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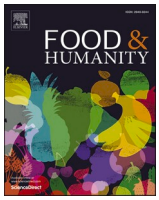

Revalorization of brewer's spent grain through solid-state fermentation with *Pleurotus ostreatus*

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ABSTRACT

Brewer's spent grain (BSG), a protein-rich agro-industrial byproduct, is underutilized in food and feed industries due in part to the presence of antinutrients such as phytic acid, which impair nutrient bioavailability. This study aimed to reduce antinutrients, improve the nutritional profile, and increase levels of bioactive compounds through solid-state fermentation (SSF) using the edible mushroom *Pleurotus ostreatus* over 14 and 21 days. After 21 days, mycelial growth resulted in a 39.4 % reduction in the dry mass of BSG, leading to significant nutrient shifts: protein concentration increased by 66 % (to 25.4 g/100 g), while carbohydrates and lipids decreased by 11 % and 78 %, respectively, suggesting their role as primary energy substrates. SSF achieved an 80 % reduction in phytic acid content within 14 days (from 10.4 to 2.0 mg/g), associated with increased acid-soluble phosphorus by 112 %. Additionally, the process generated significant nutraceutical value, with ergothioneine (EGT) content reaching 3.50 mg/g, a concentration similar to that observed in mushroom fruiting bodies, which showed a strong correlation with mycelial growth and with the antioxidant capacity (CUPRAC assay). In contrast, the total phenolic content rose modestly by 37 % and the antioxidant capacity measured by the ABTS method did not increase at 21 days. These findings demonstrate that *P. ostreatus* SSF can biotransform BSG into value-added products with potential for use as ingredients in the food and feed industries. Overall, this process supports circular bioeconomy initiatives by reducing industrial waste, promoting the sustainable use of agro-industrial byproducts, and facilitating the upcycling and valorization of BSG.

1. Introduction

The need to reduce the accumulation of contaminating residues has driven researchers to study the valorization of agro-industrial wastes into new products. Brewer's spent grain (BSG) is a contaminant byproduct of beer production generated in significant quantities worldwide, which retains high energy levels and valuable nutritional components. Therefore, its suitability as a substrate for the circular economy has been the subject of several studies (Bigdeloo et al., 2021; Rachwał et al., 2020; Zeko-Pivač, Tišma et al., 2022). BSG contains up to 30 % protein and is rich in essential amino acids, vitamins, minerals, phenolic compounds, and fiber (Chetrariu & Dabija, 2023; Devnani et al., 2023). However, the presence of antinutrients in BSG, as in most plant-derived substrates, makes its use as a food or feed ingredient challenging (Karlsen & Skov, 2022; Ktenioudaki et al., 2015). Particularly, phytic acid, the principal phosphorus storage form in grains and

Abbreviations: ABTS,, 2,2′-azinobis 3-ethylbenzthiazoline-6-sulfonic acid; ASP,, acid-soluble phosphorus; CUPRAC,, cupric reducing antioxidant capacity; DM,, total dry mass; EGT,, L-ergothioneine; GAE,, gallic acid equivalent; SC%,, substrate consumption; SSF,, solid-state fermentation; TEAC,, trolox equivalent antioxidant capacity; TP,, total phosphorus; TPC,, total phenolic content.

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legumes, is an antinutrient that complexes with proteins, including digestive enzymes, and minerals, hindering digestion and nutrient absorption (Chondrou et al., 2024; Feizollahi et al., 2021). The limited information available indicates that phytic acid concentration in BSG can vary with both the barley variety and the environmental, malting, and brewing processes (Dai et al., 2007; Kumar & Anand, 2021). Phytic acid levels in grains and bran can be effectively reduced through fermentation with microorganisms that possess phytase activity, including yeast, lactic acid bacteria, and fungi, which release inorganic soluble phosphorus (Fang et al., 2023; Jatuwong et al., 2020; Özkaya et al., 2017).

A straightforward, low-cost method for improving the suitability of agro-industrial byproducts for the circular economy is solid-state fermentation (SSF), which involves transforming a solid substrate with microorganisms (Yafetto, 2022). This method is less susceptible to contamination than submerged fermentation, as it takes place in the absence of free water. It consumes less water and energy, generates less wastewater, and allows the microorganism to exchange gases with air. Additionally, SSF allows for the biotransformation of 20–35 % of the substrate mass, in contrast to 5 % in submerged fermentation (Abu Yazid et al., 2017; Pascual et al., 2025; Soccol et al., 2017). SSF mediated by various microorganisms enhances BSG nutritional value and broadens its potential applications, e.g., by increasing protein and phenolic compounds content and improving amino acid profiles and antioxidant capacity (Da Costa Maia et al., 2020; Ibarruri et al., 2019; Mitri et al., 2022; Tan et al., 2019).

Edible fungi-based SSF incorporates the well-known nutritional benefits of mushrooms in the production of environmentally friendly, nutritious food products (Garrido-Galand et al., 2021; Holt et al., 2024; Sánchez et al., 2024, for reviews). Besides, the mushroom's metabolism degrades the substrate's complex molecules, including the antinutrients lignin and phytic acid, and produces beneficial bioactive molecules, thereby enhancing the product's digestibility, palatability, and nutritional value (Pascual et al., 2025; Timm et al., 2022; Yang et al., 2021).

Pleurotus ostreatus (Basidiomycetes) and other species of the same genus, known as oyster mushrooms, are consumed worldwide and are cataloged as GRAS (generally recognized as safe). These mushrooms have good organoleptic and nutritional properties, providing healthy bioactive compounds (Raman et al., 2021; Ritota & Manzi, 2023). SSF with *Pleurotus spp.* on lentils, quinoa, kidney beans, oats, chickpeas, soybeans, millet, and wheat grains results in increased protein content, digestibility, antioxidant capacity, and phenolic content, and less phytic acid content (Asensio-Grau et al., 2020; Ayllón-Parra et al., 2025; Espinosa-Páez et al., 2017; Lin et al., 2024; Pascual et al., 2025; Sánchez-García et al., 2022, 2023).

Fungal metabolism produces beneficial compounds known as mycochemicals (Cateni et al., 2022), such as L-ergothioneine (2-mercapto-histidine trimethyl-betaine, EGT). EGT, which so far is known to be produced only by fungi, cyanobacteria, and some mycobacteria, is particularly effective as a free-radical scavenger, compared to other antioxidants, e.g., glutathione (Fu & Shen, 2022; Stoffels et al., 2017; Yadan, 2022), and modulates the antioxidant defense at multiple levels (Dare et al., 2019; Halliwell et al., 2023; Hseu et al., 2015, 2020). Besides, it has therapeutic potential in neurodegenerative and age-associated diseases (reviewed by Chen et al., 2024; Kondoh et al., 2022) and helps prevent the formation of undesirable oxidation products in processed foodstuffs (Bao et al., 2008, 2010; Encarnacion et al., 2011; Tao et al., 2021; see Xiong et al., 2024 for a review on EGT industrial applications). Edible mushrooms are recognized as the most significant dietary source of ergothioneine by a considerable margin (Beelman et al., 2020; Tian et al., 2023, for a review). Among cultivated mushrooms, *Pleurotus spp*. exhibit the highest EGT concentrations in both the fruiting body (Dubost et al., 2007; Tian et al., 2023) and the mycelium produced through submerged fermentation (Chen et al., 2012a; Lin et al., 2015). Nevertheless, data regarding EGT production through SSF remain scarce.

To our knowledge, only Eliopoulos et al. (2022) have analyzed the biotransformation of BSG by SSF with *P. ostreatus*. Thus, an in-depth analysis of the biotechnological application of *P. ostreatus* for SSF of BSG remains necessary to assess key information, such as improvements in nutritional quality and EGT production. This study was conducted with the following objectives: i) to determine whether SSF with *P. ostreatus* improves the BSG nutritional quality by degrading the antinutrient phytic acid, thereby enhancing phosphorus bioavailability, ii) to evaluate the effect of the biotransformation process on nutritional composition, antioxidant capacity, and content of phenolic compounds and EGT after 14 and 21 days of fermentation.

2. Materials and methods

All procedures involving the manipulation of mycelium, culture media, or BSG were performed under laminar flow conditions (Biobase BBS-V800, China). For this purpose, a portable balance (Libercam RBCO-02, China) was installed inside the laminar flow hood.

2.1. Chemicals

Agar, ammonium acetate, $CaCO_3$, $FeNH_4SO_4{\cdot}12H_2O$, Folin-Ciocalteu reagent, gallic acid, and $K_2S_2O_8$ were from Biopack (Argentina). $CaSO_4$, $CuCl_2{\cdot}2H_2O$, ethanol, HCl, methanol, Na_2CO_3, NaOH, and $(NH_4)_2MoO_4$ were from Anedra (Argentina). 2,2'-bipyridine, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Na-phytate from rice, thioglycolic acid, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were from Sigma-Aldrich (USA). L-ergothioneine was from Tetrahedron (France). Neocuproine was from Cayman Chemical (USA). Glucose and NH_4VO_3 were from Stanton (Argentina). The malt extract was from Flebor (Argentina). The yeast extract was from Britania (Argentina).

2.2. Brewer's spent grain

BSG obtained from San Carlos de Bariloche's local brewery (Argentina) was dried in a forced-convection oven with temperature control (MiLab 101–1AB, China) at 120 °C for 2 h in a 1cm-thick layer of product (Santos et al., 2024) and preserved until use. In the laboratory, the BSG was rehydrated to 65 % wet basis, and 1 % $CaSO_4$ and 0.25 % $CaCO_3$ were added to prevent particles from sticking and ensure an adequate pH for fermentation. Portions of this mixture were transferred to glass recipients to prepare spawns or polypropylene bags for the SSF process, autoclaved at 121 °C for 45 min, and cooled to room temperature.

2.3. Solid-state fermentation

Pleurotus ostreatus var. *florida*, strain A01 mycelium, was provided by CISPHoCoMe (Neuquén, Argentina) and kept in slant tubes at 4 °C until use. Then, the mycelium was grown in Petri dishes with MYDA medium (2 % glucose, 1.5 % malt extract, 0.3 % yeast extract, and 1.5 % agar) at 25 °C and 60–70 % relative humidity in an incubator (Dalvo Instrumentos CP797, Argentina).

For the first spawn, we transferred three 2 × 2 cm mycelium portions to 350 mL glass jars, each containing 50 g of BSG. After 10 days of incubation at 25 °C and 60–70 % relative humidity, we weighed 10 g portions of the first spawn and cultured them in 750 mL glass jars containing 140 g of BSG for 7 days under the same conditions (second spawn). This way, the second spawn obtained consists of *P. ostreatus* mycelium cultured on the same substrate used for the SSF experiment.

SSF was conducted in 15 × 25 cm polypropylene bags, each with a 2 × 4 cm window covered with microporous tape to allow gas exchange, filled with 140 g of BSG. We weighed the bags, inoculated them with 10 g of second spawn each, and left four bags uninoculated (non-fermented). Bags were then closed with an impulse sealer and kept under

the same conditions as the spawns for 14 or 21 days. Then, we dried each bag's content on an aluminum tray at 55 ºC for 24 h in a convection oven (Eyela Windy Oven WFO-600ND, Japan). Non-inoculated bags were removed from the stove after 4 days and frozen at −18 °C. After thawing, their contents were dried under the same conditions as those of the inoculated bags. The dried bags' contents were weighed, ground into powder (Tecnodalvo TMDC, Argentina), sieved (300 μm mesh), and stored at −18 °C. Each treatment (non-fermented, 14, and 21 days) consisted of four replicates (n = 4). These time points coincide with those in our previous work (Pascual et al., 2025) and represent the rapid initial substrate biotransformation and an advanced phase preceding the highest level of transformation, where results variability increases, as observed in preliminary experiments.

2.4. Substrate consumption

Since a recent study of our laboratory has shown an excellent correlation between *P. ostreatus* mycelial growth on wheat grains estimated by quantifying glucosamine, derived from the chitin of the fungal cell wall, and dry mass decrease related to substrate consumption by the mycelial metabolism (Pascual et al., 2025), we refer the results of the present study to Substrate consumption, which is a straightforward and low-cost variable for estimating mycelial growth. Substrate consumption (SC%), which represents the portion of the total dry mass (DM) lost (as CO_2 and H_2O) along the SSF process, was calculated through Eq. (1):

$$Substrate\ consumption = \left(\frac{DM_{non-inoculated} + DM_{spawn} - DM_{myceliated}}{DM_{non-inoculated} + DM_{spawn}}\right) * 100 \quad (1)$$

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

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

2.5. Proximate composition

We analyzed the proximate composition of the non-inoculated and myceliated BSG samples using Association of Official Analytical Chemists (AOAC with Helrich, Kenneth, 1990) standardized methodologies, including residual moisture (935.29), crude protein (984.13), crude lipid (945.16), and ash (942.05). The total carbohydrate content was calculated by subtracting the protein, lipid, and ash contents from the total dry mass. The results are presented as g/100 g DM. Total phosphorus (TP) was determined according to the AOAC 965.17 method. The ashes of 1 g of sample, previously incinerated for 4 h at 600 °C, were diluted in 20 mL HCl (1:3 with distilled water), and several drops of HNO_3 were added before boiling for 5 min. After cooling, the solution was diluted to 100 mL with distilled water, centrifuged at 3800 ×*g* for 10 min, and an aliquot was used for colorimetric quantification as described by Pascual et al. (2025). Absorbance was measured at 400 nm using a spectrophotometer (Shimadzu UV-1201, Japan). The calibration curve ranged from 0.83 to 13.3 μg/mL of a phosphorus standard. Results are expressed as g/100 g DM.

2.6. Phytic acid and acid-soluble phosphorus (ASP)

According to Pascual et al. (2025), we added 10 mL of 0.2 N HCl to 100 mg of sample, incubated it at 30 ºC for 30 min with agitation, and then centrifuged for 15 min at 3800 ×*g* (Precision Durafuge-100, USA). The supernatants were stored at −18 ºC. Phytic acid concentration was measured in 0.5 mL of supernatant after incubation for 30 min with 1 mL of 0.2 g/L $FeNH_4SO_4{\cdot}12\ H_2O$ in 0.2 N HCl at 100 °C in a dry bath (DLab HB120-S, China). After centrifugation at 12,000 ×*g* for 10 min at room temperature (Eppendorf Minispin-5452, Germany), we added 100 μL of supernatant to 150 μL of a 10 % *w/v* 2,2-bipyridine solution in thioglycolic acid (Haug & Lantzsch, 1983). Absorbance was read at 519 nm in a microplate reader (Thermo Scientific Varioskan LUX, USA). We made a calibration curve with 2–32 mg/L of Na-phytate in 0.2 N HCl to calculate the phytic acid concentration and presented the results as mg/g DM. ASP was measured in the same supernatant as phytic acid, following AOAC 965.17, as described in Pascual et al. (2025).

2.7. Ergothioneine and phenolic compounds

Ergothioneine (EGT) was extracted according to Zhang et al. (2016) with slight modifications. Each sample (100 mg) was mixed with 8.0 mL of deionized water and heated at 95 °C for 60 min, with occasional manual stirring. After cooling, the samples were centrifuged at 3800 x*g* for 10 min at room temperature and stored at −18 °C for 24 h. Once thawed, the samples were centrifuged again, and the supernatants were filtered through a 0.22 μm polyether sulfone-membrane syringe filter (Thermo Scientific Nalgene, USA) and stored at −18 °C until use.

According to Lee et al. (2009), with modifications, the supernatant was thawed and injected into an HPLC (Waters 600 pump system with a Waters 2998 diode-array detector, Waters Corporation, MA, USA) with a Luna C18 column (4.6 × 250 mm, 5 μm), preceded by a 4.0 × 3.0 mm, 5 μm guard column (Phenomenex, CA, USA). The flow rate was 0.5 mL/min, and the injection volume was 50 μL. The separation gradient system involved two solvents: A, 50 mM sodium phosphate adjusted to pH 6.0, and B, methanol. This gradient was 90 % A at 0 min, switching to 80 % A at 15 min, 70 % A at 22 min, 50 % A at 25 min, 10 % A at 30 min, and then back to 90 % A at 33 min, followed by a post-run time of 10 min. Simultaneous monitoring was conducted at 254 nm, 257 nm, and 280 nm, and UV spectra (210–400 nm) were recorded for peak characterization. Peaks were identified using retention time and UV spectra, and compared to authentic EGT. Quantification was performed using an external standard method with concentrations ranging from 2.3 mg/L to 45.4 mg/L, yielding a coefficient of determination (R^2) of 0.997.

To analyze total phenolic content (TPC), we incubated (in duplicates) 1 g of sample with 10 mL of distilled water for 2 h with magnetic agitation at room temperature, in the dark, and then centrifuged at 12,000 *xg* for 15 min. The supernatants were fractionated into at least three tubes and stored at −20 °C until use. TPC was measured according to Santos et al. (2024), using extracts previously diluted 5-fold. Quantification was performed using a gallic acid calibration curve, ranging from 50 to 300 mg/L. Absorbance was measured at 725 nm using a spectrophotometer (Dlab SP-UV1100, Malaysia), and the results were expressed as mg of gallic acid equivalent (GAE)/g DM.

2.8. Antioxidant capacity

We used aliquots of the same supernatants obtained for EGT analysis to assess the antioxidant capacity by the CUPRAC (Cupric Reducing Antioxidant Capacity) method, as described by Apak et al. (2004), with minor modifications. A volume of 180 μL of CUPRAC reagent (comprising equal volumes of 10^{-2} M $CuCl_2{\cdot}2H_2O$, 1 M ammonium acetate at pH 7.0, and 7.5 × 10^{-3} M neocuproine in absolute ethanol) was mixed with 20 μL of supernatant and 46 μL of distilled water in a 96-well microtiter plate. The mixture was shaken and incubated for 60 min at 30 °C in the microplate reader. Absorbance was measured at 450 nm and compared to a calibration curve prepared with gallic acid ranging from 2 to 60 mg/L. Results are expressed as mg GAE/g DM.

As an additional technique to analyze the antioxidant capacity of fermented and non-fermented BSG, we employed the ABTS method, as described by Re et al. (1999), with minor modifications. We prepared an

ABTS stock solution by mixing equal volumes of 7 mM ABTS and 2.45 mM potassium persulfate, both dissolved in distilled water. We kept this mixture in the dark for 16 h before use. At the time of the assay, we diluted the stock solution with distilled water until it reached an absorbance of 0.700 ± 0.03 at 734 nm. We then incubated 10 μL of the same extract used for the TPC analysis with 60 μL of distilled water, 30 μL of ethanol, and 3.9 mL of the diluted ABTS solution for 45 min at room temperature. The absorbance was then measured at 734 nm using a spectrophotometer (Dlab SP-UV1100, Malaysia). Absorbance values were referred to a Trolox calibration curve ranging from 50 to 800 μM, incubated in the same conditions as the samples. Trolox was dissolved in ethanol and then diluted with water to a final ethanol:water ratio of 30:70. Results were expressed as μmol of Trolox Equivalent Antioxidant Capacity (TEAC)/g DM.

2.9. Statistics

Results are expressed as mean ± standard deviation (n = 4), or otherwise stated. Statistical differences were tested by unpaired *t*-test, one-way ANOVA followed by Tukey's *post hoc* comparisons ($p < 0.05$ for all the analyses), and Pearson correlation analyses. Normality and homoscedasticity were checked using the Kolmogorov-Smirnov and Bartlett's tests, respectively. In addition, a principal component analysis (PCA) was performed on the correlation matrix after standardizing all variables to zero mean and unit variance. The PCA was used to summarize multivariate structure, inspect variable loadings, and visualize sample clustering on the first two components. Statistical analyses were performed using GraphPad Prism version 8.0.2 and PCA was carried out in Statistica v.8 (StatSoft).

3. Results

3.1. Mycelial growth and nutrient composition

The growth of *P. ostreatus* mycelium on BSG resulted in a significant reduction in substrate dry matter. SC% differed significantly between the non-fermented and fermented samples, as well as between the two fermentation time points ($p < 0.0001$). While there was no change in the dry mass of non-fermented BSG (0.0 ± 0.2 %) throughout the experiment, the growth of *P. ostreatus* mycelium consumed 35.0 ± 0.4 % of the BSG dry mass in 14 days and 39.4 ± 0.3 % in 21 days.

Mycelial growth also altered the proportions of all the analyzed nutrients throughout the fermentation process (ANOVA, $p < 0.0001$; Table 1**)**. Carbohydrate and lipid contents significantly decreased in myceliated BSG samples compared to the non-fermented ones, with significant differences also observed between the fermentation times ($p < 0.05$). Conversely, protein, ash, and TP contents increased significantly in myceliated BSG samples, with significant differences also observed between the fermentation times ($p < 0.05$).

The correlation analysis between each nutrient's content and mycelium growth estimated by SC% revealed significant linear correlations with a negative slope for carbohydrates and lipids (r = -0.997 for both nutrients; n = 12, $p < 0.0001$). On the contrary, proteins, ash, and TP showed significant positive correlations with SC% (r = 0.998, 0.995, and 0.992, respectively; n = 12, $p < 0.0001$). See supplementary table S1 for all SC% correlations. Fig. 1 shows the change in the contents of the principal nutrients in myceliated BSG as a percentage of the non-fermented samples' content after 14 and 21 days of fermentation. Complete data sets are available in Table S2.

3.2. Phytic acid and acid-soluble phosphorus

The phytic acid content was significantly reduced by SSF, decreasing from 10.4 ± 0.3 mg/g in non-fermented BSG to 2.0 ± 0.7 mg/g after 14 days and remaining at 2.0 ± 0.6 mg/g after 21 days ($p < 0.05$ between non-fermented and fermented samples). In turn, the ASP content significantly increased throughout fermentation time, from 1.80 ± 0.08 mg/g in non-fermented BSG to 3.57 ± 0.07 and 3.82 ± 0.17 mg/g after 14 and 21 days, respectively ($p < 0.05$). The ASP/TP ratio was significantly higher in myceliated BSG (55.4 ± 1.4 % and 55.3 ± 2.9 % after 14 and 21 days) than in non-inoculated BSG (41.6 ± 1.1 %, $p < 0.05$), but did not differ between fermentation time points. In addition, ASP and TP showed a significant negative correlation with phytic acid content (r = -0.977 and −0.960, respectively, n = 12, $p < 0.0001$). Fig. 2 shows the change in the contents of phytic acid, ASP, and ASP/TP as a percentage of the non-inoculated samples' content after 14 and 21 days of SSF (see Table S2 for the complete data set).

3.3. Ergothioneine and phenolic compounds

The SSF process significantly increased the EGT content, from 0.68 ± 0.02 mg/g in non-fermented samples to 2.45 ± 0.16 and 3.50 ± 0.08 mg/g after 14 and 21 days of fermentation, with significant differences among all treatments ($p < 0.0001$**,** Fig. 3**a**). Representative chromatograms for the EGT standard and samples are available in

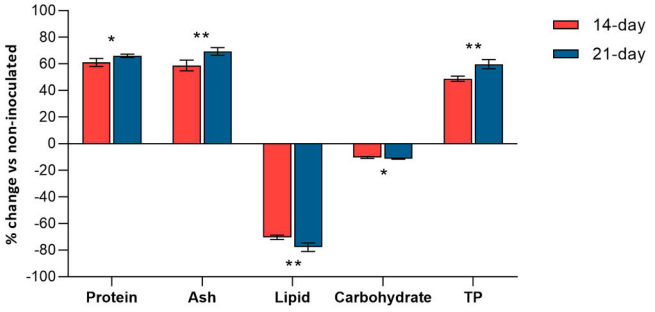

Fig. 1. Percentage change in nutrient composition between non-inoculated BSG and *Pleurotus ostreatus*-myceliated BSG. Values are expressed as mean ± SD (n = 4), * $p < 0.05$ and ** $p < 0.01$ unpaired *t*-test between 14 and 21 days of fermentation. TP, total phosphorus.

Table 1
Proximate composition of Brewer's Spent Grains (BSG) after 0, 14, and 21 days of solid-state fermentation by *Pleurotus ostreatus* mycelium.

		BSG											
		Non-inoculated				14-day				21-day			
Moisture	% WB	64.4	±	0.3	a	74.7	±	0.9	b	73.9	±	1.0	b
Protein	g/100 g DM	15.3	±	0.2	a	24.7	±	0.4	b	25.4	±	0.2	c
Ash	g/100 g DM	3.31	±	0.07	a	5.25	±	0.13	b	5.60	±	0.10	c
Lipid	g/100 g DM	4.70	±	0.09	a	1.39	±	0.08	b	1.04	±	0.15	c
Carbohydrate	g/100 g DM	76.7	±	0.2	a	68.7	±	0.6	b	67.9	±	0.2	c
TP	g/100 g DM	0.43	±	0.02	a	0.64	±	0.01	b	0.69	±	0.01	c

Values are expressed as means ± SD (n = 4).
Different letters indicate significant differences (Tukey's test, $p < 0.05$)
DM, dry mass; TP, total phosphorus; WB, wet basis

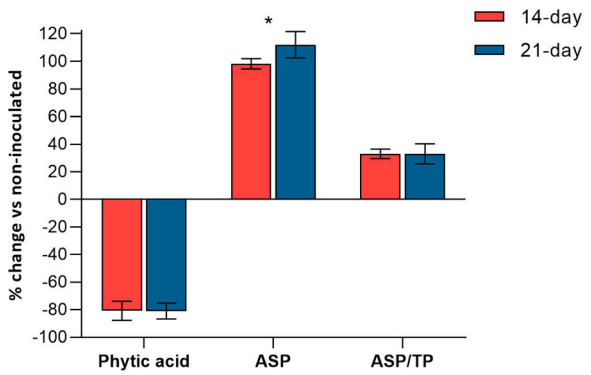

Fig. 2. Percentage change in phytic acid, acid-soluble phosphorus (ASP), and acid-soluble phosphorus / acid-total phosphorus ratio (ASP/TP) between non-inoculated BSG and *Pleurotus ostreatus*-myceliated BSG. Values are expressed as mean ± SD (n = 4), * $p < 0.05$ unpaired *t*-test between 14 and 21 days of fermentation.

Figure S1. The total phenolic content (TPC) did not differ between non-fermented BSG and after 14 days of fermentation (10.1 ± 0.6 and 10.4 ± 0.5 mg GAE/g, respectively). However, the concentration of these compounds significantly increased to 13.8 ± 0.4 mg GAE/g at the longest fermentation time ($p < 0.0001$, Fig. 3**b**).

3.4. Antioxidant capacity

Antioxidant capacity, measured as CUPRAC in the same extracts as EGT, increased from 0.68 ± 0.02 mg/g in the non-fermented samples to 2.45 ± 0.16 and 3.50 ± 0.08 mg/g after 14 and 21days of fermentation, respectively. There were significant differences among all the treatments ($p < 0.001$, Fig. 4**a).** The antioxidant capacity (CUPRAC) correlated strongly with EGT content (r = 0.972, n = 12, $p < 0.0001$). In addition, the correlation analysis of these variables with mycelium growth (SC%) revealed significant positive correlations for EGT (r = 0.932, n = 12, $p < 0.0001$) and antioxidant capacity, as measured by CUPRAC (r = 0.952, n = 12, $p < 0.0001$).

Antioxidant capacity, assessed by the ABTS method in the same extracts as TPC, decreased by 40 %, from 51.5 ± 1.9 μmol TEAC/g DM in non-fermented samples to 30.9 ± 4.5 μmol TEAC/g DM after 14 days of fermentation ($p < 0.0001$, Fig. 4**b**). At 21 days, antioxidant capacity returned to near-initial values (54.6 ± 1.9 μmol TEAC/g DM). ABTS showed no correlation with TPC, SC%, or CUPRAC.

3.5. Principal component analysis

The analysis based on the correlation matrix showed that principal component 1 (PC 1) and 2 (PC 2) explained 86.8 % and 12.3 % of the total variance, respectively, accounting for 99.1 % of the overall

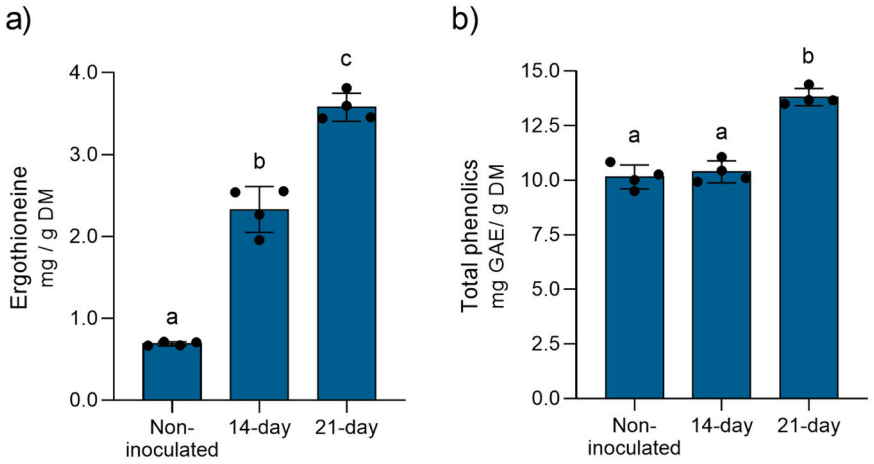

Fig. 3. Ergothioneine (a) and total phenolic contents (b), at different times of fermentation of BSG with *Pleurotus ostreatus* mycelium. Values are expressed as mean ± SD (n = 4). Different letters indicate significant differences (Tukey's *post hoc* test, $p < 0.0001$).

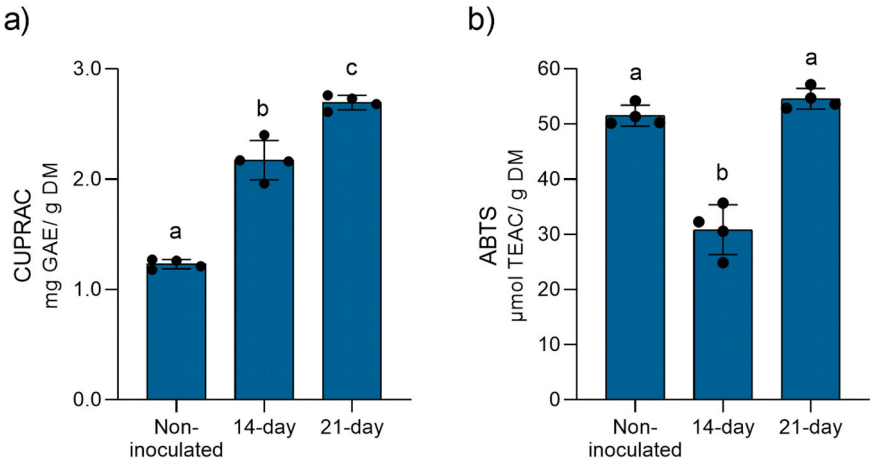

Fig. 4. Antioxidant capacity measured by CUPRAC (a) and ABTS (b), at different times of fermentation of BSG with *Pleurotus ostreatus* mycelium. Values are expressed as mean ± SD (n = 4). Different letters indicate significant differences (Tukey's *post hoc* test, $p < 0.001$).

variability in the dataset (Fig. 5).

PC 1 showed a strong positive association with fermentation time, SC %, proteins, ash, TP, ASP, EGT, and the antioxidant capacity measured by CUPRAC while it was strongly negatively associated with lipids, carbohydrates and phytic acid. All these variables showed loadings greater than 0.968 on PC 1 (Supplementary Table 3). PC 2 accounted for a smaller proportion of the variance (12.3 %) and was primarily associated with TPC and the antioxidant capacity measured by ABTS, with loadings of 0.703 and 0.963, respectively (Supplementary Table 3). ABTS loaded almost exclusively on PC 2, together with TPC, indicating that ABTS variability was more strongly associated with phenolics than with fermentation time.

4. Discussion

This study examines the revalorization of an agro-industrial byproduct, BSG, as a valuable ingredient in food and animal feed industries through its SSF with the edible mushroom *P. ostreatus*. Besides the increase in protein content at the expense of carbohydrates and lipids, our results show a strong correlation between the reduction of BSG phytic acid concentration and the increase in soluble phosphorus availability. We also detected a strong correlation between the antioxidant capacity measured by the CUPRAC assay, the EGT content, and mycelial growth, as estimated by substrate consumption. These relationships were further supported by the PCA, in which variables related to SC%, ASP, proteins, and antioxidant capacity clustered together along PC 1, indicating that these variables vary together because of the SSF. The high proportion of variance explained by PC 1 indicates a strong underlying structure in the dataset, suggesting that most variables are governed by a common biochemical transformation

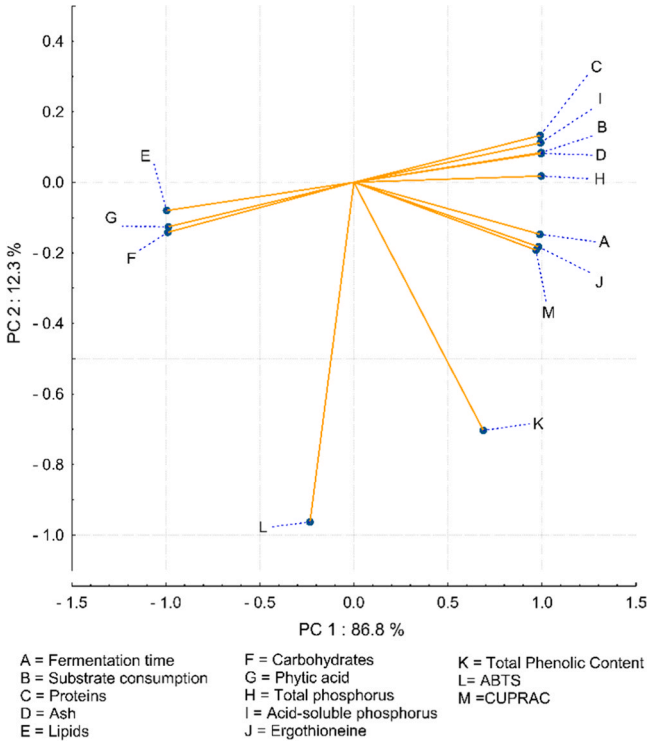

Fig. 5. Principal component analysis (PCA) biplot showing the projection of the fermentation time, substrate consumption, proximal composition, phytic acid, acid-soluble phosphorus, and antioxidant variables onto the first two principal components (PCs). PC 1 explained 86.8 % of the total variance, while PC 2 accounted for 12.3 %. Blue points represent the variables, while orange vectors indicate the loading direction and magnitude of each variable on the two components. Variable codes (A–M) correspond to the descriptors listed below the plot.

during SSF. Although PC 2 explained a smaller proportion of variance, it captured a distinct axis of antioxidant-related variation, separating ABTS and total phenolics from the traits more directly linked to substrate utilization.

4.1. Solid-state fermentation

Pleurotus ostreatus mycelium grew efficiently on BSG with no contamination issues under the conditions of this study. A comparison with a previous study from our laboratory, which used wheat grains and soybeans as substrates under similar conditions (Pascual et al., 2025), reveals that the mycelium consumes 35 % of the BSG dry mass in 14 days, the same percentage consumed by the mycelium with wheat as substrate in 21 days. This process is even less efficient with soybeans as a substrate. Besides the differences in proximate composition between BSG and wheat grains, differences in physical properties, such as higher water-holding capacity and smaller particle size, could make BSG a better substrate for mycelium growth than wheat grains (Chang & Miles, 2004; Naibaho & Korzeniowska, 2021; Stamets, 1993). The macronutrient composition of BSG, as recently reviewed by Devnani et al. (2023), indicates its suitability for SSF using *P. ostreatus*. The BSG matrix is primarily composed of lignocellulosic fiber (50–80 %), which consists of complex polymeric structures of cellulose, hemicellulose, and lignin. Additionally, it contains a considerable amount of proteins (15–30 %, predominantly prolamins and glutelins), along with minor amounts of lipids (3–10 %), starches (1–12 %), and ash (2–5 % minerals).

Zeko-Pivač, Bošnjaković, et al. (2022) observed that SSF of BSG by the medicinal mushroom *Trametes versicolor* achieved similar levels (~30 %) of organic-matter degradation to those observed in the present study. We could not make an accurate comparison of our results on *P. ostreatus* growth on BSG with other studies performed on BSG fermented with *Pleurotus spp.* (Eliopoulos et al., 2022; Stoffel et al., 2019) because the spawn used for substrate inoculation and SSF conditions, and the methods used for measuring mycelial growth in those studies differed from ours. Nevertheless, their results also show rapid substrate colonization, accompanied by changes in BSG chemical composition. Therefore, further studies aimed at improving and standardizing fermentation conditions would contribute to achieving shorter fermentation times.

Substrate consumption by mycelial growth was associated with significant changes in the proximal composition of the BSG. The capability of *P. ostreatus* to grow on a variety of woody and nonwoody lignocellulosic substrates relies on enzymatic systems to degrade cellulose, hemicellulose, and lignin, releasing carbohydrates and other valuable molecules. These systems include a complex of oxidative and hydrolytic enzymes involved in the degradation of plant cell wall polysaccharides, such as cellulase, xylanase, lacasse, and peroxidase (El Enshasy et al., 2019; Kaur et al., 2023).

Among the macronutrients, the decrease in carbohydrate and lipid contents was strongly correlated with substrate consumption, indicating that these fermentable nutrients act as a primary energy source for mycelial growth and development (Chang & Miles, 2004). The decrease in lipid content after SSF has been previously observed for BSG using the fungus *Rhizophus sp.* (Ibarruri et al., 2019) and for wheat grains fermented with *P. ostreatus* (Pascual et al., 2025). The remaining macronutrients became concentrated as substrate consumption progressed, primarily due to the release of CO_2 and H_2O from the metabolization of energetic nutrients (Terebiznik & Pilosof, 1999). Our results indicate that water was initially retained within the substrate (Table 1), suggesting that the metabolic water generated during fermentation contributes to increasing moisture levels within the substrate (Nagel et al., 2001). This excess water evaporates during the drying process; thus, it does not affect the calculations based on the final dry mass. Protein, ash, and total phosphorus in the final product exhibit a strong positive correlation with SC%, as observed for wheat grains and soybeans by Pascual et al. (2025). Notably, the protein content increased by 66 %, from 15.4

to 25.4 g/100 g DM after 21 days of fermentation. A previous study using *P. ostreatus* for BSG SSF (Eliopoulos et al., 2022) shows a similar protein increase and maximum value (49.5 %, 25 g/100 g DM after 12 days). In contrast, Stoffel et al. (2019) obtained a lower protein content increase (42 %) and a lower maximum protein content of 22.6 g/100 g DM after 35 days of fermentation with *P. albidus*. Although these comparisons are not entirely accurate, as the inoculation and fermentation conditions in those studies differ from ours, the results highlight the ability of *Pleurotus spp.* to concentrate BSG protein. As remarked in the cited papers, this effect increases the commercial value and potential use of BSG as animal feed. Additionally, mushrooms and mycelial proteins are gradually being used as alternative protein source in the food processing industry due to their well-balanced amino acid composition, high digestibility and sustainability, as recently reviewed by Guo et al. (2025). However, it is important to consider that in the present study and those of Eliopoulos et al. (2022) and Stoffel et al. (2019), the initial BSG protein contents were relatively low, as compared to those reported by Naibaho and Korzeniowska (2021) for BSG from eight different breweries (22–30 g/100 g DM). Thus, there is a need for further studies on the *P. ostreatus* mycelium's capacity to grow on BSGs with higher protein contents, which would establish the limits of the protein concentration capacity of this process.

4.2. Phytic acid reduction and soluble phosphorus

Another critical benefit of BSG SSF with *P. ostreatus*, not previously reported, is the reduction of the antinutrient phytic acid content. This compound is particularly relevant for carnivorous and omnivorous species that lack phytase activity, as it forms insoluble complexes with proteins and minerals, thereby reducing their bioavailability and absorption in the intestine (Gupta et al., 2015; Lynch et al., 2016; Selle et al., 2000). Information on phytic acid regarding BSG is limited to the paper by Ktenioudaki et al. (2015), who reported a content of 10.1 mg/g DM, which is similar to the value obtained herein, 10.4 ± 0.3 mg/g DM. These authors obtained a reduction of approximately 30 % in phytic acid levels through BSG sourdough fermentation, as measured in the resulting breads. In contrast, in our study, the mycelial growth reduced phytic acid levels by 80 % in samples corresponding to substrate consumption of approximately 35 % over 14 days, with no further reduction at 21 days. This reduction is also higher than the 45 % reduction of phytic acid content reported for wheat grains fermented by the same *P. ostreatus* strain (Pascual et al., 2025). This comparison is noteworthy given the comparable initial phytic acid content (11.4 ± 0.5 mg/g DM for wheat) and similar fermentation conditions and substrate consumption achieved (30–35 %) in both studies, which warrants further investigation into the phytase activity involved.

In addition, our results show that the reduction in phytic acid content in myceliated BSG correlates with the increase in ASP. Phytase hydrolyzes phytic acid, releasing low-phosphorylated *myo*-inositols and ASP, which is more readily available for microbial and animal uptake (Jatuwong et al., 2020; Karp et al., 2012). Phytic acid degradation, particularly during SSF with *P. ostreatus*, is associated with an increase in inorganic ASP content (da Luz et al., 2013; Pascual et al., 2025). However, to the best of our knowledge, this is the first study that effectively correlates both variables for BSG. SSF with *P. ostreatus* increased the BSG ASP content by 100 % and 112 % at 14 and 21 days of fermentation. This increase nearly doubles that observed for TP content (49 % and 60 % at the same time points). These changes result in a significant increase in the ASP/TP ratio in myceliated BSG at 14 days. Interestingly, there are no differences in the ASP/TP ratio between 14 and 21 days of SSF, which is consistent with the lack of further change in phytic acid content after 14 days. In line with this, da Luz et al. (2013) found no significant differences in phytic acid, phytase activity, or phosphorus levels between 15 and 30 days of SSF using *P. ostreatus* on *Jatropha curcas* seed cake. These results support the role of fungal phytase activity in the release of soluble phosphorus from vegetal substrates (Collopy & Royse, 2004; da Luz et al., 2013; Li et al., 2013). In contrast, the significant increase in absolute ASP and TP contents from 14 to 21 days is a result of the concentrating effect of substrate consumption, as previously described for the changes observed in nutrient composition.

The increase in phosphorus solubility in myceliated BSG should be valued, not only for the enhanced bioavailability of this nutrient in foods and feeds, but also for its potential to reduce nutrient release into the environment (Brownlie et al., 2021). This aspect is critical, as the release of nutrients, such as phosphorus and nitrogen, contributes to the eutrophication of water bodies (Brownlie et al., 2022). These nutrients often act as limiting factors in ecosystems, underscoring their critical role in maintaining ecological balance (Target 7 of the Kunming-Montreal Global Biodiversity Framework, adopted during the fifteenth meeting of the Conference of the Parties (COP 15).

4.3. EGT and phenolic compounds

Our results show that SSF of BSG with *P. ostreatus* yields 3.50 ± 0.08 mg/g EGT after 21 days, a content comparable to that of *Pleurotus spp.* fruiting bodies (3.0–3.9 mg/g, Choi et al., 2022; Kalaras et al., 2017; Liu et al., 2020; Rodriguez Estrada et al., 2009). The EGT yields from the SSF of BSG with *P. ostreatus* at 14 and 21 days surpass the EGT level reported by Chen et al. (2012b) for the 17-day SSF of adlay and buckwheat with *P. eryngii,* 0.796 and 0.786 mg/g. This advantage is even greater when the EGT yield of the present study is compared to the 0.12 μg/g reported for BSG fermented by *P. albidus* (Stoffel et al., 2019). The *Pleurotus* species or strain, growth conditions, substrate composition (e.g., carbon/nitrogen sources, particle size, moisture), and environmental factors (temperature, pH, O_2) critically affect EGT yield in both mycelial biomass (Liang et al., 2013; Lin et al., 2015) and fruiting bodies (Rodriguez Estrada et al., 2009; Tsiantas et al., 2021). The observed strong correlation (r = 0.958) between EGT yield and mycelium growth (expressed as SC%), in concordance with the findings of Chen et al. (2012b) for SSF with *P. eryngii,* suggests the need to systematically analyze cultivation variables and substrate combinations to maximize EGT production. The optimization of SSF using *Pleurotus spp.* combined with the use of low-cost byproduct-derived substrates (e.g., BSG, grape or olive marc; Tsiantas et al., 2021) presents a promising avenue to enhance EGT production, particularly when compared to synthetic biology (Liang et al., 2025) or submerged fermentation methods (Weng et al., 2024). Han et al. (2021) argue that the slow growth cycle of cultivated mushrooms limits the industrial-scale production of EGT. In this sense, our findings show that the mycelial growth of *P. ostreatus* on BSG could yield EGT values comparable to those found in fruiting bodies. This approach bypasses the fructification phase, thereby reducing the overall process duration and increasing commercialization potential. Future studies should quantify the economic advantages and cost-effectiveness of SSF to highlight its potential applications (or to accelerate its adoption) in the food and nutraceutical industries.

In contrast to EGT, BSG phenolic compounds show little or no increase upon SSF. There is no change in TPC content at 14 days, and only a 37 % (10.1–13.8 mg GAE/g) increase at 21 days, which is likely due to the concentration effect of substrate consumption. Accordingly, Stoffel et al. (2019) report a decrease of TPC content (2.5–1.7 mg GAE/g) in BSG fermented with *P. albidus* for 15 days. Both studies suggest that *P. ostreatus* does not produce a net increase of phenolics, due either to a lack of lignocellulosic complexes degradation or to enzymatic degradation of part of the original BSG phenolic content (Giacobbe et al., 2019; Zerva et al., 2021). Nevertheless, Pascual et al. (2025) reported TPC increases of 0.55–4.16 and 1.18–4.90 mg GAE/g after 28 days, for wheat and soybeans, respectively, SSF with *P. ostreatus*. Beyond differences in the substrates' chemical composition, the lack of TPC increase in myceliated BSG in the present study may be explained by the fact that BSG already contained high initial TPC levels, which resulted from the substrate preparation. Specifically, BSG was dried at 120 °C for preservation before SSF, a process known to increase TPC levels, as reported by

Santos et al. (2024). In addition, autoclaving produces a similar effect (Naibaho & Korzeniowska, 2021; Sánchez-García et al., 2022). Consequently, these elevated initial levels may have either inhibited the production of new phenolic compounds or promoted their consumption by the mycelium.

4.4. Antioxidant capacity

Although the literature offers a wide array of methods for assessing the antioxidant capacity, there is no consensus among specialists on a single one that is adequate for different matrices. The CUPRAC antioxidant capacity method is a relatively objective assay that provides reproducible and precise assessments at physiological pH. This method is capable of measuring a wide variety of physiologically relevant antioxidants, including thiol-containing compounds like EGT and glutathione. Thus, it allows for the classification of food materials based on their antioxidant values (Özyürek et al., 2011).

Using the CUPRAC method, we demonstrated that SSF with *P. ostreatus* significantly increases BSG antioxidant capacity. Furthermore, this increase in antioxidant capacity positively correlated with the progression of fermentation and EGT content, suggesting an enhancement in the BSG bioactive compound concentration along with the production of primary and secondary metabolites by the mycelium. The resulting bioactives could include EGT, phenolic compounds, vitamin C, and flavonoids, among others (Almeida et al., 2017; Hur et al., 2014; Stastny et al., 2022). The observed increase in antioxidant capacity following SSF aligns with previous studies on BSG SSF using various filamentous fungi (Chin et al., 2022; Da Costa Maia et al., 2020; Ibarruri et al., 2019; Zeko-Pivač, Bošnjaković et al., 2022) and bacteria (Tan et al., 2019; Verni et al., 2020). In addition, enhancements in antioxidant capacity have also been observed in other myceliated substrates using *P. ostreatus* (Espinosa-Páez et al., 2017; He et al., 2024; Pascual et al., 2025).

The ABTS assay revealed a transient decrease in antioxidant capacity at 14 days, followed by a recovery to initial levels after 21 days. The discrepancy with the results of the CUPRAC assay is likely due to different chemical specificities of the two methods. While CUPRAC is particularly sensitive to electron-transfer and thiol-type antioxidants such as EGT, which increased significantly along with mycelial growth, ABTS also detects compounds with hydrogen-donating antioxidant properties, including certain phenolics (Ilyasov et al., 2020; Özyürek et al., 2011). Consistent with this distinction, the PCA showed that variables closely associated with fermentation progression—such as SC %, EGT, and antioxidant capacity by CUPRAC—clustered along PC1, whereas TPC and ABTS activity loaded primarily on PC 2. This separation suggests that phenolic-related antioxidant responses followed a pattern that was not strictly aligned with fermentation time, which may help to contextualize the different temporal behaviors observed for CUPRAC and ABTS.

As reported by Santos (2024), both TPC and ABTS values increase in BSG with higher drying temperature, suggesting that the initial TPC levels and antioxidant capacity detectable by ABTS were already high in non-fermented samples. Sánchez-García et al. (2022) reported that some phenolics show strong antioxidant activities, but others may not; therefore, the lack of TPC increase at 14 days may reflect the degradation of antioxidant phenolics at the early stage of SSF and/or a low antioxidant activity of the biocompounds present at 14 days after fermentation. In contrast, the subsequent synthesis of new compounds with high antioxidant activity by the mycelium, together with the concentration effect caused by substrate consumption, would account for the significant TPC increase and the recovery of antioxidant capacity at 21 days, as detected by ABTS. Accordingly, Stoffel et al. (2019) report a decrease of ABTS content in BSG fermented with *P. albidus* for 15 days, and Sawada et al. (2024) observed a similar pattern in soybeans SSF with *P. ostreatus* with a decrease in antioxidant capacity detected by the DPPH assay after 10 days, followed by an increase at 20 and 30 days.

5. Conclusion

SSF with *P. ostreatus* can significantly improve the nutritional profile of BSG by increasing protein and phosphorus content, reducing antinutrients, and generating bioactive compounds, such as EGT. These improvements result from the concentrating effect of dry mass loss and the production of metabolites by the mycelium. The strong correlations between the beneficial effects and mycelial growth further highlight the need for systematic analysis of cultivation variables to maximize the benefits of SSF using *Pleurotus spp*. and to fully exploit the potential of low-cost, nutritious substrates like BSG.

The upcycling of BSG aligns with the principles of the circular economy, which seeks to reduce waste and enhance resource efficiency. SSF with *P ostreatus* causes a notable reduction in BSG phytic acid, enhances phosphorus bioavailability, and allows for better assimilation of proteins and essential mineral nutrients. Particularly, used as an ingredient for aquafeeds, myceliated BSG would help minimize phosphorus delivery into the environment and thus make aquaculture more sustainable. In addition, myceliated BSG offers a similar EGT content to that of mushroom fruiting bodies, which makes it a high value-added ingredient for food and feed industries.

CRediT authorship contribution statement

Mariano M. Pascual: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Julio C. Painefilú:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Natalia Ranalli:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Santos María V.:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Mariana E. Langenheim:** Validation, Methodology, Formal analysis, Data curation. **Marilina Campos:** Methodology, Investigation. **Valeria Jurski:** Methodology, Investigation. **Carlos M. Luquet:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.foohum.2025.100984.

Data Availability

Data supporting this study's findings are available at CONICET Digital, http://hdl.handle.net/11336/258529.

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