid classes	*He1*	*He4*	*He5*	*He2* (Spano et al., 2024)
TAA (mg/100 g)	2748.4	2127.5	3371.8	3004.6
EAA (mg/100 g)	738.2	111.4	423.3	748.3
EAA/TAA	0.27	0.05	0.13	0.25
FAA (mg/100 g)	515.4	368.7	971.9	722.0
FAA/TAA	0.19	0.17	0.29	0.24
SAA (mg/100 g)	1066.7	1162.7	497.4	1063.6
SAA/TAA	0.39	0.55	0.15	0.35

TAA (Total amino acids) measured as sum of all detected free amino acids; EAA (Essential amino acids) measured as sum of Leucine, Isoleucine, Valine, Threonine, Lysine, Phenylalanine, Tryptophan, Histidine; FAA (Flavor amino acids) measured as sum of Glutamate and Aspartate; SAA (Sweet amino acids) measured as sum of Threonine, Alanine, Glutamine, Phenylalanine, Tryptophan, Histidine.

survive in natural habitats. This adaptability reflects the cells' responses to different chemical and physical environments and stimuli (Berger et al., 2022). These findings may inform the selection of specific strains, growth stages, and growth conditions of *H. erinaceus* for targeted nutritional applications.

3.4.2. Sugars and polyols

This class of metabolites exhibited substantial variability across the samples, both qualitatively and quantitatively, Fig. 7A, suggesting that these compounds are characteristic of specific *H. erinaceus* strains. Notably, trehalose and maltose were detected at the highest concentration in sample He1 (9% and 4% on dry weight, respectively), which, conversely, contained the lowest level of arabitol. The highest concentrations of arabitol and glucose were observed in He4 and He5, respectively. Glucose levels were relatively consistent across all analyzed mycelial samples. Mannitol was present at similar concentration across all current samples but was not detected in the previously analyzed He2 strain (Spano et al., 2024).

As for amino acids, the pronounced variability among strains is a critical factor when considering *H. erinaceus* for the isolation and/or production of specific nutritional formulations. In particular, the different qualitative and quantitative profiles of sugars and/or polyols in each strain can represent a marker used for their identification and classification. From an application standpoint, the identified polyols are of considerable interest to the food industry as established sweeteners and nutraceutical ingredients (Rice et al., 2020), and they occur in these mycelial matrices at relatively high levels.

Literature data has been mainly focused on the extraction and analysis of *H. erinaceus* polysaccharides, with no attention put on monosaccharides, oligosaccharides, or polyols, thus underlining the need to improve the knowledge in this context. The only exception is represented by the NMR study of D. Liu et al. (2019), allowing a qualitative comparison due to different analysis approaches. In particular, it is noteworthy to underline that fructose, mannose, and xylose have been measured in the cited text and not detected in the present work. On the contrary, mannose was measured only here. Also in this case, differences in terms of metabolite profile and so nutritional and/or biological properties can be attributed to several factors, namely analytical protocols, strain, growth stage, and growth conditions.

3.4.3. Organic acids

NMR spectrseveral organic acids, including acetate, citrate, formate, fumarate, lactate, malate, and succinate, Fig. 7B. The analyzed strains exhibited variable profiles for this class of metabolites. Sample He1 was characterized by the highest concentrations of acetate, citrate, lactate, and succinate. Malate was found in high concentrations in He5, consistent with the level previously reported for He2 under the same experimental conditions (Spano et al., 2024). Comparative literature data, primarily focused on the sporophore stage of *H. erinaceus* (Gąsecka et al., 2020), indicate a generally higher content of organic acids compared to the mycelial samples analyzed in this study. Qualitative comparison with the NMR results obtained for *H. erinaceus* sporophorus analysis underline some differences in the detected metabolites, with acetate and succinate being detected only in the present paper, whereas pyruvate, 3-hydroxybutyrate, and 4-aminobutyrate being identified only in the sample of D. Liu et al. (2019).

3.4.4. Nitrogen-containing compounds

No distinct trends were observed for this class of metabolites, as each sample exhibited a unique profile, Fig. 7C. However, in the case of choline and phosphatidylcholine, samples He1 and He4 showed similar concentrations, both lower than those measured in the other samples. The presence of these metabolites underlines the potential of medicinal mushrooms not only for nutritional purposes, but also for biological ones, namely neurotransmission and lipid metabolism (Kansakar et al., 2023; Z. Li and Vance, 2008). Uridine-based nucleotides were not detected in He1, whereas the nucleosides adenosine and uridine were absent in samples He4 and He5. This class of molecules presents a variable distribution in each considered strain, as also demonstrated by the comparison with literature data (D. Liu et al., 2019).

3.4.5. Apolar fraction

As expected, the Bligh-Dyer organic extracts were primarily composed of lipids, with both qualitative and quantitative profiles exhibiting distinct characteristics across the analyzed samples, Fig. 8. Total saturated fatty acids (TOT SFA) were most abundant in samples He1 and He4, each with a molar percentage of 62%. In contrast, He5 displayed a three times lower content of TOT SFA. An opposite trend was observed for total unsaturated fatty acids (TOT UFA), primarily due to the contribution of mono-unsaturated fatty acids (MUFA). Among other UFA, di-unsaturated fatty acids (DUFA) were most concentrated in He1 and least in He4. A very low molar percentage of tri-unsaturated fatty acid (TUFA), 0.3%, was detected only in He5.

Ergosterol, the main sterol in mushrooms, was measured in the range of 3–5% across all samples. Similarly, the concentration of the glycerophosphatidylcholine (GPC) polar head was approximately 7% in all samples. Another lipid polar head, namely glycerophosphatidylethanolamine (GPE), was detected exclusively in He5. Comparable levels of lipid polar heads and ergosterol were previously reported for He2 (Spano et al., 2024), although the proportions of TOT SFA and TOT UFA were different, being present at 46% and 51%, respectively.

Literature data regarding the fatty acids content of *H. erinaceus* have shown a great quantitative variation among the considered strains, as here demonstrated. In particular, Rodrigues et al. (2015) have analyzed *H. erinaceus* growth in Portugal (growth stage not specified) with a completely different fatty acid content (27.6% SFA, 32.9% MUFA, 39% DUFA, 0.30% TUFA) respect to the here analyzed one. Differently, Sande et al. (2019) have underlined a prevalence of MUFA respect to DUFA in *H. erinaceus* sample (other information not available).

Although mushrooms are not characterized by high amounts of fatty acids, the lipidic fraction can anyway be interesting from a nutritional point of view due to the presence of ω-6 polyunsaturated fatty acids, and mainly of ergosterol, a precursor of vitamin D2 (Sun et al., 2022).

3.4.6. General considerations

The obtained metabolite profiling results underlined great variability among the considered strains characterized by peculiar qualitative and quantitative profiles. In particular, He1 and He2 showed good similarity in terms of metabolite content, as well as protein expression, probably related to the same collection area. Anyway, although He4 was collected in the same macro-area of He1 and He2, a different profile was observed, underlining how potential variations of the micro-area conditions can

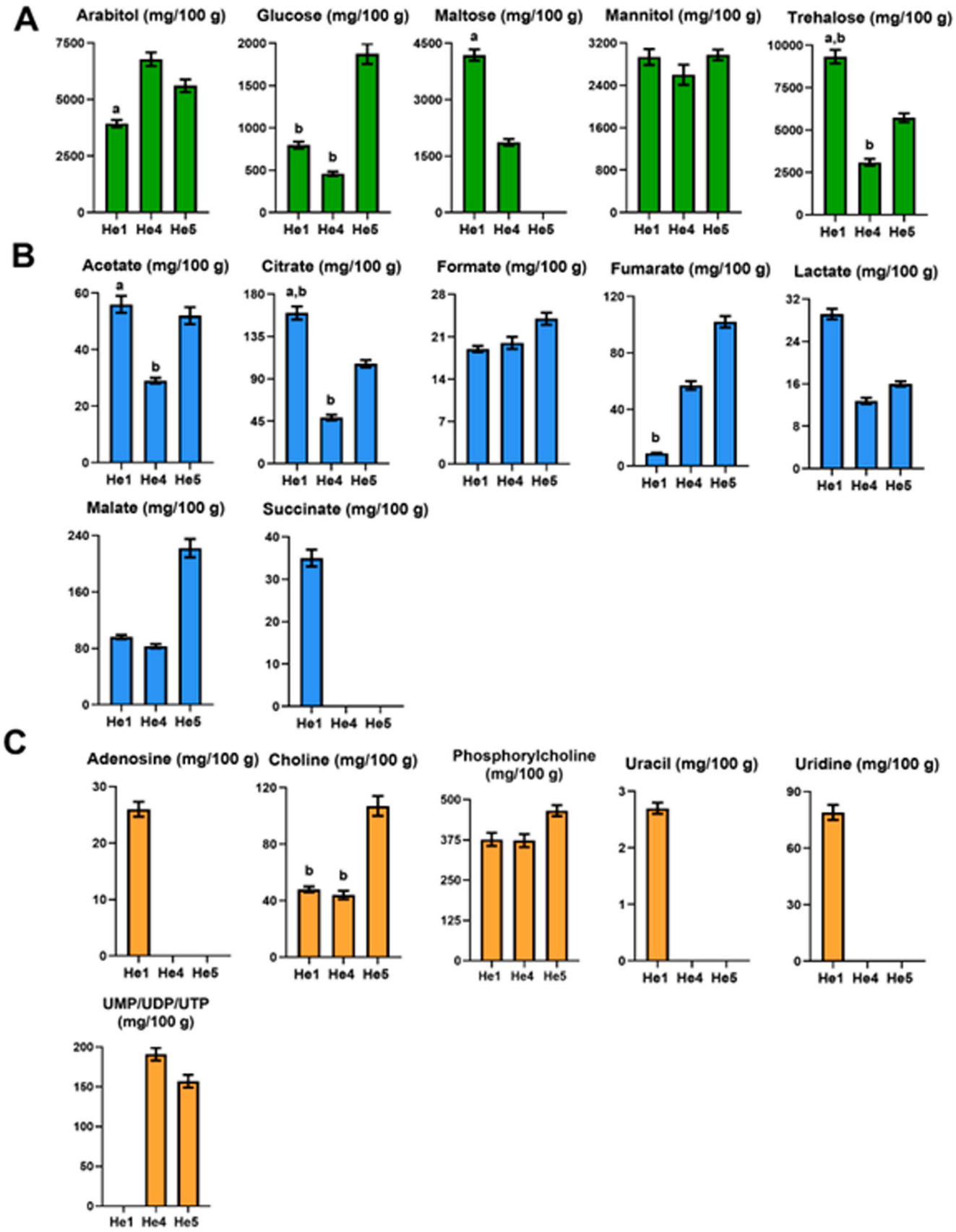

Fig. 7. Histograms relative to A. Sugars and polyols, B. Organic acids, C. Nitrogen-containing compounds (mg/100 g of dried sample ± SD, $n = 3$) in the Bligh–Dyer hydroalcoholic extracts. One-way ANOVA, followed by Tukey's multiple comparisons test, was applied to underline significant differences ($p < 0.0001$): a) *vs* He4, b) *vs* He5.

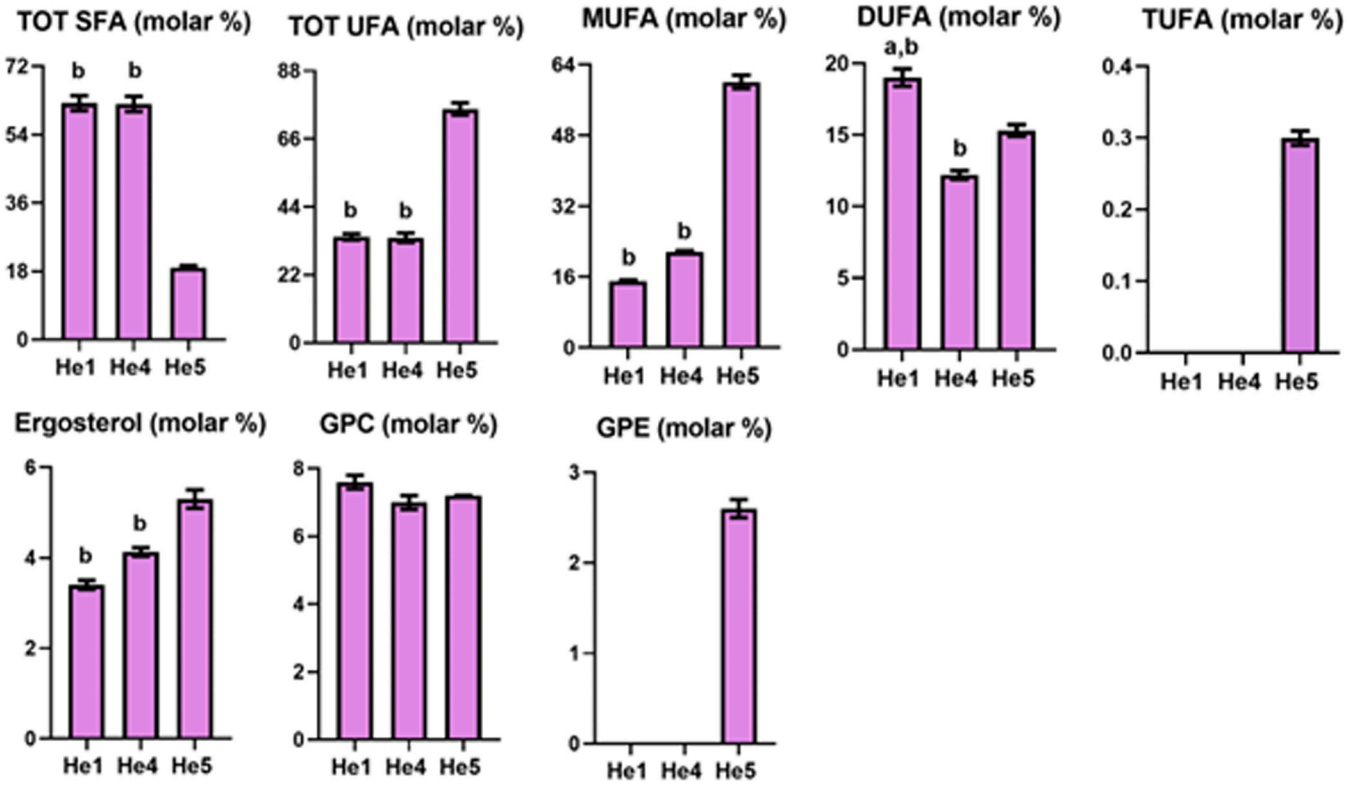

Fig. 8. Histograms relative to the metabolites quantified (relative molar percentage ± SD, n = 3) in Bligh–Dyer organic extracts. One-way ANOVA, followed by Tukey's multiple comparisons test, was applied to underline significant differences ($p < 0.0001$): a) *vs* He4, b) *vs* He5.

strongly affect the chemical profile of this type of matrix. As expected, He5, collected in a different area, was characterized by a unique and different profile.

In this context, each strain presents different features and potential applications in the food field. He1 and He2 samples could represent a source of EAA and proteins to use as well or as ingredients for fortified food matrices (Maseko et al., 2025; Ionescu et al., 2025). Moreover, the high amounts of sugars and polyols (at least 20% on total dry weight) make these strains both a source of energy and prebiotics, as well as important ingredients in the food industry (sweeteners, stabilizers). He5 also showed to be a good source of free amino acids, with the main feature of this strain being represented by the particularly high content of mono-unsaturated fatty acids, ergosterol, and glucose. Finally, He4 was characterized by the lowest amounts of quite all the considered metabolites. These results underline that inter-strain differences related to different collection areas, and thus development conditions, can strongly affect the chemical profile of mushrooms. These peculiar features can be used to produce and use each strain for specific purposes, as single matrix or a mixture of them,

4. Conclusions

The growing demand for sustainable food sources has positioned mushrooms as promising alternatives to conventional protein sources owing to their rich chemical composition, characterized by high levels of essential nutrients and low contents of fat and digestible carbohydrates.

In this study, multiple mycelial strains of *H. erinaceus*, officially recognized as Novel Foods by the European Union since 2015, were comprehensively analyzed using proteomics and metabolomics approaches. These complementary approaches offer a broader understanding of the sample's chemical profiles. The integration of both datasets supports a more comprehensive characterization of both shared and strain-specific features.

Functional analyses revealed significant enrichment in key biological processes, including protein synthesis and energy metabolism. Additionally, marked qualitative and quantitative variations in metabolite profiles among strains highlighted the influence of genetic variability on the nutritional and biochemical properties of this emerging food source.

Overall, the findings reinforce the potential of *H. erinaceus* mycelium as a sustainable and nutritionally valuable food source. The observed strain-specific metabolic traits suggest that targeted strain selection could be strategically employed to enhance specific nutritional attributes, since each analyzed strain showed a unique protein and metabolite profile. The integration of proteomic and metabolomic data provides a robust framework for guiding future strain selection and product development, aligning with the growing consumer demand for nutritious, environmentally conscious food options. These data represent a starting point in the research field regarding food use of *H. erinaceus* mycelia since, together with the potential food/nutraceutical properties, other aspects have to be deeply clarified, namely safety (i.e. presence of heavy metals, toxic compounds) and technological properties (i.e. storage preservation, production yields).

CRediT authorship contribution statement

Mattia Spano: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lorenzo Goppa:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carlo Santambrogio:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Emanuele Ferrari:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Elena Savino:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Paola Rossi:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Erika Ponzini:** Writing –

review & editing, Methodology, Formal analysis, Data curation. **Stefania Brocca:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Luisa Mannina:** Writing – review & editing, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Selene Chinaglia:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2026.109103.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD066941

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