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Mushroom species-specific ergothioneine and phenolic enrichment in solid-state fermented wheat grains as drivers of enhanced antioxidant capacity

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Abstract

Edible and medicinal mushrooms are valued for their bioactive compounds with established health benefits. Mycelium-enriched cereals are an emerging source of mycochemicals with natural antioxidant activity for functional food applications. To assess species-specific potential for antioxidant enrichment and secondary metabolite accumulation, we evaluated eight commercially cultivated fungal species using solid-state fermentation of wheat grains under standardized conditions. We quantified total antioxidant capacity (QUENCHER-CUPRAC), ergothioneine (EGT), total phenolic content (TPC), and flavonoids in myceliated wheat and uninoculated controls (n = 4 per species). All species significantly increased total antioxidant capacity 1.5- to 8.7-fold compared with controls ($p < 0.0001$). This variable showed a moderate positive correlation with mycelial growth (substrate consumption; r = 0.513; $p < 0.001$). Among secondary metabolites, *Pleurotus citrinopileatus* had the highest EGT content (2.33 ± 0.02 mg/g, near sporocarp levels); *P. citrinopileatus* and *Trametes versicolor* had the highest TPC (~6.8 mg GAE/g); and *Pleurotus djamor* accumulated the highest flavonoid content (1.00 ± 0.02 mg QE/g). EGT, TPC, and flavonoids accounted for most of the variability in antioxidant capacity (multiple linear regression, $R^2 = 0.979$). *Post hoc* analysis revealed two distinct EGT vs. TPC regression slopes across phylogenetic groups, suggesting that some species favor EGT while others prioritize phenolic compound accumulation. These results indicate that species-specific EGT and phenolic accumulation largely drive the enhancement of antioxidant capacity in myceliated wheat grains, supporting the use of fungal SSF as a biotechnological tool to enrich the bioactive profiles of cereals for functional food development.

Keywords

Solid-state fermentation; ergothioneine; phenolics; antioxidant capacity; *Pleurotus*; mycelium

Introduction

Mushrooms are rich sources of bioactive compounds, known as mycochemicals, with antioxidant, anti-inflammatory, and immunomodulatory properties, among other health-relevant benefits, and show promise for applications in nutraceuticals, functional foods, and cosmeceuticals [1]. These beneficial metabolites have been documented predominantly in sporocarps (fruiting bodies), yet growing evidence shows their presence and accumulation in fungal biomass (mycelium) as well [2]. Cultivating mycelium on solid substrates through solid-state fermentation (SSF) is a proven biotechnological approach to enhance the nutritional quality of staple cereals and legumes. Through their versatile enzyme systems, fermenting fungi degrade complex substrates, reducing antinutrient levels and improving protein content, amino acid profiles, mineral bioavailability, dietary fiber, and vitamin content [3–7]. Edible and medicinal fungi provide high-value antioxidant secondary metabolites, including L-ergothioneine (EGT) and phenolic compounds, a broad class encompassing phenolic acids and flavonoids [2, 8, 9]. Despite the widespread cultivation of mushrooms via SSF, mycelium-enriched substrates remain an underexplored source of bioactive compounds for functional food development [4].

EGT is a thiol-containing amino acid derivative with potent antioxidant activity and well-established health benefits. The European Union approved it as a novel food, and the United States Food and Drug Administration (FDA) recognized it as generally recognized as safe (GRAS). This compound exerts protective effects by modulating antioxidant defense systems and inflammatory pathways through multiple interrelated mechanisms operating across biological scales, from mitochondria to cells, tissues, and organs [10, 11]. Mammals obtain EGT from dietary sources and actively transport it via the organic cation transporter novel 1 (OCTN1), which is broadly expressed across most tissues. It accumulates preferentially in organs with high metabolic demand and susceptibility to oxidative stress (e.g., liver, kidney, erythrocytes) and has a markedly long tissue half-life, unlike many conventional antioxidants [11]. A dedicated, evolutionarily conserved transporter ensures high tissue retention and metabolic stability, underscoring EGT's distinct biological significance. Emerging evidence links altered EGT blood levels to aging, neurodegeneration, and chronic inflammation [10, 12].

EGT is a secondary metabolite produced by most fungi, certain Actinobacteria, and Cyanobacteria [13, 14]. In humans, the primary dietary sources of EGT are edible and medicinal mushrooms, with particularly high concentrations in species of the orders Agaricales and Polyporales (Phylum Basidiomycota, Class Agaricomycetes). These taxa include many of the most widely cultivated mushrooms globally, such as oyster mushrooms (*Pleurotus spp*.), shiitake (*Lentinula edodes*), and medicinal polypores (*Ganoderma lucidum* and *Trametes versicolor*) [9, 14, 15]. A recent

bibliometric analysis identifies EGT biosynthesis and the antioxidant potential of edible fungi as a current research hotspot [16].

Numerous studies have documented EGT content in the sporocarps and mycelia of commercially cultivated mushrooms, revealing wide-ranging levels that are strongly influenced by species-specific factors [9, 15, 17]. However, conventional mushroom cultivation is inefficient for industrial EGT production due to long fructification times (37-45 days) and low yields [14]. The potential of commercial mushrooms to enrich substrate's EGT in SSF remains largely unexplored. Although some species, such as *Pleurotus spp*. and *L. edodes,* accumulate EGT in myceliated substrates, quantitative data from SSF remain scarce and are scattered across species-specific studies [6, 12, 18].

Phenolic compounds are another critical class of antioxidant secondary metabolites enriched in myceliated substrates [7, 19, 20]. They are widespread across the plant and fungal kingdoms and have attracted increasing interest in health science and the food industry as natural antioxidants. They modulate cellular redox balance and inflammatory pathways and inhibit lipid peroxidation and quality deterioration in foods and beverages [1]. In substrates such as wheat and other cereals, phenolic compounds are predominantly localized in the aleurone layer and bran as insoluble, esterified bound forms linked to cell wall polysaccharides, which severely limits their bioaccessibility and *in vivo* antioxidant efficacy compared with free or readily extractable forms [3, 21]. During SSF of wheat grains, fungal enzymes release these bound phenolics from the cereal matrix, increasing the proportion of bioavailable antioxidants [4, 5, 20, 22]. However, no studies have systematically compared secondary metabolite enrichment and antioxidant capacity across fungal species under standardized SSF conditions that bypass the fructification phase.

This study evaluates eight edible and medicinal fungal species cultivated on wheat grains under standardized SSF conditions. We quantified EGT, total phenolics, and flavonoids in the myceliated substrate and correlated metabolite accumulation with substrate consumption and total antioxidant capacity. These results may inform the selection of high-performing fungal species for functional ingredient development and enrichment of cereal-based products.

Materials and methods

Chemicals

We purchased agar, ammonium acetate, $CaCO_3$, ethanol, Folin-Ciocalteu reagent, $AlCl_3$, gallic acid, and glucose from Biopack (Argentina). $CaSO_4$, $CuCl_2{\cdot}2H_2O$, HCl, methanol, Na_2CO_3, and NaOH were from Anedra (Argentina). Neocuproine and quercetin were from Cayman Chemical (USA). Trolox was from Sigma-Aldrich (USA). Ergothioneine (EGT) was from Tetrahedron (France). The malt and yeast extracts were from Flebor (Argentina) and Britania (Argentina), respectively.

Fungal species

We obtained *Trametes versicolor* (strain 303), *Ganoderma lucidum* (strain 811), *Hericium erinaceus* (strain 813), and *Pleurotus eryngii* (strain 812) from CIEFAP (Chubut, Argentina). *Lentinula edodes*

(strain B13), *P. djamor* (strain A17), *P. citrinopileatus* (strain A16), and *P. ostreatus* var. *florida* (strain A01) were purchased from CISPHoCoMe (Neuquén, Argentina).

Substrate conditioning

The substrate for spawns and experimental SSF consisted of organically certified wheat grains (*Triticum aestivum*, Molino Campo Claro, Argentina) that were conditioned by soaking in excess tap water for 24 h, followed by boiling for 10 min to achieve a hydration level of 49-54 %. After sieving and cooling, the grains were mixed with 1 % $CaSO_4$ (to prevent clumping) and 0.25 % $CaCO_3$ (to maintain proper pH). The substrate was placed in glass jars for spawns or polypropylene bags (described below) for SSF experiments, then autoclaved at 121 °C for 45 min.

Culture conditions and spawn production

All mycelium and culture media manipulations were performed according to standard protocols under laminar flow conditions (Biobase BBS-V800, China). We performed inoculations with a sterile spoon and weighed the material on a portable balance (Libercam RBCO-02, China) placed inside the laminar-flow hood. All cultures were conducted at 25 °C, 60-70 % relative humidity, in the dark, using an incubator (Dalvo Instrumentos CP797, Argentina).

For the first spawn of each strain, three 2 × 2 cm pieces of mycelium from MYDA Petri dishes were placed in 350 mL glass jars containing 50 g of substrate and incubated for 7-10 days. To generate a second spawn, 20 g of the first spawn were added to 200 g of substrate in 750 mL glass jars; after 7 days of incubation, this second spawn was used to inoculate the experimental bags [6].

Experimental Solid-State Fermentation

SSF was conducted in 15 × 25 cm polypropylene bags containing 120 g of substrate and a 2 × 4 cm microporous tape opening for gas exchange. Each bag was inoculated with 12 g of spawn (10 %) in quadruplicate, while non-inoculated bags served as controls (CTRL). After sealing, the bags were incubated for 21 days and then stored at -18 °C until analysis. For sample numbers and spatial considerations, the experiment was performed in three batches, each with its own set of control bags (n = 2). Before drying, bags were equilibrated to room temperature. The contents were then dried at 55 °C for 15 h on an aluminum tray in a convection oven (EYELA WFO-600ND, Japan), weighed, ground to a powder, sieved through a 300 µm sieve, and stored at -18 °C until analysis (henceforth, myceliated substrate).

Mycelial growth estimation

According to Pascual et al. (2025), mycelial growth during the fermentative process can be estimated as the percentage of substrate consumption (SC %), which represents the fraction of the total dry mass (DM) lost during SSF. This is calculated using Equation (1):

$$Substrate\ consumption = \left(\frac{DM_{non-inoculated} + DM_{spawn} - DM_{myceliated}}{DM_{non-inoculated} + DM_{spawn}}\right) * 100$$

(1)

Where, $DM_{non-inoculated}$ is the mean DM of non-inoculated control samples (CV < 1 %), and $DM_{myceliated}$ is the DM after the fermentation process. DM_{spawn} is the DM contributed by the inoculated spawn, obtained by subtracting the spawn's % humidity from the total inoculated spawn mass. Each DM value was calculated as the mass of the sample after the drying process at 55 °C (partial dry mass), corrected for residual moisture (loss on drying at 105 °C for 3 h) using Equation (2):

$$DM = \frac{partial\ dry\ mass * 100}{(100 - \%\ residual\ moisture)} \quad (2)$$

Antioxidant capacity

The total antioxidant capacity of the samples was measured directly on solid samples using the QUENCHER (QUick, Easy, New, CHEap and Reproducible) approach, coupled with the Cupric Reducing Antioxidant Capacity (qCUPRAC) method [23](Tufan et al., 2013). Each 10 mg sample was mixed with 100 µL of EtOH:H_2O (1:1, v/v) and 4 mL of CUPRAC reagent in EtOH:H_2O (1:1, v/v), prepared by combining equal volumes of 10 mM CuCl2·2H_2O, 1 M ammonium acetate buffer (pH 7.0), and 7.5 mM neocuproine. The mixture was incubated at 52 °C for 60 min with agitation every 15 min, cooled to room temperature, and centrifuged at 12,000 × g for 10 min. We measured the absorbance of the supernatant at 450 nm using a Synergy HTX microplate reader (BIOTEK, USA), which was used for all absorbance measurements unless otherwise stated.

Total antioxidant capacity was quantified using a Trolox calibration curve (0.02 to 2 mM; $R^2 = 0.999$) prepared in EtOH:H_2O (1:1, v/v). Results are reported primarily as micromoles of Trolox equivalents (µmol TE)/g DM.

Secondary metabolites

Ergothioneine

Ergothioneine was extracted and quantified as described by Pascual et al. [6]. Each 100 mg sample was mixed with 8.0 mL of deionized water, incubated at 95 °C for 60 min, cooled, and then stored at -18 °C for 24 h. After thawing, samples were centrifuged at 3,800 × g for 10 min, and the supernatants were filtered through a 0.22 µm polyethersulfone syringe filter (Thermo Scientific Nalgene, USA) and stored at -18 °C until use. Chromatographic analysis was performed on a Waters 600 HPLC system equipped with a Waters 2998 diode-array detector (Waters Corporation, MA, USA). Separation was achieved on a Luna C18 column (4.6 × 250 mm, 5 µm) with a guard column (Phenomenex, CA, USA). Detection was performed at 254, 257, and 280 nm, and UV spectra (210–400 nm) were recorded to characterize the peaks. EGT was identified by retention time and UV

spectral matching with an authentic standard. Quantification was performed using an external calibration curve ranging from 2.6 to 52.9 mg/L (R^2 = 0.998). Results are expressed as mg/g DM.

Total phenolic compounds

Phenolic compounds were extracted by incubating 45 mg of each sample with 1.8 mL of deionized water at 60 °C for 60 min, with agitation every 10 min. After cooling to room temperature, we centrifuged the sample at 12,000 × g for 30 min, aliquoted the supernatants, and stored them at 4 °C until analysis. Total phenolic content (TPC) was determined using the Folin–Ciocalteu assay in a 96-well microplate. We mixed 20 µL of the supernatant with 185 µL of distilled water and 15 µL of 1 N Folin-Ciocalteu reagent, which had been diluted 1:1 (v/v) with distilled water. After 6 min of incubation, we added 30 µL of 10 % (w/v) Na_2CO_3 and incubated the mixture in the dark at room temperature for 60 min. Absorbance was measured at 750 nm. Results were calculated against a gallic acid calibration curve (0.4 to 8 mg/L in deionized water, R^2 = 0.999) and expressed as mg of gallic acid equivalents (GAE)/g DM.

Flavonoids

Extraction was performed by incubating 45 mg of the sample with 1.8 mL of EtOH:H_2O (80:20 v/v) at 60 °C for 10 min. The mixture was sonicated in an ultrasonic bath at 80 W and 40 kHz for 10 min, then incubated at 60 °C for 10 min. After cooling to room temperature, the samples were centrifuged at 12,000 × g for 10 min, and the resulting supernatants were collected and stored in aliquots at -18 °C until analysis. Flavonoid content was determined according to Pękal and Pyrzynska [24] with minor modifications. Briefly, 100 µL of the supernatant was mixed with 50 µL of 2 % w/v $AlCl_3$ solution and 50 µL of deionized water. The mixture was vigorously shaken, incubated at 30 °C for 30 min, and absorbance was measured at 425 nm. Sample absorbance values were corrected by subtracting the absorbance of the corresponding blank prepared without $AlCl_3$. Quantification was performed using a quercetin calibration curve spanning 2.5 to 40 mg/L in EtOH:H_2O (80:20, v/v; R^2 = 0.999). Results were expressed as mg of quercetin equivalent (QE)/g DM. Note: The $AlCl_3$ method selectively quantifies flavonoids with 5-hydroxy-4-keto or ortho-hydroxy-carbonyl chelating moieties; flavan-3-ols and flavanones lacking these groups may be underrepresented [25].

Statistics

Results were expressed as mean ± SEM (n = 4 unless otherwise stated). After assessing normality and homoscedasticity with the Kolmogorov-Smirnov and Bartlett's tests, we compared the results using Welch's ANOVA with the Games-Howell *post hoc* multiple-comparison test. Then, we performed correlation analyses among multiple variables using Pearson correlation coefficients (r) calculated across all samples. A multiple linear regression model was applied to identify metabolite predictors of total antioxidant capacity. Data were fitted to a linear model using least-squares regression, followed by an F-test to compare slopes between groups. The global significance level

was set to α = 0.05 for all analyses. All statistical analyses were conducted using GraphPad Prism version 8.0.2.

Results and Discussion

Mycelial growth on wheat grains

In SSF systems, the mycelium cannot be separated from the substrate for direct gravimetric analysis, so we estimated mycelial growth by dry mass loss (SC%), a simple and cost-effective estimator of mycelial metabolism. A previous study from our laboratory validated SC% as a robust metric for *P. ostreatus* growth across different substrates, based on its strong correlation with glucosamine content [5]. In the present study, all mushroom species achieved full surface colonization. *G. lucidum* and *P. ostreatus* showed visibly faster mycelial expansion. Consequently, we harvested these two species on Day 14, seven days before the other species, to prevent mycelial overgrowth over the gas-exchange openings. Thus, we excluded *G. lucidum* and *P. ostreatus* from the ANOVA-based interspecies comparison.

All the studied species reduced the substrate's initial dry mass (Figure 1A). Among the six species compared (ANOVA, $p < 0.0001$), *L. edodes*, *P. djamor*, and *T. versicolor* were the most efficient substrate utilizers (SC% 14.9 ± 0.8 to 20.0 ± 1.8 %). In contrast, *H. erinaceus*, *P. eryngii,* and *P. citrinopileatus* showed lower SC% (2.5 ± 0.4 to 8.1 ± 0.8 %). Additionally, *G. lucidum* and *P. ostreatus* achieved high SC% (24.0 ± 1.1 % and 21.2 ± 1.1 %), consistent with our previous work on the same *P. ostreatus* strain and substrate, which showed SC% of 18 % at Day 14 and 33 % at Day 21 [5]. Direct comparisons with published SSF values remain challenging due to heterogeneity in indirect growth metrics (glucosamine, ergosterol, dry mass loss), substrate formulations, and culture conditions [26]; by contrast, the standardized conditions applied in this work provide a directly comparable ranking of mycelial growth across the species studied. Collectively, this broad range in SC% (2.5 to 24.0 %) suggests either interspecies differences in mycelial growth kinetics or suboptimal substrate or culture conditions that constrain metabolic activity or nutrient acquisition in some species [2, 4, 27].

Figure 1

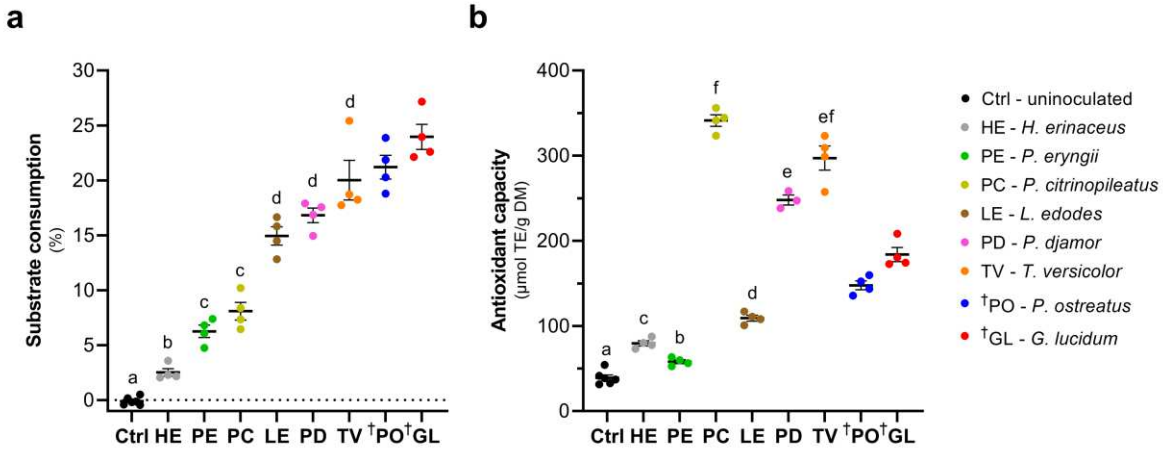

Fig. 1 Substrate consumption (a) and total antioxidant capacity (b) in myceliated wheat grains after 21 days of solid-state fermentation across fungal species († 14 days). Colored circles represent individual replicates (n = 3-6); horizontal lines with error bars represent the mean ± SEM. Different letters indicate significant differences (Games–Howell test following Welch's ANOVA, $p < 0.05$). DM = dry mass; TE = Trolox equivalent

Total antioxidant capacity

SSF significantly increased the substrate's total non-enzymatic antioxidant capacity (qCUPRAC) across all species tested (Figure 1B). Among the six species compared (ANOVA, $p < 0.0001$), *P. citrinopileatus* and *T. versicolor* had the highest values (341.2 ± 6.7 and 297.1 ± 14.1 µmol TE/g, respectively), followed by *P. djamor* (247.9 ± 5.9 µmol TE/g). These values were significantly higher than those of *L. edodes*, *H. erinaceus*, and *P. eryngii* (109.0 ± 3.3, 79.5 ± 3.0, and 57.9 ± 2.3 µmol TE/g, respectively). Additionally, *G. lucidum* and *P. ostreatus* showed intermediate values of 183.8 ± 8.3 and 147.6 ± 5.2 µmol TE/g, respectively, after a shorter fermentation time.

Conventional solvent extraction methods fail to extract reactive antioxidants that remain entrapped within the food matrix, are chemically bound to macromolecules (e.g., fiber-bound phenolics), or are present in insoluble forms. In contrast, the QUENCHER approach used here enables direct solid-liquid measurement and avoids time-consuming extractions, detecting interactions among accessible antioxidant forms that react with the coupled colorimetric assay [28]. It also aligns with green chemistry principles by reducing solvent consumption. This methodology has been successfully coupled with ABTS, DPPH, FRAP, and CUPRAC to evaluate insoluble fractions in wheat-based substrates, where conventional extractions underestimate the total antioxidant potential by 40-60 % [23]. When coupled with CUPRAC, this approach detects reducing compounds in both hydrophilic and lipophilic domains, as well as thiols such as EGT, at physiological pH (7.0), with linear 1:1 additivity of electron stoichiometry [29].

Compared with the unfermented substrate (39.1 ± 3.3 µmol TE/g), antioxidant capacity increased 1.5-fold in *P. eryngii* to 8.7-fold in *P. citrinopileatus*. This broad range reflects clear species-specific variation in the ability to synthesize fungal-derived bioactives or mobilize matrix-bound antioxidants, likely through distinct enzymatic portfolios [3, 22]. This enrichment in myceliated wheat is consistent with previous studies showing that SSF of wheat grains enhances antioxidant capacity regardless of the fungal species and the analytical method employed [5, 7, 20, 30], although the magnitude of the effect is not directly comparable across studies. To determine whether the variation observed reflected differences in fungal colonization, we related qCUPRAC levels to SC% for all samples across the eight species and found a moderate positive association ($r = 0.513$; $p < 0.001$), indicating that although antioxidant capacity and mycelial growth tend to increase together, their relationship is not tightly coupled across species. For example, *P. citrinopileatus* had the highest qCUPRAC but the third-lowest SC%, whereas *T. versicolor* and *P. djamor* exhibited high levels of both qCUPRAC and SC%.

Secondary metabolites

Myceliated wheat grains showed significant increases in EGT, TPC, and flavonoid content across the tested fungal species compared with control grains (ANOVA $p < 0.0001$; Table 1). *P. citrinopileatus* had the highest EGT (2.33 ± 0.02 mg/g) and TPC (6.86 ± 0.33 mg GAE/g), with the second-highest flavonoid level (0.795 ± 0.027 mg QE/g). *T. versicolor* had TPC nearly identical to *P. citrinopileatus* but lower EGT and flavonoid contents (1.50 ± 0.11 mg/g and 0.641 ± 0.015 mg QE/g, respectively). In turn, *P. djamor* had the highest flavonoid content (0.995 ± 0.017 mg QE/g), the second-highest EGT (1.69 ± 0.03 mg/g), and an intermediate TPC (3.95 ± 0.24 mg GAE/g). Exceptions were *P. eryngii*, which did not significantly increase either TPC or flavonoids, and *H. erinaceus*, which showed no significant increase in flavonoids and produced the lowest significant TPC yield (1.39 ± 0.55 mg GAE/g). Consistently, these two species showed low EGT contents (0.55 ± 0.03 and 0.68 ± 0.03 mg/g, respectively), whereas *L. edodes* had a similarly low EGT but moderate TPC and flavonoid levels. In turn, *P. ostreatus* and *G. lucidum*, sampled at 14 days, yielded intermediate levels of all three metabolite classes.

Table 1. Ergothioneine, total phenolic, and flavonoid content in myceliated wheat grains after 21 days of solid-state fermentation across fungal species

Species	Ergothioneine (mg/g DM)	Total phenolic content (mg GAE/g DM)	Flavonoids (mg QE/g DM)
Uninoculated wheat	0.26 ± 0.04 ^{a}	0.99 ± 0.07 ^{a}	0.341 ± 0.012 ^{a}
Hericium erinaceus	0.68 ± 0.03 ^{b}	1.39 ± 0.05 ^{b}	0.333 ± 0.015 ^{a}
Pleurotus eryngii	0.55 ± 0.03 ^{b}	1.03 ± 0.01 ^{a}	0.408 ± 0.015 ^{a}
Pleurotus citrinopileatus	2.33 ± 0.02 ^{d}	6.86 ± 0.33 ^{e}	0.795 ± 0.027 ^{c}
Lentinula edodes	0.52 ± 0.01 ^{b}	2.30 ± 0.05 ^{c}	0.696 ± 0.017 ^{bc}
Pleurotus djamor	1.69 ± 0.03 ^{c}	3.95 ± 0.24 ^{d}	0.995 ± 0.017 ^{d}
Trametes versicolor	1.50 ± 0.11 ^{c}	6.78 ± 0.52 ^{e}	0.641 ± 0.015 ^{b}
† *Pleurotus ostreatus*	1.04 ± 0.07	2.34 ± 0.10	0.546 ± 0.020
† *Ganoderma lucidum*	0.91 ± 0.08	4.34 ± 0.12	0.784 ± 0.014

Values are expressed as mean ± SEM (n = 3-6). Species are ordered by increasing SC%. Different superscript letters within a column indicate significant differences (Games–Howell test following Welch's ANOVA, $p < 0.05$)
† Species harvested at 14 days and excluded from statistical comparisons. DM = dry mass; GAE = gallic acid equivalent; QE = quercetin equivalent

To date, studies on EGT production via SSF are largely species- and substrate-specific, including reports on the genus *Pleurotus spp.* on brewer's spent grain (*P. ostreatus*) [6], adlay and buckwheat (*P. eryngii*) [18], and soybeans (*P. citrinopileatus* and *P. ostreatus*) [31], as well as *Ganoderma resinaceum* on an optimized substrate [32]. Among these, Sawada et al. [31] reported comparatively higher EGT production with *P. citrinopileatus* than with *P. ostreatus* in soybean SSF at 40 days (0.8 vs. 0.16 mg/g, respectively). However, both values remain considerably lower than those in this study, consistent with our previous results, which showed much slower growth of *P. ostreatus* in soybean than in wheat [5]. Our results show that wheat-SSF with fungal mycelium markedly

increases EGT, with intensity varying by species. To our knowledge, this is the first report comparing EGT production across edible and medicinal basidiomycetes using wheat-SSF.

Previous studies in sporocarps consistently assign *P. citrinopileatus* the highest EGT content among cultivated mushrooms, ranging from 2.4 to 4.0 mg/g DM, whereas the other species analyzed herein were reported to have lower values (~0.4 to 2.0 mg/g) [15, 17, 33]. Together, these reports indicate a sporocarp EGT range from approximately 0.4 to 4.0 mg/g across the species evaluated, providing a benchmark for comparison. The EGT content of *P. citrinopileatus* obtained in this study after 21 days of wheat SSF (2.3 mg/g) falls close to the lower limit of the sporocarp benchmark range for the same species, despite no systematic optimization of SSF parameters, suggesting substantial room for enhancement through process refinement, given that EGT yields are highly influenced by cultivation practices and post-harvest processing [12]. Key variables documented to influence EGT levels in *Pleurotus spp*. include growth stage and maturation time [6, 31], substrate composition, temperature, light exposure, post-harvest storage and drying, and extraction protocols [18, 32, 33]. To enable *P. citrinopileatus*-SSF to reach or exceed current EGT benchmarks in fermented grain systems, future work should prioritize optimizing these cultivation factors alongside strain screening [12, 14].

SSF with different mushroom species consistently increased soluble TPC in wheat grains. Although the Folin-Ciocalteu TPC assay is nonspecific and may be affected by interfering compounds, proper identification studies confirm the presence of both substrate-native phenolics (predominantly phenolic acids) and fungal-derived compounds [7, 19, 20, 34]. In this study, aqueous TPC enrichment ranged from 1.4-fold (*H. erinaceus*) to 6.9-fold (*P. citrinopileatus*) relative to controls. Compared with earlier wheat SSF studies, *P. ostreatus* yielded less phenolic content here (2.34 mg GAE/g) than the 3.0-4.1 mg GAE/g reported for the same species and time point [5, 30], whereas *G. lucidum* yield was higher in this study (4.34 mg GAE/g) than the 3.40 mg GAE/g reported after 53 days by Weng et al. [7]. These discrepancies in TPC may reflect differences in strains, fermentation conditions, extraction protocols, and/or solvents used [4, 19, 20]. TPC enrichment by SSF proceeds through a dual mechanism: 1) mobilization of bound phenolics from cell walls and 2) their subsequent biotransformation or, to a lesser extent, *de novo* synthesis by fungal metabolism. This process involves the concerted action of fungal oxidative and hydrolytic enzymes that release bound and polymeric phenolics from the cereal cell wall matrix [3, 22]. Carbohydrate-cleaving enzymes break down the polymeric matrix, and ligninolytic enzymes degrade lignin barriers that sequester phenolics in the aleurone and bran [27]. In addition, feruloyl esterases cleave the ester bonds linking phenolics to arabinoxylans, releasing ferulic and p-coumaric acids, which are detected as increases in TPC [19]. The high variability in TPC enrichment likely reflects species-specific differences in metabolic capacity, as supported by differential lignocellulolytic enzyme production across fungi [22, 27].

We used the $AlCl_3$ colorimetric assay to quantify flavonoids, a nonspecific screening method that detects flavanols (e.g., quercetin, rutin) and flavones (e.g., luteolin, apigenin) via formation of a flavonoid-Al(III) complex. It is widely used because it is affordable, simple, and fast. While LC-MS/MS provides compound-specific identification, it requires highly sophisticated instrumentation

[25]. Because these flavonoid classes include most flavonoids in wheat grains, particularly flavones [21], the $AlCl_3$ method offers a practical, representative screen for flavonoid enrichment in wheat-SSF systems. Regarding mushrooms, although there is ongoing debate about whether fungal cells synthesize flavonoids *de novo* or absorb them from the substrate, their presence has been confirmed in sporocarps through proper identification and has been observed to vary significantly among species [8, 9]. Like other phenolics, flavonoids may be liberated from the substrate matrix during SSF through fungal enzymatic hydrolysis and cleavage of glycosidic bonds, yielding more reactive aglycone forms [19, 34]. In this study, while most species increased flavonoid levels relative to the control, the ranking diverged notably from those of EGT and TPC, with *P. djamor* ranking highest (2.9-fold), followed by *P. citrinopileatus* and *L. edodes* (2.3-fold and 2.0-fold, respectively). This result suggests that flavonoid accumulation depends on species-specific metabolic pathways that mobilize and modify wheat kernel flavonoids during SSF, and/or synthesize flavonoids.

Beyond the species-wide ranking, *P. ostreatus* and *G. lucidum* require separate analysis and careful interpretation because their culture period was abbreviated. Although these species produced intermediate levels of EGT, TPC, and flavonoids compared with the other species, their 14-day culture period likely prevented them from reaching higher secondary metabolite levels, closer to those of the most productive species harvested at 21 days. Preliminary analysis of the relationship between EGT content and SC% in wheat fermented with *P. ostreatus* over time (Supplementary Figure 1) supports this assumption, revealing a strong positive linear correlation ($r = 0.993$; $n = 12$; $R^2 = 0.986$). This finding aligns with previous SSF studies using *Pleurotus spp.* on other substrates: *P. ostreatus* in brewer's spent grain (SC% vs. EGT; $r = 0.958$) [6] and *P. eryngii* in adlay and buckwheat (biomass vs. EGT; $r = 0.888$-0.921) [18]. Collectively, these high within-species correlations suggest that EGT is a growth-associated metabolite in *Pleurotus spp*. In contrast, across all eight species, we observed a weaker correlation ($r = 0.346$; $p < 0.05$), suggesting that EGT accumulation is species-specific rather than a universal function of mycelial growth. Similarly, TPC and flavonoids showed modest positive correlations with SC% across all tested species ($r = 0.515$, $p < 0.001$; $r = 0.647$, $p < 0.001$). These correlations align with those described above for qCUPRAC vs. SC% ($r = 0.513$), suggesting that the mechanisms driving secondary metabolite enrichment are only modestly coupled to substrate consumption. These findings have practical implications for species selection: prioritizing mycelial colonization alone may not optimize secondary metabolite enrichment, as metabolic traits also influence accumulation patterns. Therefore, screening protocols relying solely on substrate consumption cannot reliably predict EGT or phenolic yields across diverse species; metabolite profiling should accompany these measurements. Yet this metric remains pragmatic for preliminary interspecies screening under standardized conditions. It can predict biotransformation outcomes that correlate linearly with it, such as increases in crude protein, ash content, and acid-soluble phosphorus, and decreases in crude lipids, carbohydrates, and phytic acid [5].

Correlations between secondary metabolites and antioxidant capacity

Figure 2 shows positive correlations between secondary metabolites and antioxidant capacity across all samples. EGT (r = 0.942; $p < 0.0001$) and TPC (r = 0.957; $p < 0.0001$) show the strongest correlations, followed by flavonoids (r = 0.761; $p < 0.0001$). A multiple linear regression model with EGT, TPC and flavonoids as predictors explained 97.9 % of the variability in antioxidant capacity ($R^2 = 0.979$; $F_{(3, 29)} = 449.4$; $p < 0.0001$; n = 33). All three were significant contributors (EGT: $\beta = 66.9$, $p < 0.0001$; TPC: $\beta = 24.7$, $p < 0.0001$; flavonoids: $\beta = 59.4$, $p < 0.004$), indicating that these metabolites were key determinants of overall antioxidant capacity in wheat grain SSF. Because qCUPRAC measures reducing compounds directly in the solid matrix, its results reflect the cumulative contributions of all three compound classes to the antioxidant signal without solvent-extraction bias. Thus, qCUPRAC may serve as a rapid screening tool for ranking total antioxidant capacity without requiring individual quantification of each metabolite class. We next examined pairwise correlations among secondary metabolites.

Figure 2

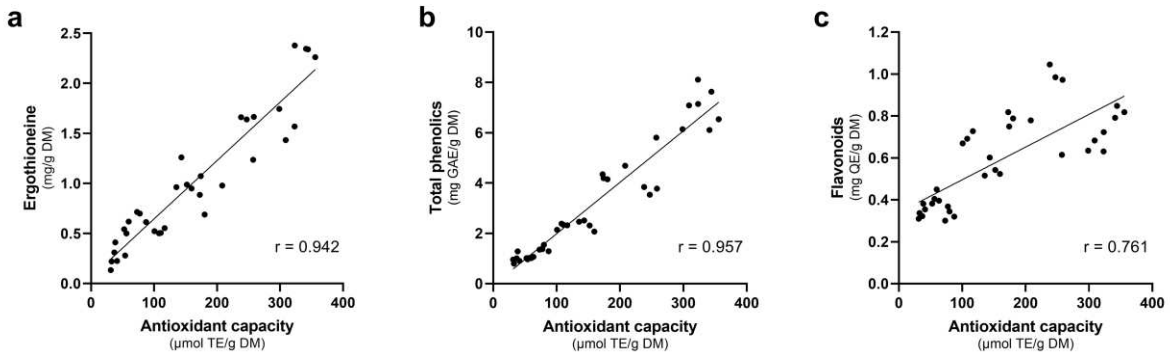

Fig. 2 Correlation between antioxidant capacity and ergothioneine (a), total phenolics (b), and flavonoids (c) in myceliated wheat grains after solid-state fermentation across the tested fungal species. Each circle represents an individual sample (n = 35-37); solid lines indicate linear regression. Pearson's r is shown in each panel. DM = dry mass; GAE = gallic acid equivalent; QE = quercetin equivalent; TE = Trolox equivalent

EGT and TPC showed a strong positive correlation across all fungal species (r = 0.807; $p < 0.001$; $R^2 = 0.651$). EGT-flavonoid (r = 0.594; $p < 0.001$; $R^2 = 0.353$) and TPC-flavonoid (r = 0.590; $p < 0.001$; $R^2 = 0.348$) were also significant but weaker (Supplementary Figure 2). A *post hoc* analysis across phylogenetic groups revealed distinct linear regression slopes ($F_{(1,26)} = 10.62$; $p < 0.01$; n = 30; Figure 3): Group 1 (order Agaricales, *P. eryngii*, *P. ostreatus*, *P. citrinopileatus*, and *P. djamor* plus *H. erinaceus*, the only species of the order Russulales studied); slope 0.301 ± 0.016, n = 18, $R^2 = 0.957$; Group 2, (order Polyporales, *T. versicolor* and *G. lucidum* plus *L. edodes* slope 0.206 ± 0.026, n = 12, $R^2 = 0.864$. Notably, *L. edodes* was the only Agaricales species that aligned with the Polyporales regression pattern. The steeper slope of Group 1 indicates preferential EGT accumulation relative to TPC, whereas the Group 2 accumulates relatively higher TPC. These differential accumulation patterns suggest that EGT and TPC production may be co-regulated, yet the proportions of each metabolite differ between the groups analyzed, consistent with reported interspecies differences in *egt* gene cluster architecture and phenolic metabolic pathways among

Basidiomycota [13, 22]. Yet both white-rot groups show robust antioxidant capacity and remarkable efficiency in wood degradation [27], consistent with the top qCUPRAC performers observed in both lineages (*P. citrinopileatus*, Agaricales, Group 1; *T. versicolor*, Polyporales, Group 2). Given the exploratory nature of this analysis, modest per-group sample sizes (n = 12-18), and phylogenetically limited sampling, these observations require confirmation through comparative studies across a larger and more representative set of fungal species.

Figure 3

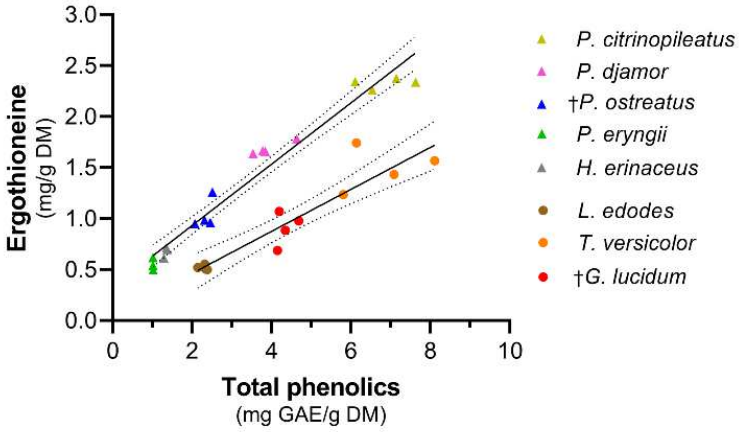

Fig. 3 Relationship between total phenolic content and ergothioneine across fungal species after 21 days of solid-state fermentation of wheat grains (†14 days). Different colors and symbols denote species. Linear regression slopes differed significantly between order-based groups ($F_{(1,26)} = 10.62$; $p < 0.01$): Agaricales, family Pleurotaceae plus *H. erinaceus* (Russulales; triangles): slope = 0.301 ± 0.016 (n = 18; $R^2 = 0.957$); Polyporales plus *L. edodes* (Agaricales, Omphalotaceae; circles): slope = 0.206 ± 0.026 (n = 12; $R^2 = 0.864$). Dashed lines indicate 95% confidence intervals. DM = dry mass; GAE = gallic acid equivalent

Conclusions

Solid-state fermentation of wheat grains with eight edible and medicinal basidiomycetes proved an effective strategy for enhancing the cereal's antioxidant profile, with total antioxidant activity increasing 1.5- to 8.7-fold. *P. citrinopileatus* emerged as the top performer, ranking highest in antioxidant capacity and EGT content, confirming its status as one of the richest EGT sources among cultivated mushrooms. The enrichment patterns of EGT, phenolics, and flavonoids strongly correlated with antioxidant capacity measured by qCUPRAC, indicating that they are the principal contributors to total antioxidant capacity. Therefore, qCUPRAC, a straightforward and inexpensive assay, appears to be a suitable alternative for selecting species and SSF conditions to produce bioactive compounds with antioxidant properties. In turn, SC% showed a weak cross-species correlation with metabolite enrichment; thus, it seems insufficient for selecting fungal species to enrich bioactive compounds.

Notably, the regression analysis between EGT and TPC revealed two distinct slopes, indicating differential accumulation of EGT and phenolic compounds, and suggesting group-specific metabolic

strategies. However, this interpretation requires broader species sampling, better representation of the Agaricomycetes, and species-tailored optimization of substrate, harvest time, and cultivation parameters.

Statements and Declarations

Competing Interest

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

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Data Availability

Data supporting this study's findings are available at CONICET Digital, http://hdl.handle.net/11336/286556.

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