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Nutritional improvement of wheat grains and soybeans by solid-state fermentation with *Pleurotus ostreatus* mycelium

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ABSTRACT

The nutritional quality of plant-based substrates can be improved through fungi-based fermentation. This study evaluates changes in the nutritional and non-nutritional composition of food substrates biotransformed with the edible mushroom *Pleurotus ostreatus* over time. The mycelium was cultivated on wheat grains or soybeans using solid-state fermentation, and the myceliated substrates were analyzed. Mycelium grows on both substrates but at a higher rate in wheat grains. Protein and phosphorus contents increase linearly with mycelial growth in both substrates, with final values up to 53 % and 36 % higher than the initial contents in wheat, while carbohydrates decrease linearly. Phytic acid shows a linear decrease, coinciding with 3- and 2-fold increases in wheat and soybean's acid-soluble/total phosphorous ratio. Phenolic content and antioxidant capacity increase in both myceliated substrates, fitting logistic growth models, with 7.8- and 4.8-fold increases in wheat. This study shows that solid-state fermentation with *P. ostreatus* can be applied to wheat and soybeans to enhance their content of valuable nutrients and bioactive compounds and to decrease their antinutrient content. Changes in both nutritional and non-nutritional composition are linked to the extent of mycelial growth, which can be reliably evaluated by simply measuring the percentage of substrate consumption.

1. Introduction

Fermentation is a process carried out by microorganisms to modify biological substrates that serve as nutrient sources (Garrido-Galand, Asensio-Grau, Calvo-Lerma, Heredia, & Andrés, 2021). As microorganisms degrade substrates, they may alter molecule levels and release bioactive metabolites, enhancing their nutritional profile, digestibility, palatability, and prebiotic properties (Yang, Zeng, & Qiao, 2021). Solid-state fermentation (SSF) is the growth of microorganisms on insoluble substrates in the near absence of free (Yafetto, 2022). SSF is a robust and straightforward process with low energy requirements. It is environmentally friendly, as low-cost materials or agro-industrial residues can be used as substrates, and it consumes less water and generates less wastewater than submerged fermentation (Garrido-Galand et al., 2021; Mitchell, Krieger, & Berovic, 2006). Specifically, SSF with edible fungi has biotechnological applications in the food industry that produce sustainable and nutrient-dense food products known as mycofoods (Holt et al., 2024), as well as novel healthy ingredients and functional foods (Espinosa-Páez et al., 2021; Ritota & Manzi, 2023). A disadvantage of SSF is the difficulty to directly estimate the fungal growth, as the fermented substrate forms an inseparable mass (myceliated substrate) that prevents direct fungal biomass determination. Therefore, the evaluation of the growth process is limited to indirect methodologies, such as the quantification of glucosamine from the chitinous fungal cell wall that is absent in plant-based substrates (Aidoo, Hendry, & Wood, 1981; Steudler & Bley, 2015).

The oyster mushroom, *Pleurotus ostreatus* (Basidiomycetes), is one of the most popular species among edible mushrooms worldwide and is cataloged as GRAS (Generally Recognized As Safe). Oyster mushrooms offer many advantages, including good taste and textural properties, high nutritional quality, bioactive compounds with beneficial effects on human health, and they can also be incorporated into food products

Abbreviations: ASP, acid-soluble phosphorus; CUPRAC, Cupric reducing antioxidant capacity; DM, total dry mass; GAE, gallic acid equivalent; SSF, solid-state fermentation; TP, total phosphorus; TPC, total phenolic content.

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(Deepalakshmi & Mirunalini, 2014; Raman et al., 2021). *Pleurotus ostreatus* mycelium has a high capacity for substrate bioprocessing and can be used to biotransform plant-based foods to modify their nutritional and functional properties (Ritota & Manzi, 2023). However, there are only a few reports that show that SSF of lentils, quinoa, kidney beans, and oats with this mushroom results in a myceliated substrate with enhanced protein content and digestibility, antioxidant profile, and phenolic compounds or with reduced phytic acid content (Asensio-Grau, Calvo-Lerma, Heredia, & Andrés, 2020; Espinosa-Páez et al., 2017; Sánchez-García, Asensio-Grau, García-Hernández, Heredia, & Andrés, 2022; Sánchez-García, Muñoz-Pina, García-Hernández, Heredia, & Andrés, 2023).

Wheat grains *(Triticum spp.)* and soybeans *(Glycine* max*)* are central in worldwide plant-based food and feed industries. Both are rich in nutrients and provide health benefits due to their high content of bioactive compounds with antioxidant capacity, such as isoflavones and phenolic acids (Kim, Kim, & Yang, 2021; Tian et al., 2022). Among several other health benefits, phenolic compounds protect against oxidative damage and inflammation (Ma, Wang, Feng, & Xu, 2021). Despite these benefits, soybeans are yet to be adopted as a primary protein source in the human diet, partly due to antinutritional factors such as phytic acid, lectins, protease inhibitors, and tannins, which affect protein digestion and nutrient bioavailability and absorption. Soybeans and other pulses can also contain saponins, which interfere with bile acids and cholesterol absorption; raffinose family oligosaccharides, which cause flatulence; and allergenic proteins, among others. Furthermore, soybean meal is a key ingredient in the production of animal feeds, which can also negatively affect monogastric animals, such as pigs and poultry, and farmed fish, such as salmonids (Garrido-Galand et al., 2021; Gilani, Wu Xiao, & Cockell, 2012 Harahap, Suliburska, Karaca, Capanoglu, & Esatbeyoglu, 2024; Krogdahl, Penn, Thorsen, Refstie, & Bakke, 2010, for reviews).

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate) is the principal storage form of phosphorus in cereal grains and legumes (Cabrera-Orozco, Jimenez-Martinez, & Davila-Ortiz, 2013; Elhalis, Chin, & Chow, 2023). It is also one of the main factors that limit the absorption of phosphorus, divalent cations, and proteins from plant-based foods in the gastrointestinal tract of single-stomached animals, leading to poor mineral status. This problem drives increased interest in developing strategies to reduce phytic acid content during food processing, aiming to enhance mineral and protein bioavailability (Gupta, Gangoliya, & Singh, 2015; Selle, Ravindran, Caldwell, & Bryden, 2000). Further processing of pulses and grains by diverse methods, such as soaking, germination, thermal treatment, acid or alkaline treatment, and fermentation by microorganisms, has been shown to reduce their contents of antinutrients (Garrido-Galand et al., 2021; Jayachandran & Xu, 2019; Kaur & Purewal, 2023; Li, Manickavasagan, & Lim, 2024).

Numerous studies aim to improve the nutritional, antinutritional, and bioactive compound composition of plant-based ingredients, including whole soybeans or soybean products by SSF with bacteria and fungi (Harahap et al., 2024 for a review). For example, soybean SSF was performed with different lactic bacteria, *Bacillus spp.,* and combinations of lactic bacteria and *B. subtilis* (Juan & Chou, 2010; S. Li et al., 2020; Liu, Guo, Zhu, Yolandani, & Y., & Ma, H., 2024; Medeiros et al., 2018; Zhang et al., 2014) resulting in lowered proportion of high molecular weight proteins and other antinutrient factors, increased nutrient content and improved isoflavone profile, among other benefits. Fungi like *Saccharomyces cerevisiae, Aspergillus spp.*, *Eurotium cristatum*, and *Rhizopus oryzae* have also been reported to improve the nutritional composition and bioactive compounds content and quality of soybeans and soybean products, *e.g.*, increasing aglycone isoflavones, decreasing glycoside isoflavones, and modulating the gut microbiota (Chen et al., 2023; Chen, Shih, Chiou, & Yu, 2010; Queiroz Santos et al., 2018; Xiao et al., 2024). In contrast, the suitability of Basidiomycetes for soy SSF remains scarcely explored. As far as we know, soybean SSF with *P. ostreatus* has only been studied by Sawada et al. (2023) and, more recently, by He, Peng, Xu, Shi, and Qiao (2024). These studies show improvements in nutritional composition, antioxidant capacity, and content of bioactive compounds.

This study aims to increase the knowledge of SSF with *P. ostreatus* by assessing the biotransformation process of wheat grains and soybeans. We evaluate SSF with *P. ostreatus* over time as a feasible strategy to positively modify the proximate composition, reduce phytic acid content, and enhance these substrates' phosphorus bioavailability, phenolic compound content, and antioxidant capacity.

We also provide new information to improve the evaluation of fungal SSF processes by analyzing two methodologies of mycelial growth estimation: glucosamine quantification, which is a commonly used yet laborious method (Aidoo et al., 1981; Mitchell et al., 2006; Sánchez-García et al., 2022, 2023) and overall dry mass loss due to substrate consumption by the fungal metabolism, which is a simpler and cheaper method (Nicolini, Von Hunolstein, & Carilli, 1987; Terebiznik & Pilosof, 1999).

2. Materials and methods

2.1. Chemicals

Agar, $CaCO_3$, EtOH, $FeNH_4SO_4{\cdot}12\ H_2O$, Folin-Ciocalteu reagent, $FeCl_3$, and H_3NSO_3 were from Biopack (Argentina). $CaSO_4$, $ClNH_4$, HCl, Na_2CO_3, $NaHSO_4$, $NaNO_2$, NaOAc, NaOH, and $(NH_4)_2MoO_4$ were from Anedra (Argentina). Ascorbic acid, gallic acid, glucose, HNO_3, and NH_4VO_3 were from Stanton (Argentina). The malt extract was from Flebor (Argentina). The yeast extract was from Britania (Argentina). 2,2′-bipyridine, 3-methyl-2-benzo thiazolinone hydrazine (MBTH), *N*-acetyl-b-D-glucosamine hydrochloride, phytic acid sodium salt from rice, and thioglycolic acid were from Sigma-Aldrich (USA).

2.2. Edible mushroom strain

Pleurotus ostreatus var. florida (strain A01) was purchased from CISPHoCoMe (Neuquén, Argentina). The mycelium was maintained in slant tubes at 4 °C and cultured in Petri dishes at 25 °C with MYDA (2 % glucose, 1.5 % malt extract, 0.3 % yeast extract, and 1.5 % agar) until its use to produce the spawn.

2.3. Substrate and spawn preparation

Organic certified wheat grains (*Triticum aestivum*) and soybeans were obtained from Molino Campo Claro and Dietética Científica SACIFI, Argentina. Wheat grains and soybeans were soaked in excess tap water for 24 and 8 h, respectively, to achieve the minimum hydration level (49–54 %) in both since wheat grains hydrate more slowly (Stamets & Chilton, 1983) and then boiled for 10 min to soften the grains and improve their final hydration (Chang & Miles, 2004). Additionally, this combined process reduces the loads of unwanted bacteria and molds and endospore viability (Stamets & Chilton, 1983), thereby reducing autoclaving time. After sieving and cooling to room temperature, the "wheat substrate" and the "soy substrate" were produced by mixing wheat grains or soybeans with 1 % $CaSO_4$ to prevent the grains from sticking together and 0.25 % $CaCO_3$ to maintain proper pH for mycelial growth. These substrates were sorted into glass jars for spawns or polypropylene bags for the experiment, as detailed in section 2.4, autoclaved for 45 min at 121 °C, and cooled back to room temperature. Each bag had a 2 × 4 cm opening covered with microporous tape for gas exchange.

The first spawn was produced by transferring 4 cm^2 portions of mycelium from Petri dishes to 350 mL glass jars with 100 g of wheat substrate or soy substrate (three portions per jar) under a laminar flow cabin and incubating for 7–10 days at 25 °C and 60–70 % relative humidity, in a growth stove (Dalvo Instrumentos, CP797). Subsequently, under laminar flow, 10 % *w*/w of the first spawn was weighed with a portable balance, transferred to 750 mL glass jars containing 250 g of

wheat or soy substrate, and cultivated for 7 days under the same conditions (second spawn). This procedure allowed the production of spawns consisting of mycelium grown on the same substrates used for the mycelial growth step.

2.4. Mycelial growth

Two wheat and one soy batches were prepared on different days. In wheat batches 1 and 2, each polypropylene bag (25 × 40 cm) contained 400 g (batch 1, 12 bags) or 350 g (batch 2, 16 bags) of wheat substrate, respectively ($n = 4$ for each time of incubation). The soy batch consisted of 16 polypropylene bags (15 × 25 cm) containing 150 g of soy substrate each, n = 4, except for 14 days ($n = 3$ due to contamination of one bag). Inoculation was performed by weighing the substrate-containing bags with a portable balance and adding 10 g of *P. ostreatus* spawn per 100 g of substrate with a sterile spoon inside a laminar flow cabin. Then, bags were sealed with an impulse sealer and incubated inside the growth stove at 25 °C, 60–70 % relative humidity, and darkness. Inoculated bags were removed from the stove at 14 and 21 days (wheat batch 1), at 10, 14, and 21 days (wheat batch 2), and 7, 14, and 21 days (soy batch). The content of each bag was dried at 55 °C for 24 h on an aluminum tray in a convection oven (EYELA Windy oven WFO-600ND). After drying, each bag's content was weighed, ground to powder, passed through a 300 μm mesh size sieve, and stored at −18 °C until analysis (henceforth, myceliated substrate). Non-inoculated bags were submitted to the same processes as the inoculated bags for each batch as controls (0 days, n = 4 each).

2.4.1. Glucosamine content

The chitin content of the myceliated substrate was first hydrolyzed into glucosamine, adapted from Ekblad and Näsholm (1996), by incubation of 20 mg of sample with 1.5 mL of 1 M NaOH at 25 °C with agitation for 10 min. After centrifugation at 12,000 xg for 15 min, the supernatants were discarded, 1.5 mL of 1 M NaOH were added to the pellets, and autoclaved at 121 °C for 40 min. After a second centrifugation, the pellets were washed twice with 1 mL of 0.1 M phosphate buffer pH 7.0, hydrolyzed with 1.5 mL of 50 % HCl at 100 °C for 6 h in a dry bath, cooled at room temperature, and centrifuged at 12,000 x*g* for 15 min.

Glucosamine content was detected according to Plassard, Mousain, and Salsac (1982) with minor modifications by adding 6 μL of hydrolyzed sample (supernatant) to 30 μL of 1.25 M NaOAc and 64 μL of buffer (1 M HCl, 0.208 M NaOAc, pH 3.0). Then, 100 μL of 5 % $NaHSO_4$ and 100 μL of 5 % $NaNO_2$ were sequentially incorporated. After 15 min at 25 °C, we added 100 μL of 11 mM ammonium sulfamate solution (11 mM H_3NSO_3, 11 mM NaOH, 11 mM $ClNH_4$, pH 2.6), and 5 min later, 100 μL of 5 % MBTH, and then incubated for 60 min at 30 °C. Finally, we added 100 μL of 0.5 % $FeCl_3$. After 10 min, absorbance was measured at 650 nm in a microplate reader (Thermo Scientific Varioskan LUX) and compared to a calibration curve of *N*-acetyl-b-D-glucosamine hydrochloride. Results are expressed as g/100 g DM (total dry mass). Each sample's DM was calculated as the weight 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. 1:

$$DM = \frac{partial\ dry\ mass * 100}{(100 - \%residual\ moisture)} \quad (1)$$

2.4.2. Substrate consumption

Substrate consumption was defined as the percentage of dry mass consumed during the fermentation process and calculated using Eq. (2):

$$Substrate\ consumption = \left(\frac{DM_{non-inoculated} + DM_{spawn} - DM_{myceliated}}{DM_{non-inoculated} + DM_{spawn}}\right) * 100 \quad (2)$$

Where $DM_{non-inoculated}$ represents the average DM of non-inoculated samples (CV% < 1 %), DM_{spawn} refers to the DM of the spawn used as inoculum and $DM_{myceliated}$ is the DM after the fermentation process. DM_{spawn} was calculated by measuring the fresh and dry weights of the spawn remaining after inoculation to calculate the spawn % humidity and subtracting it from the inoculated weight.

2.5. Proximate composition

The proximate composition of dried samples was determined according to standardized methodologies of the Association of Official Analytical Chemists (AOAC (with Helrich, Kenneth)., 1990). We analyzed crude protein (984.13; Jones factor for nitrogen content: 5.7), crude lipid (945.16), and ash (942.05). Total carbohydrate content was calculated by subtracting protein, lipid, and ash contents from total DM. Results are expressed as g/100 g DM. We used the defatted samples obtained from lipid composition determination to quantify glucosamine, phytic acid, acid-soluble phosphorus, and total phenolic content in soybeans.

2.6. Phytic acid and phosphorus

Phytic acid and acid-soluble phosphorus (ASP) were extracted by incubating 100 mg of sample with 10 mL of 0.2 N HCl at 30 °C with agitation for 30 min, followed by centrifugation at 3800 x*g* for 15 min. Extracts (supernatants) were stored at −18 °C until quantification.

We determined the samples' phytic acid content according to Haug and Lantzsch (1983). After thawing, 0.5 mL of the extracts were incubated with 1 mL of ammonium iron (III) sulfate solution (0.2 mg/mL $FeNH_4SO_4 \cdot 12\ H_2O$ in 0.2 N HCl) at 100 °C for 30 min in a dry bath. Tubes were centrifuged at room temperature at 12,000 x g for 10 min, and 100 μL of supernatant were combined with 150 μL of 10 % *w/v* 2,2′-bipyiridine solution (10 % *v*/v thioglycolic acid) in a 96-well microtiter plate. Absorbance was measured at 519 nm in a microplate reader. The calibration curve ranged from 2 to 32 μg/mL of Na-Phytate in 0.2 N HCl. Results are expressed as mg/g DM.

ASP content was quantified colorimetrically (AOAC 965.17). One mL of the extract obtained for phytic acid analysis was diluted in 5 mL of distilled water, and 1.5 mL of molybdovanadate reagent was added (prepared by dissolving 2.0 g NH_4VO_3 in 250 mL of hot distilled water, adding 250 mL of 70 % $HClO_4$, and then mixing with 40 g $(NH_4)_2MoO_4$ dissolved in 400 mL of hot distilled water). After 5 min, absorbance was measured at 400 nm. The calibration curve ranged from 0.83 to 13.3 μg/mL of phosphorus standard, and results are expressed as mg/g DM. Total phosphorus (TP) was determined according to AOAC 965.17. Ashes from 1 g of sample (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 with distilled water to 100 mL, centrifuged at 3800 x*g* for 10 min, and an aliquot was used for colorimetric quantification, as described above for ASP. Results are expressed as mg/g DM.

2.7. Phenolic compounds and antioxidant capacity

2.7.1. Total phenolic content

Phenolic compounds were extracted by incubating 45 mg of each sample with 1.8 mL of distilled water for 60 min at 60 °C in a dry bath, with agitation every 10 min. After cooling to room temperature, samples were centrifuged at 13,000 x*g* for 30 min.

Total phenolic content (TPC) was measured according to Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, and Jacobo-Velázquez (2013) by mixing 20 μL of the supernatants with 185 μL of distilled water and 15 μL of Folin-Ciocalteu reagent (1 N) in a 96-well microplate and incubating for 6 min at room temperature in the dark. Absorbance was measured in this acidic condition at 750 nm and compared against a calibration curve of 0 to 20 μg/mL ascorbic acid in distilled water. The

assay was continued by adding 30 μL of 10 % Na_2CO_3 to the microplate and incubating for 60 min at room temperature in the dark. Absorbance was then measured in this alkaline condition at 750 nm and compared to a calibration curve of 0 to 12 μg/mL gallic acid in distilled water. The obtained conversion factor between ascorbic and gallic acid was 0.809. Corrected TPC results were calculated according to Sánchez-Rangel et al. (2013) and expressed as mg of gallic acid equivalent (GAE)/g DM.

2.7.2. Antioxidant capacity

The antioxidant capacity was measured by qCUPRAC (QUENCHER approach coupled to the Cupric Reducing Antioxidant Capacity method) according to Tufan, Çelik, Özyürek, Güçlü, and Apak (2013), with minor modifications. Briefly, 4 mL of CUPRAC reagent in EtOH:H_2O (1:1, *v*/v) were mixed with 10 mg of each sample plus 100 μL of EtOH:H_2O (1:1, v/v). This mixture was incubated for 60 min at 52 °C in a dry bath with manual agitation every 15 min. After cooling to room temperature, the mixture was centrifuged at 12,000 x*g* for 10 min. The absorbance in the supernatant was measured at 450 nm in the microplate reader and compared to a calibration curve of 20 to 600 μg/mL gallic acid in EtOH:H_2O (1:1, v/v). Results are expressed as mg GAE/g DM.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean ($n = 4$). Statistical differences were determined using one-way ANOVA followed by Tukey's *post hoc* comparisons. Normality and homoscedasticity were checked using the Kolmogorov-Smirnov and Bartlett's tests, respectively. Data were fitted using least squares regressions to the most appropriate model for each set. The global significance level was 0.05 for all analyses, and all data sets in the figures are shown with 95 % confidence bands. Fitting and statistical analyses were performed using GraphPad Prism version 8.0.2.

3. Results

3.1. Mycelial growth

Glucosamine content increased over time, indicating *P. ostreatus* growth, but differently for both substrates. Glucosamine content per day in wheat grains follows a quadratic model ($R^2 = 0.915$), with a 9.2-fold increase in 21 days (1.85 ± 0.14 g/100 g), while in soybeans, it follows a linear model ($R^2 = 0.939$), with a 6.6-fold increase, up to 0.75 ± 0.06 g/100 g at day 21 (Fig. 1 A). Regression analyses of substrate consumption per day show similar behavior to glucosamine with quadratic ($R^2 = 0.981$) and linear ($R^2 = 0.966$) models for wheat and soy, respectively (Fig. 1 B). These regression curves and the mean data suggest that *P. ostreatus* mycelium consumed 10.3 ± 0.9 % of the wheat mass in 10 days, while an equal percentage of soybeans (10.2 ± 0.3 %) was consumed in 14 days. By the end of the fermentation process, 33.3 ± 1.1 % of wheat and 13.6 ± 0.8 % of soybeans were consumed. The mycelial growth estimators show a quadratic relationship between them for wheat ($Y = -5.51 \times X^2 + 31.7 \times X - 6.17$; $n = 28$; $R^2 = 0.993$) and a linear relationship for soybeans ($Y = 21.2 \times X - 2.59$; $n = 15$; $R^2 = 0.972$; Fig. 1 C). Results henceforth are presented as a percentage of substrate consumption.

3.2. Proximate composition

Regression analyses of nutrient composition in myceliated substrates show significant linear relationships between substrate consumption and protein, carbohydrate, lipid, and ash contents for wheat and soybeans (Fig. 2; regression parameters in Table 1). Protein and ash contents show positive slopes, while carbohydrate content shows a negative slope. After 21 days of fermentation, protein and ash contents in wheat showed significant increases of 52 % (up to 17.6 ± 0.6 g/100 g) and 65 % (up to 3.95 ± 0.07 g/100 g), respectively. In soybeans, protein content increased significantly by 13 % (up to 44.8 ± 0.4 g/100 g), while ash increased by 27 % (up to 6.82 ± 0.07 g/100 g). Lipid content *vs.* substrate consumption in soybeans fits a linear regression model ($R^2 = 0.895$, $p < 0.0001$) with a positive slope, showing a significant increase of 21 % after 21 days. In contrast, the lipid content in wheat grains shows a negative slope ($R^2 = 0.571$, $p < 0.01$), with a 42 % significant decrease at 21 days compared to time 0. Supplementary Table S1 summarizes the linear regression data for nutrient content variables *vs.* glucosamine content, and Table S2 presents the average proximal composition at different fermentation days.

3.3. Phytic acid and phosphorus

The SSF process significantly reduced the phytic acid content as the substrates were consumed (Fig. 3, $p < 0.0001$), showing a linear relationship in both wheat grains ($R^2 = 0.977$) and soybeans ($R^2 = 0.763$). The initial phytic acid content in soybeans was 21.1 ± 0.4 mg/g and significantly decreased to 14. 9 ± 1.2 mg/g during the 21-day experiment (29 % reduction). In wheat, phytic acid content decreased significantly by 44 %, from 11.4 ± 0.2 to 6.4 ± 0.2 mg/g. Conversely, in both substrates, ASP, TP, and the proportion of ASP/TP increased linearly with substrate consumption (Fig. 3, $p < 0.0001$; regression parameters in Table 2). For wheat, the initial values were 0.79 ± 0.04 mg/g (ASP), 3.89 ± 0.04 mg/g (TP), and 20.4 ± 1.3 % (ASP/TP), and increased significantly after 21 days of fermentation to 3.09 ± 0.11 mg/g (ASP), 5.20 ± 0.06 mg/g (TP) and 59.4 ± 1.6 % (ASP/TP). For soybeans, these variables increased significantly after 21 days from 1.82 ± 0.08 to 4.13 ± 0.15 mg/g (ASP), from 6.14 ± 0.04 to 7.38 ± 0.03 mg/g (TP); and, for ASP/TP, from 29.6 ± 1.1 to 54.4 ± 1.8 %. Supplementary Table S3 summarizes the linear regression data for these variables *vs.*

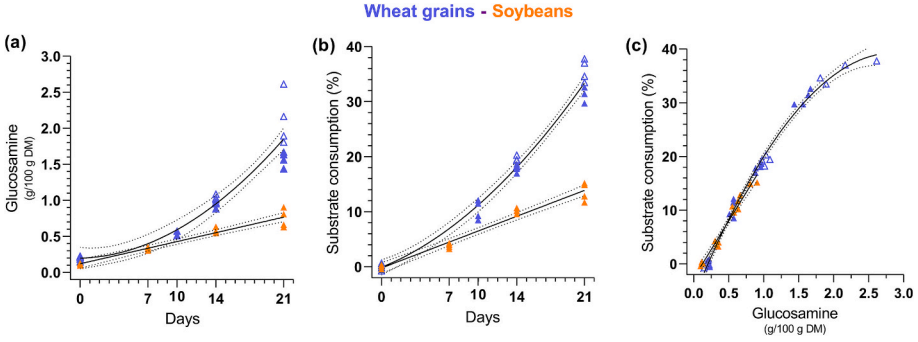

Fig. 1. Glucosamine content and substrate consumption in myceliated (*Pleurotus ostreatus*) wheat grains and soybeans. (a) Glucosamine content and (b) substrate consumption *vs.* fermentation days, and (c) substrate consumption *vs.* glucosamine content. Wheat grains: blue triangles (empty for batch 1, filled for batch 2). Soybeans: orange triangles. Continuous lines represent quadratic models for wheat grains and linear regressions for soybeans. Bands show 95 % confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

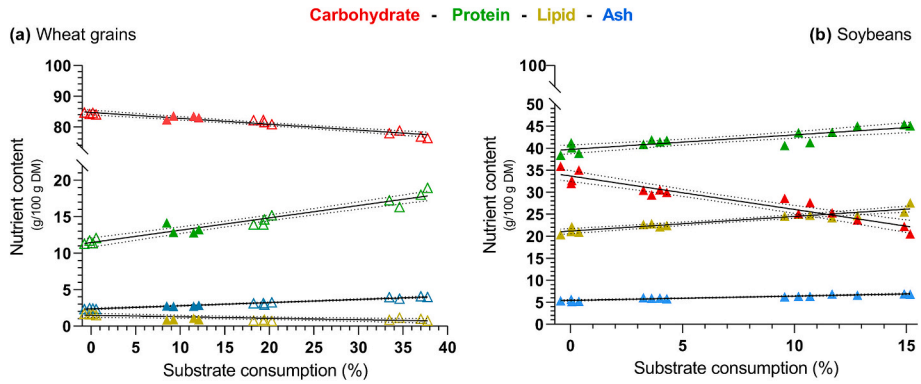

Fig. 2. Proximate composition of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans. Nutrient content *vs.* substrate consumption of myceliated (a) wheat grains and (b) soybeans. Wheat grains: empty triangles batch 1, filled triangles batch 2. Nutrients: carbohydrate (red), protein (green), lipid (yellow), ash (blue). Continuous lines: linear regressions. Bands show 95 % confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Linear regression parameters for the proximate composition of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans as a function of substrate consumption.

		Wheat grains					Soybeans				
		a		b	n	R^2	a		b	n	R^2
Carbohydrate	g/100 g	−0.192	****	84.7	12	0.941	−0.760	****	33.7	15	0.910
Protein	g/100 g	0.169	****	11.5	12	0.951	0.329	****	39.7	15	0.740
Lipid	g/100 g	−0.0202	**	1.48	12	0.571	0.332	****	21.2	15	0.895
Ash	g/100 g	0.0433	****	2.36	12	0.970	0.0981	****	5.43	15	0.902

** $p < 0.01$; **** $p < 0.0001$: regression slope statistically different from zero. Regression parameters: a, slope, b, y-intercept.

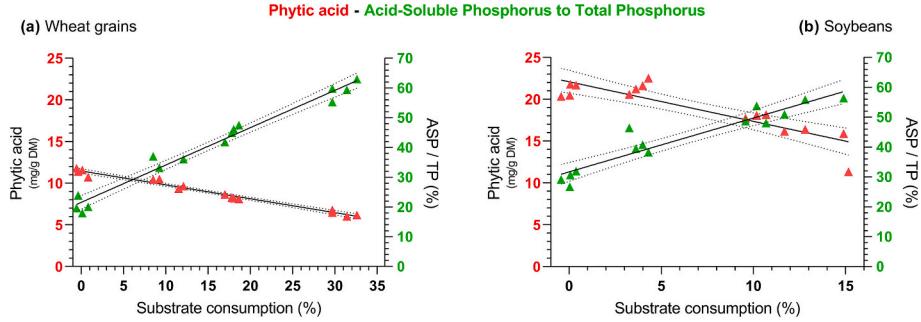

Fig. 3. Phytic acid and phosphorus contents of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans. Phytic acid (red) and the proportion of acid-soluble phosphorus to total phosphorus (ASP/TP; green) *vs.* substrate consumption of (a) wheat grains and (b) soybeans. Continuous lines: linear regressions. Bands show 95 % confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Linear regression parameters for phytic acid, acid-soluble phosphorus (ASP), total phosphorus (TP), and the proportion of acid-soluble phosphorus to total phosphorus (ASP/TP) of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans as a function of substrate consumption.

		Wheat grains					Soybeans				
		a		b	n	R^2	a		b	n	R^2
Phytic acid	mg/g	−0.167	****	11.5	16	0.977	−0.473	****	22.1	15	0.763
ASP	mg/g	0.0752	****	0.787	15	0.983	0.163	****	1.93	15	0.945
TP	mg/g	0.0450	****	3.86	16	0.929	0.0911	****	6.17	14	0.958
ASP/TP	%	1.25	****	21.6	15	0.975	1.80	****	31.7	14	0.892

**** $p < 0.0001$: regression slope statistically different from zero. Regression parameters: a, slope, b, y-intercept.

glucosamine content, and Table S4 presents the average values measured at different fermentation days.

3.4. Phenolic compounds and antioxidant capacity

Total phenolic content (TPC) and antioxidant capacity (qCUPRAC) of wheat grains and soybeans increased following logistic growth models (Fig. 4, Table 3). The Y_{max}/Y_0 quotients obtained for wheat show increases of 7.6-fold in TPC content and 4.8-fold in qCUPRAC. In soybeans, the relative increases are lower, 4.1 and 1.8-fold, for TPC and qCUPRAC, respectively. TPC and qCUPRAC correlate significantly for both substrates (Pearson's correlation test $r = 0.982$ and 0.909 for wheat and

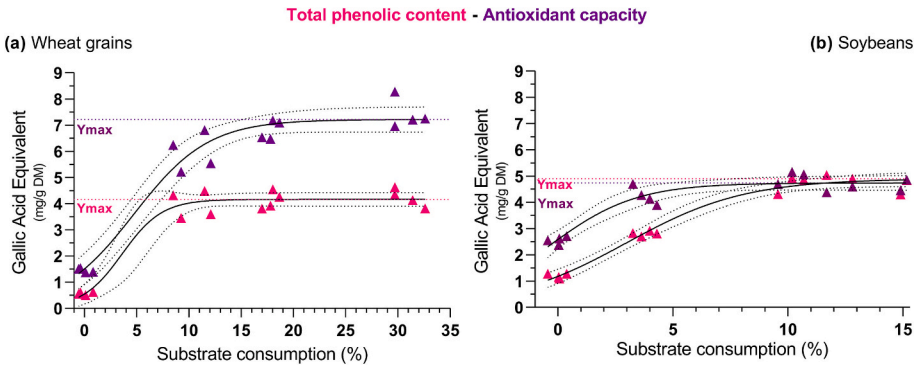

Fig. 4. Phenolic compounds content and antioxidant capacity of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans. Total phenolic content (pink) and antioxidant capacity (violet) *vs.* substrate consumption of (a) wheat grains and (b) soybeans. Continuous lines: logistic growth models. Bands show 95 % confidence intervals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Logistic growth model parameters for total phenolic content (TPC) and antioxidant capacity (qCUPRAC) of myceliated (*Pleurotus ostreatus*) wheat grains and soybeans as a function of substrate consumption.

		Wheat grains					Soybeans				
		Y_0	Y_{max}	k	n	R^2	Y_0	Y_{max}	k	n	R^2
TPC	mg GAE/g	0.548	4.16	0.523	16	0.958	1.18	4.90	0.387	15	0.977
qCUPRAC	mg GAE/g	1.51	7.22	0.280	16	0.956	2.59	4.74	0.532	15	0.907

Regression parameters: Y0, initial value; Ymax, carrying capacity; k, growth rate.

soybeans, respectively, $p < 0.0001$). Supplementary Table S5 shows the model parameters of both variables *vs.* glucosamine content, and Table S6 presents the average values at different fermentation days.

4. Discussion

Estimating fungal biomass in SSF by indirect analytical methods, such as measuring cell components (*e.g.*, glucosamine content) or secondary metabolites, can be laborious, time-consuming, and requires specialized reagents and sophisticated equipment (Manan & Webb, 2018; Steudler & Bley, 2015). According to Mitchell et al. (2006), any growth-related variable that can be experimentally linked or coupled to the fermentation processes is suitable for evaluating the growth rate of filamentous fungi, such as the dry mass reduction ratio (Wang et al., 2010). In this sense, using edible fungi, Nicolini et al. (1987) observed a decrease in the final dry mass following fungal growth. Still, they only found a correlation between dry mass loss and glucosamine content in a few cases. In contrast, in the present work, substrate consumption and glucosamine content exhibit a strong correlation for both substrates, indicating that substrate consumption is a valid indirect variable for assessing the growth profile of *P. ostreatus* in SSF processes, which may be advantageous for designing and scaling these processes. Notably, this variable only requires the complete dryness of samples, eliminating the need for complex analytical methods.

Regarding nutrients, as fermentation progressed, protein, ash, and carbohydrate contents changed consistently between substrates. The observed decrease in carbohydrates suggests their use as a primary energy source for mycelial metabolism and growth, which leads to a loss of dry matter mass as fermentation produces CO_2 and H_2O that are volatilized and released into the environment (Terebiznik & Pilosof, 1999; Viccini, Mitchell, & Krieger, 2003). This loss of dry matter increases the proportion of the remaining nutrients, as observed for proteins and ash in both substrates and lipids in soybeans, as described for wheat flour fermented with *P. albidus* (Stoffel et al., 2019). This effect is particularly favorable regarding protein resources and indicates an enhancement of the nutritional properties of the substrates by SSF. The increase in protein content in other cereals and legumes through SSF with *P. ostreatus* has been described in previous studies, although it was not related to the extent of mycelial growth or evaluated along the time of fermentation (Asensio-Grau et al., 2020; Espinosa-Páez et al., 2017; He et al., 2024; Wang et al., 2023). In our work, the enrichment in protein content in myceliated wheat and soybeans directly correlates to substrate consumption. During the 21-day fermentation process, we obtained a 53 ± 3 % improvement in crude protein for wheat and a 11 ± 1 % for soybeans, which agrees with previous reports for SSF of wheat flour and soybean meal with *Pleurotus spp.* (Stoffel et al., 2019; Wang et al., 2023). In a recent paper, He et al. (2024) report a 28 % increase in soybean protein content, which is more than two-fold higher than the reported herein; however, besides these authors fermented the soybeans for a longer period (31 days), the final protein content was almost identical in both studies (45.3 % *vs.* 44.8 %). Nevertheless, to make an accurate comparison, both studies should include the measurement of mycelial growth estimators.

It is interesting to highlight the discrepancy in crude lipid content between the substrates. In wheat grains, lipid content decreased significantly after fermentation, while in soybeans, it increased proportionally to substrate consumption. This difference indicates that *P. ostreatus* consumes wheat lipids but not soybean lipids, which could be explained by the fact that several oilseed species, such as soybeans, possess high lipase inhibitory activity (Wang & Huang, 1984), found harmful for filamentous fungi such as *Rhizopus* and *Aspergillus* (Huang & Wang, 1992), and could also inhibit *P. ostreatus* lipases (Piscitelli et al., 2017).

The initial phytic acid content in soybeans was higher than in wheat, measuring 21.1 ± 0.4 mg/g and 11.4 ± 0.2 mg/g, respectively. This content is near the upper range reported in Schlemmer, Frølich, Prieto, and Grases's (2009) review for both substrates. However, the precipitation method (Haug & Lantzsch, 1983) used in the present study has limitations (Marolt & Kolar, 2020). The stoichiometric ratio between iron(III) and phytic acid in the precipitate can vary with the pH, ionic strength, and other multivalent metals that may be present. Additionally, other compounds, such as dephosphorylated inositol phosphate analogs, may lead to overestimations if they are present. Nevertheless, according to Raboy, Gibson, Bailey, and King (2020), this technique can estimate phytic acid and inositol polyphosphates with results

comparable to those of high-performance liquid chromatography (HPLC), and it is efficacious for most unprocessed plant-based foods. Our results show that the *P. ostreatus* mycelial growth reduces the phytic acid content in both substrates, coinciding with previous reports, which show that SSF by filamentous fungi can reduce the phytic acid content of plant-based substrates through the action of microbial phytases that hydrolyze it into acid-soluble orthophosphates and myo-inositol (Jatuwong et al., 2020). Collopy and Royse (2004) have described phytases in some edible mushrooms. Notably, SSF with *P. ostreatus* has been reported to reduce the phytic acid content in quinoa (45 %) and lentils (27 %) (Sánchez-García et al., 2022). In our work, we observed a similar reduction of 44 % in phytic acid content for a 30 % substrate consumption in wheat, leading to a final value of 6.4 ± 0.2 mg/g. In soybeans, phytic acid content did not change until 5 % substrate consumption, suggesting a delay in the onset of the process, and then reached a reduction of 29 %. This phytic acid reduction is modest compared with the 72 % obtained by Yao, Li, Li, Zhu, and Gao (2021) for soybean meal anaerobic fermentation with *B. subtilis* but near to the 34.8 % reduction obtained with the fermentation of the same substrate with the fungus *A. oryzae* (Gao, Wang, Zhu, & Qian, 2013).

The reduction of phytic acid content is correlated with the release of phosphorus in the bioavailable form, as shown by the ASP increase in both substrates along mycelium growth. The ASP/TP proportion increased 3-fold from the initial content in wheat (c.a. from 20 to 60 % ASP/TP) and almost 2-fold in soybeans. In addition, we observed an enrichment of total phosphorus content in both substrates, which can be attributed to the effect of substrate consumption, as described above for proteins. These results support the idea that SSF enhances phosphorus bioavailability, improving plant-based substrates' nutritional value. Furthermore, reducing phytic acid enables more efficient absorption of minerals and proteins from foods and feeds, decreasing nutrient release to the environment (Gupta et al., 2015; Selle et al., 2000).

Total phenolic content showed a significant increase associated with *P. ostreatus* growth in both substrates, reaching its maximum at around 12 % substrate consumption, which suggests that the process leading to its increase is not sustained throughout mycelium growth. The TPC of wheat grains and soybeans before the fermentation (Y_0) was within the range previously reported for these substrates, 0.5–0.9 and 1.2–1.8 mg GAE/g, respectively (Alghamdi et al., 2018; Podio, Baroni, & Wunderlin, 2017). The TPC of myceliated wheat grains (Y_{max}) was 7.6-fold higher than Y_0, surpassing ratios reported for other edible mushrooms: *Agaricus bisporus* (3.6-fold), *A. brasiliensis* (2.9-fold), *A. blazei* (4.0-fold), and *P. albidus* (3.2-fold) (Stoffel et al., 2019; Zhai, Chen, Zhang, Zhao, & Han, 2021; Zhai, Wang, & Han, 2015). Regarding myceliated soybeans, the TPC suffered a 4.1-fold increase compared to the initial content. This val ue is comparable to the reported by Sawada et al. (2023) for soybeans SSF with *P. ostreatus* (3.5-fold, after 30 days), while Suruga, Tomita, and Kadokura (2020) reported a maximum increase ratio of 1.5 for soybeans fermented with *Ganoderma lucidum* after approximately 28 days and lower values for *Hericium spp.* The increase of TPC described in myceliated food substrates may be related to fungal hydrolytic enzymes that release and enhance the solubility of phenolic compounds primarily bound to the cell walls of food grains (Hur, Lee, Kim, Choi, & Kim, 2014; Sandhu & Punia, 2017). It may also be related to the mycelial production of phenolic secondary metabolites, as described for some edible mushrooms (Cruz-Moreno, Pérez, García-Trejo, Pérez-García, & Gutiérrez-Antonio, 2023; Zhai et al., 2021).

Antioxidant capacity is a helpful variable for assessing the functional properties of foods. In our work, it increased in both substrates due to SSF, albeit in different patterns. The initial value obtained for unfermented soybeans ($Y_0 = 2.59$ mg GAE/g) was 71 % higher than for wheat grains ($Y_0 = 1.51$ mg GAE/g). However, fermentation increased the antioxidant capacity by 4.8-fold in myceliated wheat and 1.8-fold in myceliated soybeans, resulting in a 52 % higher antioxidant capacity in myceliated wheat grains ($Y_{max} = 7.22$ mg GAE/g) than in myceliated soybeans ($Y_{max} = 4.74$ mg GAE/g). This difference could be related to the fact that the antioxidant capacity of myceliated soybeans reached a plateau at the same point in substrate consumption as the TPC content, while in myceliated wheat, it continued to grow after the phenolic compounds plateaued, suggesting that non-phenolic antioxidant molecules are generated by SSF in myceliated wheat grains (Gökmen, Serpen, & Fogliano, 2009). In this sense, the antioxidant activity, measured with the ABTS method, TPC, and the antioxidant ergothioneine of soybeans fermented with *P. ostreatus,* showed similar temporal patterns of changes (Sawada et al., 2023). Although we expressed the results as a function of mycelial growth (% substrate consumption) instead of time and used a different method for antioxidant activity (qCUPRAC), our results on soybeans coincide with these authors in that the antioxidant activity of myceliated soybeans reached maximum values at the same time as TPC.

5. Conclusion

Solid-state fermentation with *P. ostreatus* significantly improves the nutritional and non-nutritional composition of the plant-based foods used as substrates, with these effects directly linked to the extent of their biotransformation. Overall, this process enhances the nutritional quality of the substrates by increasing the content of valuable macronutrients and reducing antinutritional factors. The antioxidant capacity of the myceliated substrates is increased, with phenolic compounds identified as the primary contributors to this enhancement. We consider it essential to identify these phenolic compounds for future evaluation of the myceliated substrates' functional properties and to determine whether they are released or newly produced by mycelial activity during fermentation. In addition, non-phenolic antioxidants, yet to be identified, contribute to the antioxidant capacity of myceliated wheat grains and deserve further attention. Considering the reliability of substrate consumption as a convenient proxy for mycelial growth assessment, this approach appears preferable for developing new fungal SSF-modified cereals and legumes for human and animal nutrition.

Solid-state fermentation of plant-based substrates with edible mushrooms appears promising for environmentally sustainable production of healthy, nutritious ingredients. Our research on *P. ostreatus* myceliated grains and legumes supports their potential for industrial applications and developing mycofood technologies. These products can be incorporated into food and feed as supplements or ingredients, adding their many functional compounds and nutritional content.

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CRediT authorship contribution statement

Mariano M. Pascual: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lucila T. Herbert:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marilina Campos:** Writing – review & editing, Methodology, Investigation. **Valeria Jurski:** Writing – review & editing, Methodology, Investigation. **Julio C. Painefilú:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Carlos M. Luquet:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2025.104021.

Data availability

Data supporting this study's findings are available at CONICET Digital at http://hdl.handle.net/11336/235958.

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