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In vitro protein digestion and mineral accessibility of edible filamentous fungi cultivated on winery and distillery by-products

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ABSTRACT

Edible filamentous fungi (mycoprotein) offer a sustainable protein source that supports the upcycling of agri-food industry sidestreams, stimulating circular production systems. This study evaluated the nutritional quality, including protein digestibility and mineral accessibility of three edible fungal species *Neurospora intermedia* (NI), *Aspergillus oryzae* (AO), and *Rhizopus oryzae* (RO), cultivated on winery by-products (grape marc, wine lees, and vinasse) and on synthetic glucose medium as a control. Protein content, amino acid profile, essential minerals, *in vitro* protein degree of hydrolysis (DH%), and mineral accessibility were assessed. One important hypothesis explored was whether enriched fungal biomass polyphenol levels would negatively influence protein digestibility and mineral accessibility. Wine lees supported the highest biomass protein content (27.2–30.6 % dry weight, dw), followed by grape marc, and vinasse. The amino acid profile revealed that essential amino acids comprised 40.88–51.69 % of the total protein, with lysine (8.43–14.18 %) and leucine (7.62–9.95 %) being the most abundant. Notably, RO grown in grape marc accumulated higher polyphenol level compared to NI and AO, up to 96 mg gallic acid equivalent/g dw. After *in vitro* digestion, NI and AO revealed higher protein digestibility than RO (38–80 % vs 9–53 % DH), and all fungal species cultivated in wine lees—particularly RO—exhibited the highest levels of accessible iron and zinc Grape marc-grown RO showed significantly reduced protein digestibility and mineral accessibility. These findings present a promising route to produce mycoprotein, while lowering the wine and distillery sector footprint. Polyphenol levels should be optimized to avoid hampering protein digestibility and mineral accessibility.

1. Introduction

Addressing hunger, malnutrition, and climate change simultaneously aligns with United Nations Sustainable Development Goal 2, 12, and 13, which emphasize a dietary shift toward sustainable protein sources produced in a responsible manner with minimal environmental impact. Achieving this will require maximizing resource efficiency such as minimizing waste throughout the food value chain. In this context, edible filamentous fungi have emerged as promising drivers among numerous different alternative protein sources (Shahid et al., 2024). They not only host a valuable nutritional profile characterized by high levels of essential amino acids and dietary fibers such as β-glucan, but are also capable of utilizing agri-food industry sidestreams as feedstocks, thereby promoting circularity in food production systems (Ng et al., 2024).

The winery and distillery industries play a prominent role in the European Union agri-food sector. They generate substantial amounts of both solid and liquid organic and inorganic residues, collectively known as "oenological by-products," amounting to approximately 20 million tons annually worldwide (Hoxha, Taherzadeh, & Marangon, 2025). The most abundant oenological by-product is grape marc, comprising 30 % (w/w) of processed grapes during winemaking and contain up to 60 % of solid by-products (Nanni et al., 2021). Another oenological by-product includes pre-distillation wine lees, collected after alcoholic fermentation, and the post-distillation wine lees or vinasse, collected after the distillation process. These by-products, rich in polyphenols, present both environmental challenges and opportunities for sustainable valorization in food, feed, bioenergy, cosmetics and many sectors (Hoxha, Taherzadeh, & Marangon, 2025).

There is currently an increased trend into the development and

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commercialization of mycoprotein-based foods (Lübeck & Lübeck, 2022). The mycelial biomass of the fungal species *Fusarium venenatum* is used as the main ingredient in commercial meat alternatives Quorn™ (K. Li, Yu, et al., 2023). Several other species of filamentous fungi have also been widely used for food industry applications such as soy sauce, and sake (rice wine) based on *Aspergillus oryzae* (Ito & Matsuyama, 2021), oncom based on *Neurospora intermedia* (Maini Rekdal et al., 2024); and tempeh production based on *Rhizopus oryzae* and *Rhizopus oligosporus* (Martín-Miguélez et al., 2025). These filamentous fungi are also interesting from the perspective of food system circularity since they can utilize diverse agri-food industry sidestreams, including fish processing (Sar et al., 2021), pea processing (Souza Filho et al., 2018), dairy (Mahboubi et al., 2017), and other agricultural sidestreams (Maini Rekdal et al., 2024) and at the same time building up a high crude protein content, typically ranging from 20 to 45 % dw.

An essential aspect dictating the nutritional value of fungi-based foods is however the quality of the proteins being accumulated, including their amino acid profile and digestibility. Further to this, the accessibility of essential minerals has emerged crucial when replacing e.g. red meat as a main protein source, with more environmentally sound protein sources (Latunde-Dada et al., 2023). Winery and distillery by-products also contain polyphenols (Chiappero et al., 2023), which may be further accumulated by filamentous fungi during fermentation. Polyphenols are known for their antioxidant properties, which have both food stabilizing effects (Lei et al., 2024) and potential health benefits (Rana et al., 2022). However, they can also interact with proteins and minerals, leading to precipitation, which may reduce nutrients bioavailability (Lund, 2021; Thakur et al., 2019).

The standardized INFOGEST *in vitro* food digestion protocol provides a consistent approach for evaluating macronutrient digestibility and micronutrient accessibility. Existing *in vitro* studies addressing the protein digestibility of edible filamentous fungi have examined *Fusarium venenatum* and species of *Aspergillus oryzae*, *Neurospora intermedia*, and *Rhizopus* sp. (Ariëns et al., 2021; Colosimo et al., 2020; Wang et al., 2023). Using the same model, we have previously assessed mineral accessibility of *Rhizopus oligosporus* (Wang et al., 2024). The INFOGEST *in vitro* method has also been successfully used to evaluate protein digestion of several other microbial biomasses such as bacteria and yeast (Nordlund et al., 2024).

To the best of the authors' knowledge, no previous study has investigated the protein digestibility and mineral accessibility of filamentous fungi cultivated on winery and distillery by-products. In the present study, three species of edible filamentous fungi, *Aspergillus oryzae, Neurospora intermedia,* and *Rhizopus oryzae* were cultivated on grape marc, pre-distillation wine lees, and vinasse, alongside a synthetic medium consisting of glucose and yeast extracts as a control. Following harvest, the fungal biomasses were subjected to protein, essential amino acids, essential minerals and polyphenol quantification, as well as *in vitro* digestibility and accessibility assessment. We hypothesized that both the composition of the cultivation substrates and the fungal species would influence the nutritional quality and polyphenol content of the fungal biomass. In relation to polyphenols, it was also hypothesized that a higher accumulation would hamper the fungal protein digestibility and mineral accessibility. This study provides new insights both into the nutritional potential of filamentous fungi cultivated on winery and distillery by-products, highlighting their polyphenols, proteins, and minerals, and its effects on nutrient digestibility and accessibility.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade (Sigma-Aldrich, Sweden). Nitric acid (68 % trace metal grade) was purchased from Fischer Scientific. Ultrapure water 18.2 MΩ-cm (Milli-Q IQ7000) was used for the amino acid and mineral analysis. Pepsin from porcine gastric mucosa (P6887), bile bovine, and pancreatin from porcine pancreas (8x USP, P7545) were purchased from Sigma-Aldrich, Sweden.

2.2. Winery and distillery by-products

In this study, the winery and distillery by-products were collected between November and December 2023 from the Acquavite distillery located in Vazzola, Veneto, Italy. The grape varieties used in the production of these by-products were sourced exclusively from the Veneto region. The grape marc (GM) consisted of about 80 % skins from the Prosecco/Glera grape variety, along with skins from other white and red grape varieties. GM was collected after pressing and distillation, followed by mechanical seed removal, drying, and milling as described by (Hoxha, Lennartsson, & Taherzadeh, 2025; Hoxha, Taherzadeh, & Marangon, 2025). The resulting GM was vacuum-packaged and stored at 4 °C during transport and throughout the study until use.

Pre-distillation wine lees (WL) was collected during the clarification, sedimentation, and racking steps of Prosecco wine production, prior to distillation. The wine lees used in this study consisted of a blend of both gross and fine lees, with an approximate composition of 60 % liquid and 40 % solids fractions. The WL was stored at 4 °C during transport and throughout the study until use in maximum one month.

Vinasse or post-distillation wine lees (VIN) was generated from the distillation of wine lees that were collected from multiple wineries and blended in the distillery's storage tanks. These wine lees were stored for 2–3 weeks prior to distillation. Vinasse was collected immediately after distillation and stored at 4 °C until further use in maximum six months. For the further analysis, GM was analyzed in its original form. WL and VIN were freeze-dried to obtain solid samples.

2.3. Microorganisms

Three edible filamentous fungal strains were sourced from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) for this study. These included two Ascomycetes—*Aspergillus oryzae var. oryzae* (CBS 819.72) and *Neurospora intermedia* (CBS 131.92)—and one Zygomycete, *Rhizopus oryzae* var. *delemar* (CBS 145940). The strains were maintained on Potato Dextrose Agar (PDA) composed of 4 g/L potato extract, 20 g/L glucose, and 15 g/L agar. Spore suspensions were prepared by adding 20 mL of sterile Milli-Q water to each PDA plate, followed by gently releasing the spores using a sterile L-shaped plastic spreader. The resulting spore-containing suspension was then collected for use in subsequent fungal cultivations.

2.4. Fungal cultivations in 4 L bubble column reactors

Grape marc (GM) was hydrothermally pretreated by preparing 4 % (w/v) solutions and autoclaving at 121 °C for 20 min. After cooling to room temperature, the suspension was centrifuged and filtered to obtain the GM liquor. Pre-distillation wine lees (WL) was centrifuged (4500 g for 15 min at 4 °C) and the liquid fraction was collected and used as cultivation medium diluted 1:1 (v/v) with water. Vinasse (VIN) was diluted with water into a concentration of 10 % (v/v) and thus was used as a suspension mixture for fungal cultivations. No additional nutrient supplementation was added for the fungal cultivations. In addition, fungi were cultivated in a synthetic glucose media (SYN), which was prepared using 15 g/L glucose and 5 g/L yeast extract, serving as a control.

Bubble column bioreactors (Belach Bioteknik, Sweden) were filled with 3 L of either 4 % grape marc liquor, 50 % diluted wine lees liquid fraction, 10 % diluted vinasse, and synthetic glucose media. They were sterilized at 121 °C for 20 min, then cooled at room temperature and inoculated with 20 mL/L of spore suspension. Cultivations were conducted at 35 °C and pH 5, controlled with either 2 M NaOH or 2 M H_2SO_4 according to Hoxha, Lennartsson, and Taherzadeh (2025). Antifoam BIOSPUMEX™ 200K (PMC Ouvrie SAS, France) was added to control

the excessive foams (only for GM fermentations). The cultivation lasted 48 h with a constant aeration rate of 1 vol of air per volume of liquid per minute. Biomass was harvested through a kitchen sieve 1 mm^2. Harvested biomass was washed and dried in a drying oven at 70 °C overnight. Dried samples were then milled using a ball mill (Retsch, Germany) and subsequently subjected to mineral analysis, amino acid analysis, polyphenol determination, and *in vitro* gastrointestinal digestion. Fungal cultivation was performed in duplicates (n = 2) for WL, and single replicates (n = 1) for GM, VIN, and SYN.

2.5. Static in vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was performed following the INFOGEST 2.0 protocol (Brodkorb et al., 2019), including standardized electrolyte compositions for simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), and standardized enzyme activity measurements. Pepsin and pancreatin activities were measured at 2867 U/mg and 5.1 U/mg, respectively. Bovine bile was measured to contain bile salts at 1.55 mmol/mg. Minor modifications, as described by Wang et al. (2023), included a reduction in pancreatin enzyme and bile concentration.

Briefly, 100 mg dried fungal biomass was placed in a tube, and 1 mL of water was added. While oral digestion was omitted, 1 mL of SSF (without salivary amylase) was added. Gastric digestion was initiated by adding 2 mL of SGF containing pepsin, with the final concentration adjusted to 2000 U/mL in a total gastric volume of 4 mL. The mixture was incubated at pH 3 for 2 h. The intestinal phase was initiated by adding 4 mL of SIF containing pancreatin and bile, adjusted to reach a final concentration of 10 U/mL and 1 mmol/L in the total volume, respectively. The pH was adjusted to 7, and the mixture was incubated for another 2 h. Digestion was stopped by adding 800 μL of soybean trypsin-chymotrypsin/Bowman–Birk inhibitor (0.05 g/L). All incubations were carried out at 37 °C with gentle mixing (7 rpm) using a Stuart Rotator SB3 (UK). Blank digestions were carried out by following the same procedure as above, but without adding the dried fungal biomass. The intestinal digests were then frozen and stored at −80 °C. Each sample was digested in triplicate.

2.6. Analytical methods

2.6.1. Carbon-to-nitrogen (C/N) ratio

Solid substrates, namely GM, freeze-dried WL, and VIN were analyzed for their carbon-to-nitrogen (C/N) ratio using a FlashSmart™ Elemental Analyzer (Thermo Scientific, USA).

2.6.2. Amino acid profiling

Amino acids were analyzed following the procedure outlined by Trigo et al. (2021). Briefly, 50 mg of dried fungal biomass sample was combined with 8 mL of 6 M hydrochloric acid (HCl). After purging air from the tubes and replacing it with nitrogen, the samples underwent hydrolysis at 110 °C for 24 h on a heating block. After hydrolysis, 0.5 mL aliquot of the hydrolysate was dried by flushing with air, then resuspended in 5 mL 0.2 M acetic acid and filtered using a 0.22 μm syringe filter prior to analysis with liquid chromatography–mass spectrometry (LC-MS) as previously reported by Trigo et al. (2021).

Two microliters of each sample were analyzed using an LC/MS system (Agilent 1100 HPLC and 6120B Single Quadrupole MS), equipped with a Phenomenex C18 column (250 μm × 4.6 μm × 3 μm). Chromatogram data were processed using MassHunter Quantitative Analysis software (version B.09.00, Agilent Technologies). Due to acid hydrolysis, tryptophan could not be quantified, and asparagine and glutamine were quantified alongside aspartic acid and glutamic acid, respectively. Results are shown in terms of total amino acids (% dw) and essential amino acids (% of total amino acids).

2.6.3. Degree of hydrolysis

The quantification of the degree of protein hydrolysis (DH%) for the sample of the intestinal digests was accomplished utilizing the o-phthalaldehyde reagent (OPA) to measure the primary amines. The OPA reagent was freshly prepared by mixing 10 ml of OPA stock solution (0.05M in ethanol), 10 mL of n-acyl-L-cysteine (0.057 M in water), 5 mL SDS 20 %, and 75 mL 100 mM borate buffer. Aliquot of the intestinal digests was centrifuged at 2000 rpm for 5 min. The supernatant was diluted and then added (100 μL) to the OPA reagent (1000 μL), incubated at room temperature for 10 min, and measured at 335 nm using a UV–Vis spectrophotometer (Libra, Bichrom). Sample absorbance readings were compared with the L-serine standard curve (0.15–0.8 mM).

The degree of hydrolysis was calculated by Equation (1):

$$\text{Degree of hydrolysis } (\%) = \frac{h_{sample} - h_{digestion\ blank}}{h_{total\ (sample)}}\ x\ 100 \qquad \text{Eq. 1}$$

where h is the measured value of total primary amines (mmol serine equivalents) by the OPA method, and h_{total} is the maximum number of primary amines in each sample (mmol amino acid) obtained through the amino acid analysis.

2.6.4. Mineral analysis

Minerals, including phosphorus (P), zinc (Zn), copper (Cu), magnesium (Mg), and iron (Fe) were quantified following the protocol described by Wang et al. (2024). For all solid samples, namely GM, freeze-dried WL and VIN, and oven dried fungal biomass, 100 mg samples were initially treated with 1 mL of 68 % HNO_3 at 110 °C for 2 h. Subsequently, an additional 1 mL of 68 % HNO_3 and 0.5 mL of 30 % H_2O_2 were added, and the mixture was heated for another 2 h at 110 °C. After digestion, the samples were transferred into volumetric flasks to a final volume of 10 mL with Milli-Q water and filtered through a 0.22 μm PES filter. Mineral quantification was carried out using Microwave Plasma Atomic Emission Spectroscopy (MP-AES 4200, Agilent Technologies). Analysis was conducted at wavelengths of 213.6 nm for P, 213.8 nm for Zn, 324.7 nm for Cu, 285.2 nm for Mg, 371.9 nm for Fe, and 422.7 nm for Ca. Standards were prepared using standard mixtures (Agilent Technologies) ranging from 0.05 ppm to 2 ppm of each mineral in 2 % HNO_3. All measurements were conducted in triplicates.

To assess mineral accessibility, filtered digests were acidified to 2 % HNO_3 and analyzed under the same conditions as described for the fungal biomass. Standard curves for minerals ranging from 0.05 to 2 ppm were similarly prepared using simulated digestion fluid (a 1:2:4 mixture of SSF, SGF, and SIF), and acidified to 2 % HNO_3.

Mineral accessibility was calculated using Equation (2):

$$\textit{Mineral accessibility } (\%) = \frac{\textit{total mineral in filtered digest} - \textit{total mineral in filtered blank digest}}{\textit{total mineral in the } 100\ \textit{mg sample}} x\ 100 \qquad \text{Eq. 2}$$

2.6.5. Total phenolic content

For the fungal biomass, extracts were prepared following the procedure described by Qin et al. (2013), with minor modifications. Briefly, 100 mg of fungal biomass was mixed with 8 mL of an ethanol:water:HCl

solution (80:19.9:0.1, v/v/v) and vortexed for 1 min. The mixture was incubated for 4 h at 37 °C and gently agitated at 7 rpm using a rotator (SB3, Stuart). After incubation, the samples were centrifuged at 3000×*g* for 1 min, and the supernatant was filtered using 0.22 μm filters.

Total polyphenol content was determined according to the method by Singleton and Rossi (1965). Extracts were combined with Folin–Ciocalteu reagent (1:10 v/v) and a 7.5 % Na_2CO_3 solution in a ratio of 1:5:4. The mixtures were incubated at room temperature for 30 min, centrifuged at 18,900×*g* for 2 min, and absorbance measured at 765 nm using a UV–Vis spectrophotometer (Libra, Bichrom). Polyphenol concentrations were calculated based on a gallic acid standard curve (7.5–250 ppm, $r^2 > 0.99$) and expressed in milligrams of gallic acid equivalents (GAE) per gram of dry fungal biomass. All analyses were performed in triplicate.

2.7. Statistical analysis

Statistical analysis was performed using Minitab® 21.1.1 software. One way ANOVA was applied followed by Tukey's post hoc for pairwise comparison at a significance level of 5 %. Different groups of superscripts are used in tables to indicate the statistically significant pairwise comparisons at a threshold p-value <0.05. Results in tables and figures are presented as mean values ± standard deviation. Results in tables and figures are presented as mean values ± standard deviation (n = 3), except for cultivation in wine lees (n = 5)

3. Results and discussion

This study aimed to evaluate the nutritional quality, in terms of protein content, essential amino acid profiles, mineral content, protein digestibility, and mineral accessibility—of filamentous fungi cultivated on winery by-products. Three species of filamentous fungi; *Neurospora intermedia* (NI), *Aspergillus oryzae* (AO), and *Rhizopus oryzae* (RO) were cultivated on three different winery and distillery by-product, namely wine lees (WL), grape marc (GM), and vinasse (VIN), and a synthetic glucose media (SYN) as a control. It was hypothesized that variations in the composition of cultivation medium and fungal strains would affect the fungal biomass's nutritional quality and total polyphenol content, with the latter potentially affecting protein digestibility and mineral accessibility.

3.1. Protein content and essential amino acid profile of fungal biomass

The protein content of fungal biomass was measured as total content of amino acids, and results are presented in Table 1. When cultivated on glucose media, the protein content ranged from 18.37 % to 21.5 % dw, while when cultivated on wine lees resulted in a significantly higher protein content, ranging from 27.25 % to 30.6 % dw. In contrast, when grape marc was used as the cultivation media, the protein content was significantly lower compared to wine lees and glucose media across all fungal species: NI at 16.1 %, AO at 10.98 %, and RO at 11.8 % dw. Additionally, when vinasse was used as the cultivation medium, notable species-specific differences were observed. NI showed the highest protein content at 25.1 %, followed by AO at 18.89 %, while RO had the lowest content at 9.25 % dw.

The protein content of *N. intermedia*, *A. oryzae*, and *R. oryzae* in this study was considerably lower compared to our previous study (Wang et al., 2023) that reported 37.9 % for *N. intermedia*, 45.1 % for *A. oryzae*, and 32.1 % for *R. oryzae*. This difference is primarily attributed to cultivation media composition and cultivation period. While the previous study used 30 g/L glucose and 24-h cultivation period, the present study comprised cultivation in 15 g/L glucose for 48 h. Crude protein content of filamentous fungi cultivated in synthetic glucose media are relatively high between 42 and 56 %. The discrepancy between crude

Table 1
Protein content (total amino acids) and essential amino acids profile of filamentous fungi *N.intermedia* (NI), *A.oryzae*(AO), and *R.oryzae* (RO) cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN).

Substrate	Fungi species	Total amino acids (% dw)	Amino acid profile (% total AA)								
			Lysine	Histidine	Threonine	Valine	Methionine	Isoleucine	Leucine	Phenylalanine	Essential AA
SYN	NI	19.6 ± 0.68^{de}	9.74 ± 0.1^{b}	2.87 ± 0.07^{abcd}	6.28 ± 0.1^{a}	5.83 ± 0.08^{ef}	1.31 ± 0.03^{f}	4.62 ± 0.22^{fg}	7.71 ± 0.23^{cd}	4.49 ± 0.06^{d}	42.85 ± 0.51^{de}
	AO	18.4 ± 0.69^{de}	8.9 ± 0.23^{b}	2.67 ± 0.05^{abcd}	6.22 ± 0.02^{abc}	6.77 ± 0.37^{abc}	1.48 ± 0.08^{ef}	5.2 ± 0.04^{cdef}	8.29 ± 0.12^{bcd}	5.1 ± 0.05^{abcd}	44.62 ± 0.79^{cd}
	RO	21.5 ± 1.22^{cd}	11.33 ± 0.03^{ab}	3.52 ± 0.04^{a}	6.22 ± 0.28^{abc}	6.31 ± 0.06^{cde}	1.51 ± 0.04^{def}	5.21 ± 0.23^{cdef}	7.87 ± 0.16^{cd}	5.04 ± 0.07^{abcd}	47.01 ± 0.74^{bc}
WL	NI	28.9 ± 1.75^{ab}	9.92 ± 0.57^{b}	2.59 ± 0.28^{bcd}	6 ± 0.83^{abc}	6.21 ± 0.15^{de}	1.74 ± 0.15^{abcd}	4.83 ± 0.14^{efg}	9.29 ± 0.79^{ab}	5.02 ± 0.26^{abcd}	45.61 ± 2.25^{cd}
	AO	27.3 ± 2.55^{ab}	9.69 ± 0.73^{b}	2.5 ± 0.38^{bcd}	5.76 ± 0.09^{abc}	7.04 ± 0.17^{a}	1.84 ± 0.06^{ab}	5.4 ± 0.16^{bcd}	9.35 ± 0.21^{ab}	5.28 ± 0.11^{abc}	46.87 ± 0.95^{bc}
	RO	30.6 ± 2.49^{a}	14.18 ± 3.51^{a}	3.23 ± 0.5^{ab}	5.33 ± 0.14^{c}	6.9 ± 0.35^{ab}	1.9 ± 0.18^{a}	5.83 ± 0.34^{ab}	8.92 ±1^{abc}	5.39 ± 0.45^{ab}	51.69 ± 1.55^{a}
GM	NI	16.1 ± 0.32^{ef}	9.19 ± 0.07^{b}	1.95 ± 0.38^{d}	6.05 ± 0.29^{abc}	6.82 ± 0.02^{abc}	1.73 ± 0.05^{abcde}	5.32 ± 0.17^{bcde}	9.05 ± 0.07^{abc}	4.82 ± 0.12^{bcd}	44.93 ± 0.34^{cd}
	AO	10.9 ± 0.78^{fg}	8.43±0^{b}	1.95 ± 0.24^{d}	6.35 ± 0.28^{a}	7.35 ± 0.17^{a}	1.51 ± 0.08^{def}	5.63 ± 0.15^{abc}	9.95 ± 0.29^{a}	5.34 ± 0.15^{abc}	46.5 ± 0.85^{bc}
	RO	11.9 ± 0.32^{fg}	9.59 ± 0.23^{b}	2.27 ± 0.36^{cd}	6.26 ± 0.15^{ab}	7.28 ± 0.22^{a}	1.82 ± 0.08^{abc}	6.21 ± 0.34^{a}	9.91 ± 0.25^{a}	5.49 ± 0.29^{a}	48.83 ± 1.08^{ab}
VIN	NI	25.2 ± 4.24^{bc}	8.55 ± 0.25^{b}	2.78 ± 0.06^{abcd}	5.68 ± 0.13^{abc}	5.63 ± 0.13^{f}	1.6 ± 0.03^{bcde}	4.43 ± 0.03^{g}	7.62 ± 0.15^{d}	4.59 ± 0.04^{d}	40.88 ± 0.49^{e}
	AO	18.89 ± 0.9^{de}	11.39 ± 0.35^{ab}	2.57 ± 0.65^{abcd}	5.31 ± 0.03^{bc}	6.09 ± 0.18^{def}	1.58 ± 0.07^{cdef}	4.92 ± 0.18^{defg}	7.82 ± 0.33^{cd}	4.79 ± 0.09^{cd}	44.47 ± 0.61^{cd}
	RO	9.25 ± 0.7^{g}	8.77 ± 0.28^{b}	3.06 ± 0.11^{abc}	5.57 ± 0.38^{abc}	6.44 ± 0.08^{bcd}	1.51 ± 0.07^{def}	5.51 ± 0.16^{bcd}	7.88 ± 0.11^{cd}	5.24 ± 0.14^{abc}	43.97 ± 0.63^{cde}
Adult amino acid requirement (%protein)*			4.5	1.5	2.3	3.9	1.6	3.0	5.9	3.8	

Data are expressed as mean values ± standard deviation. For all media n = 3, except wine lees n = 5.
Adult amino acid requirement is based on FAO/WHO recommendation (FAO, 2007).
Different letters in the same columns denote statistically significant differences ($p < 0.05$) among samples.
SYN: synthetic glucose media, WL: pre-distillation wine lees, GM: grape marc, VIN: vinasse.
NI: *Neurospora intermedia,* AO: *Aspergillus oryzae,* RO: *Rhizopus oryzae*.

protein content and total amino acids is most likely due to the presence of non-protein nitrogen such as RNA, and chitin and chitosan in fungal cell wall.

The significant differences in the protein content observed for fungi cultivated on different winery and distillery by-products are most likely driven by several reasons. One of the reason could be the cultivation media differs in the carbon-to-nitrogen (C/N) ratios. The fungal biomass *N. intermedia* showed the highest protein content when cultivated on wine lees (C/N ratio: 8.43), followed by vinasse (C/N ratio: 15.38) and grape marc (C/N ratio: 21.36). It is well established that the carbon, nitrogen, and phosphorus content of filamentous fungi is highly influenced by the cultivation media (Zhang & Elser, 2017). Previous study using oat as cultivation medium showed a reduction in protein content of *Rhizopus oligosporus* from 39 % in glucose media, with C/N ratio of 7.54 to protein content 21 % in oat-based media with C/N ratio of 23.2 (Wang et al., 2024).

When cultivated on vinasse, *R. oryzae* exhibited significantly lower protein content compared to *N. intermedia*. This is likely due to the mixed fungal biomass entangled with the unfermented solid particle for fungus *R.oryzae*. Vinasse also contain large amounts of dark color pigments; melanoidin, hydroxymethylfurfural, and furfural (Pant & Adholeya, 2007). These products of the Maillard reaction and sugar caramelization are known to inhibit fungal growth (Karimi et al., 2005). In our experiment, the vinasse was required to be diluted ten-fold to accommodate fungal growth. It is likely that *A. oryzae* and *N. intermedia* have higher tolerance to these inhibitory compounds (Nair et al., 2018) and capable of utilizing the solid fraction of vinasse, thus achieving more fungal growth and higher protein content.

The three fungal species cultivated on winery and distillery by-products exhibited high lysine content, ranging from 8.43 % to 14.18 % of the total amino acids dw. The highest lysine content was achieved by *R. oryzae* when cultivated on wine lees. This high lysine level of filamentous fungi is attributed to the fungi ability to synthesize lysine via the α-aminoadipate pathway (Xu et al., 2006). Histidine levels in fungi cultivated on grape marc (1.95–2.27 %) were significantly lower than those in fungi grown on synthetic media (2.67–3.62 %) and wine lees (2.50–3.23 %). In contrast, fungi grown on grape marc (9.05–9.95 %) was together with fungi grown on wine lees (8.92–9.29 %) significantly higher in leucine compared to fungi cultivated on synthetic media (7.71–8.29 %) and vinasse (7.62–7.88 %).

Based on the recommended daily protein intake of 0.66 g per kilogram of body weight, the essential amino acid requirements for adult humans, as recommended by FAO/WHO (FAO, 2007), are: histidine (15 mg/g protein), isoleucine (30 mg/g), leucine (59 mg/g), lysine (45 mg/g), methionine (16 mg/g), phenylalanine and tyrosine (38 mg/g), threonine (23 mg/g), tryptophan (6 mg/g), and valine (39 mg/g). The filamentous fungi cultivated on winery and distillery by-products had protein contents ranging from 9 % to 30 % dw, which is lower than that of conventional animal-based protein sources such as beef, chicken, and fish at about 50–90 % dw (Wang et al., 2023). However, the amino acid profiles of the fungal biomass fulfill FAO/WHO essential amino acid requirements, except for methionine, which only met or surpassed the requirement when the fungi were cultivated on wine lees and grape marc. Among the essential amino acids, lysine and leucine are particularly important from a nutritional standpoint. Lysine is the limiting amino acid in most cereal grains, and is a key amino acid in protein synthesis, collagen formation, and nutrient absorption, while leucine plays a key role in stimulating muscle protein synthesis (Young & Pellett, 1990). Given its well-balanced amino acid profile, the fungal biomass studied in this work could serve as a valuable alternative protein source, especially as a complement to plant-based diets.

3.2. Essential mineral content of fungal biomass

The essential mineral content of the fungal biomass of the three edibles filamentous fungi species cultivated on three different winery and distillery by-products were quantified and are presented in Table 2. There were no significant differences between the three fungal species cultivated in synthetic glucose media with respect to phosphate (P) content; 984–1148 mg/100 g dw. However, cultivation using wine lees significantly increased the phosphorus content in NI and RO, up to 2514 mg/100 g dw. When grape marc was used, RO had significantly higher phosphorus content compared to NI and AO, while cultivation using vinasse, NI exhibited the highest phosphorus content.

The adequate intake in adults of phosphorus is 520 mg/day and the tolerable upper limit intake per day is 3000 mg (Blomhoff et al., 2023). Fungi cultivated on wine lees and vinasse, as well as RO grown on grape marc, had significantly higher phosphorus levels, up to 2514 mg/100g dw. Excess phosphorus intake can negatively impact bone and kidney health (Blomhoff et al., 2023). Therefore, additional treatments, such as heat treatment (Vrdoljak et al., 2015) should be considered to reduce phosphorus content in harvested fungal biomass.

Iron (Fe) content of fungal biomass cultivated in synthetic media ranged from 3.3 to 5.2 mg/100 g dw. Cultivation in wine lees significantly increased iron concentrations across all fungal species, particularly in RO (117.3 mg/100 g dw) compared to NI (57.7 mg/100 g dw) and AO (45.7 mg/100 g dw). Cultivation in grape marc also resulted in higher iron contents, with RO again having the highest content (85.9 mg/100 g dw), followed by NI and AO. Fungal biomass cultivated on vinasse showed intermediate iron content, lower than those cultivated

Table 2
Content of essential minerals of filamentous fungi *N.intermedia* (NI), *A.oryzae*(AO), and *R.oryzae* (RO) cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN).

Substrate	Fungi species	Mineral content of fungal biomass (mg/100 g)					
		P	Ca	Mg	Fe	Zn	Cu
SYN	NI	984.8 ± 28.8^{f}	41.5 ± 1.25^{f}	72.3 ± 1.44^{f}	4.8 ± 0.12^{e}	10 ± 0.36^{de}	1.4 ± 0.21^{ef}
	AO	990.6 ± 6.05^{f}	6.5 ± 0.43^{f}	104.1 ± 0.57^{de}	3.3 ± 0.03^{e}	8 ± 0.03^{ef}	0.3 ± 0^{f}
	RO	1148.7 ± 69.7^{ef}	43.1 ± 0.36^{ef}	72.1 ± 3.3^{ef}	5.2 ± 0.26^{e}	11.2 ± 0.43^{de}	0.5 ± 0.01^{f}
WL	NI	2513.5 ± 75.2^{a}	215.5 ± 7.75^{cd}	224.9 ± 8.87^{a}	57.7 ± 2.43^{c}	46.8 ± 1.1^{b}	7.4 ± 0.14^{c}
	AO	1558.3 ± 91.6^{cde}	98.9 ± 3.23^{ef}	124.6 ± 5.78^{cd}	45.7 ± 2.32^{c}	9.8 ± 0.78^{def}	10.3 ± 0.27^{ab}
	RO	2333.5 ± 63.3^{a}	296.3 ± 1.96^{bc}	240.8 ± 3.64^{a}	117.3 ± 1.36^{a}	55.9 ± 0.62^{a}	11.6 ± 0.17^{a}
GM	NI	1189.4 ± 245.3^{ef}	213.3 ± 28.11^{cd}	116.3 ± 19.89^{d}	49.5 ± 2^{c}	18.3 ± 4.28^{c}	2.5 ± 0.09^{de}
	AO	1128.4 ± 47.5^{f}	148 ± 2.4^{de}	131.8 ± 3.64^{bcd}	37.6 ± 0.72^{cd}	3.7 ± 0.16^{f}	1.7 ± 0.1^{def}
	RO	1845.6 ± 256.3^{bc}	716.5 ± 99.8^{a}	157 ± 13.24^{b}	85.9 ± 21.18^{b}	19.8 ± 4.23^{c}	3.2 ± 1.32^{d}
VIN	NI	2246.1 ± 22.3^{ab}	383.6 ± 3.12^{b}	152.6 ± 2.56^{bc}	14.3 ± 0.3^{de}	21.8 ± 0.1^{c}	2.9 ± 0.12^{de}
	AO	1646.3 ± 17^{cd}	393.3 ± 1.88^{b}	155.9 ± 1.25^{bc}	17.5 ± 1.49^{de}	16.5 ± 0.1^{cd}	2.6 ± 0.02^{de}
	RO	1314.7 ± 253.19^{def}	272 ± 19.22^{bc}	163.8 ± 20.03^{b}	34.2 ± 7.39^{cd}	20.9 ± 2.62^{c}	8.5 ± 1.59^{bc}

Data are expressed as mean values ± standard deviation. For all media n = 3, except wine lees n = 5.
Different letters in the same columns denote statistically significant differences ($p < 0.05$) among samples.
SYN: synthetic glucose media, WL: pre-distillation wine lees, GM: grape marc, VIN: vinasse.
NI: *Neurospora intermedia,* AO: *Aspergillus oryzae,* RO: *Rhizopus oryzae*.

in wine lees and grape marc, yet higher than those in synthetic glucose media, with RO (34.2 mg/100 g dw) showing the highest iron content.

Based on the mineral content of the substrate, we estimated iron concentration in all the cultivation media at: 10 ppm for wine lees, 9.5 ppm for grape marc, 1.6 ppm for vinasse, and 1.1 ppm for glucose media (Table S3). The iron content of fungi thus appeared being dependent on the cultivation medium, with fungi cultivated on wine lees having the highest iron content while the lowest level was found for fungi cultivated on glucose media.

In fungi, iron serves as a critical cofactor in various essential enzymes. Among the fungal species examined, RO consistently exhibited the highest iron content, particularly when cultivated using wine lees and grape marc. The filamentous fungi from *Rhizopus* genus have unique biological capability to store excess iron in ferritin-like proteins (Canessa & Larrondo, 2013). Ferritins effectively sequester large amounts of iron, thereby mitigating potential toxicity, including damage from the Fenton reaction associated with low molecular weight iron ions. Further research is required involving direct measurements of ferritin protein abundance to validate these findings.

Regarding calcium (Ca) content, NI and RO cultivated in synthetic media contained 41.5 and 43.1 mg/100 g dw, respectively, whereas AO exhibited significantly lower calcium content at 6.5 mg/100 g dw. Cultivation in wine lees, grape marc, and vinasse markedly increased the calcium content in all fungal biomasses compared to synthetic media, with values ranging from 98 to 716 mg/100 g dw. Fungi biomass with the highest calcium content was observed in RO grown on grape marc. We estimated the calcium concentration of the cultivation media at 52, 342 and 18 ppm for wine lees, grape marc, and vinasse, respectively (Table S2). These results reflected higher calcium concentration in the media could yield fungal biomass with higher calcium content.

The zinc (Zn) content of three species of fungi biomass grown in synthetic glucose media ranged from 8.0 to 11.2 mg/100 g dw. When cultivated in wine lees, NI (46.8 mg/100 g dw) and RO (55.9 mg/100 g dw) showed much higher zinc levels, while AO remained low at 9.8 mg/100 g dw. A similar pattern was observed with grape marc: NI and RO had higher zinc contents (18.3 and 19.8 mg/100 g dw, respectively), whereas AO was again lower (3.7 mg/100 g dw). In contrast, cultivation in vinasse resulted in comparable zinc levels across all three fungal species, ranging from 16.5 to 21.8 mg/100 g dw.

The fungus *A. oryzae* consistently exhibited lower calcium and zinc accumulation compared to *N. intermedia* and *R. oryzae*. Under excess mineral conditions, fungi can maintain zinc and calcium homeostasis through two main mechanisms: efflux transport across the plasma membrane, which removes excess minerals, and intracellular storage in vacuoles (Lange & Peiter, 2020; Robinson et al., 2021). The lower zinc and calcium levels observed in *A. oryzae* suggest that this species may primarily regulate homeostasis through efflux transport. In contrast, *N. intermedia* and *R. oryzae* may rely more on vacuolar storage. These hypotheses require further investigation, including studies with defined media to examine the effects of zinc concentration on the growth and mineral accumulation of these species.

3.3. Total polyphenol content of fungal biomass

Filamentous fungi cultivated in synthetic glucose media exhibited moderate polyphenol levels (17.1–23.1 mg GAE/g biomass dw), while those grown on wine lees showed lower concentrations (7.18–12.36 mg GAE/g biomass dw), with no significant differences among fungal species (Fig. 1). In comparison, cultivation on grape marc led to significantly higher polyphenol content. RO showed the highest level (96.42 mg GAE/g biomass dw), while NI and AO displayed moderate values (26.38 and 28.92 mg GAE/g biomass dw, respectively). Fungal biomass cultivated on vinasse had relatively low polyphenol content (10.25–12.57 mg GAE/g dw), similar to wine lees, again without notable species-specific differences.

Compared to the Ascomycete fungi *N. intermedia* and *A. oryzae*, the

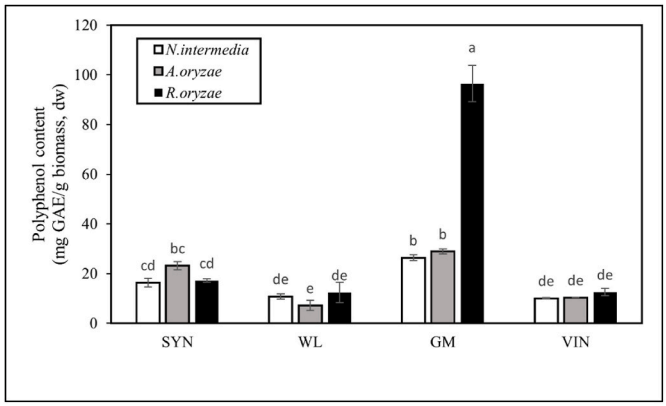


Fig. 1. Polyphenol content (mg GAE/d dw biomass) of filamentous fungi *N. intermedia*, *A.oryzae*, and *R.oryzae* cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN). Data are expressed as mean values ± standard deviation depicted as error bars. For all media n = 3,except wine lees, n = 5. Values that do not share the same subscripts denote statistically significant differences ($p < 0.05$) among samples.

Zygomycete fungi *R. oryzae* exhibited significantly greater polyphenol accumulation when cultivated in grape marc. This is could be due to differences in cell wall composition. *R. oryzae* contains chitosan in its cell wall (Svensson et al., 2021), which contains free amino groups from glucosamine that can interact with and form complexes with polyphenols (Popa et al., 2000; Sun et al., 2014; Yi et al., 2021). Another hypothesis is that filamentous fungi accumulate only specific polyphenols, and this accumulation depends on the fungal species. Additionally, wine lees, vinasse, and grape marc differ significantly in their polyphenol profiles (data is not shown). However, further studies are required to investigate the underlying mechanisms and to explain why *R. oryzae* behaves in this manner.

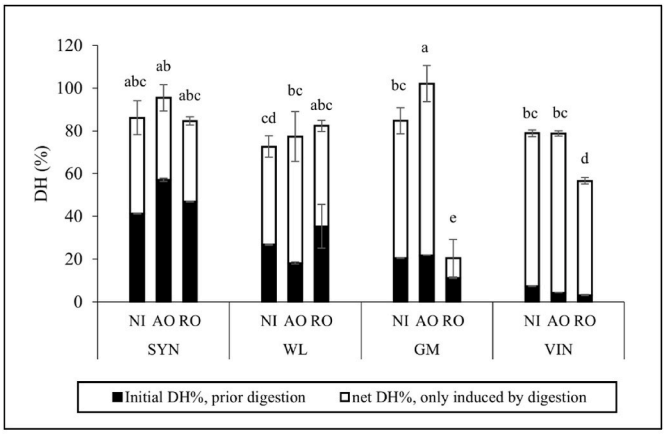
Previous studies support the capacity of filamentous fungi to grow in polyphenol-rich media, tolerating the inhibitory properties of polyphenol on certain microorganism (Wikandari et al., 2015). While *Aspergillus* and *Neurospora* biomass yields remain unaffected by high polyphenol concentrations (2.4–240 mg/L), *Rhizopus oligosporus*—another Zygomycete—has shown a three-fold increase in biomass yield when cultivated in media containing 240 mg/L ellagic acid compared to 2.4 mg/L (Bulkan, Sitaresmi, et al., 2022; Bulkan, Yudhanti, et al., 2022). This suggests that certain filamentous fungi not only tolerate, but also may benefit from polyphenol-rich substrates.

Polyphenols are known for their antioxidant properties, which can enhance the storage stability of products by preventing lipid oxidation. For example, a recent study showed that using lingonberry press cake in combination with herring rest raw materials during protein extraction improved the product's storage stability in a dose-dependent manner (Zhang et al., 2024). Since *Rhizopus oryzae* can accumulate polyphenols, this highlights its potential for use in valorizing industrial sidestreams that are rich in both polyphenols and polyunsaturated fatty acids, such as sidestreams from olive or rapeseed processing.

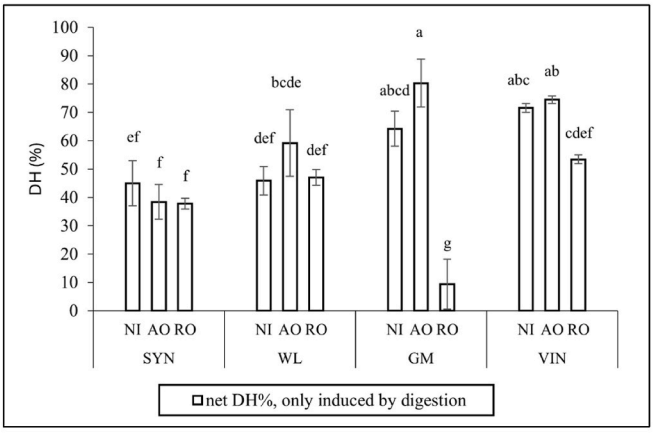
3.4. Protein degree of hydrolysis of fungal biomass following in vitro digestion

In the present study, protein digestibility was assessed after *in vitro* digestion by measuring the protein degree of hydrolysis. The degree of hydrolysis (DH%) indicates the extent of protein hydrolysis in a sample, determined by comparing the number of primary amines in the digested sample to the total amino acids. Fig. 2 depicts the total DH% before and after intestinal digestion of the three species of fungal biomass cultivated on synthetic glucose media and three different winery and distillery by-products (grape marc, wine lees and vinasse).

When grown on glucose media, all three fungal species *Rhizopus*



(a)



(b)

Fig. 2. Protein degree of hydrolysis (DH%) (a) total DH%, including initial DH %, and (b) net DH% of the filamentous fungi *N.intermedia* (NI), *A.oryzae* (AO), and *R.oryzae* (RO) cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN). "Initial DH" shows the DH % present in samples prior to the digestion. "net DH" shows the DH% obtained in the intestinal step, induced by pepsin and pancreatin after gastrointestinal digestion. Data are expressed as mean values ± standard deviation depicted as error bars. For all media n = 3,except wine lees, n = 5. Values that do not share the same subscripts denote statistically significant differences ($p < 0.05$) among samples.

oryzae, *Neurospora intermedia*, and *Aspergillus oryzae* exhibited similarly high total DH values at the end of the digestion, ranging from 84 % to 95 %, with no significant differences among species. Cultivation on wine lees resulted in a moderate but non-significant reduction in total DH%, with values between 72 % and 82 % across all fungal species. Using grape marc, a distinct pattern emerged: while *N. intermedia* and *A. oryzae* maintained high total DH% levels (86 % and 101 %, respectively), *R. oryzae* showed a dramatic decline to just 20.4 %, indicating a substantial inhibition of protein hydrolysis in this fungus species. On vinasse, total DH% values for *N. intermedia* and *A. oryzae* remained comparable at 78 %, whereas *R. oryzae* again exhibited a lower total DH at 56.6 % compared to wine lees.

As shown in Fig. 2a, there were high initial DH% in the different filamentous fungi prior to digestion, reflecting primary amines released into the aqueous phase before the addition of digestive enzymes. This may reflect the presence of non-protein nitrogen sources (e.g., RNA), shorter peptides, or free amino acids within the fungal cytoplasm.

The net protein digestibility (calculated as total DH% – initial DH%) resulting from gastrointestinal digestion ranged from 38 to 80 % for *N. intermedia* and *A. oryzae*. Pairwise comparisons showed that *N. intermedia* cultivated on grape marc had significantly higher digestibility than when cultivated on glucose medium but was not significantly different from *N. intermedia* cultivated on wine lees. In contrast, *A. oryzae* cultivated on grape marc exhibited significantly higher digestibility than when cultivated on either glucose medium and wine lees. For *R. oryzae,* vinasse resulted in highest net protein digestibility among four substrates, at about 53 %, while digestibility was still very low (<10 %) when using grape marc as cultivation media.

In comparison to conventional protein sources, the digestibility values observed for fungal biomass are within the range reported for animal-based proteins such as chicken (62 %), beef (60 %), and salmon (67 %) under similar *in vitro* conditions (Wang et al., 2023). Among alternative sources, algae protein typically shows a DH of around 30 % (Verspreet et al., 2021), while edible mushrooms reach approximately 40 % (X. Li, Yu, et al., 2023). Cereal-derived protein concentrates from oat, wheat, and barley exhibit DH values ranging from 50 to 60 % (Gong et al., 2022), and commercial plant-based burgers formulated from pea protein have reported DH values between 50 and 58 % (Cutroneo et al., 2023). These comparisons suggest that fungal proteins cultivated on winery sidestreams can achieve digestibility levels comparable to both traditional and alternative protein sources.

The notably low DH% observed in *R. oryzae* cultivated on grape marc supports our hypothesis that a high polyphenolic content in the fungal biomass may hinder the *in vitro* protein digestibility. Phenolic compounds can interfere with protein hydrolysis by interacting with both protein substrates and digestive proteases. Their binding to proteins—primarily through hydrophobic interactions—can reduce accessibility to proteases that preferentially cleave at hydrophobic residues (Cirkovic Velickovic & Stanic-Vucinic, 2018). While the specific interactions between grape-derived polyphenols and fungal proteins have not yet been investigated, the inhibitory effects of grape polyphenols on digestive enzymes are well documented. For example, procyanidins can inhibit trypsin activity depending on their degree of polymerization, and both resveratrol and anthocyanins have been shown to inhibit pepsin activity (Cirkovic Velickovic & Stanic-Vucinic, 2018). Identification of the specific polyphenols accumulating in the fungal biomass is subject for a future study.

The Ascomycete fungi, particularly *N. intermedia*, maintained relatively high protein content and protein digestibility across all winery and distillery by-products explored. This highlights its superiority as a nutritious alternative protein source among the three fungal species studied. In contrast, the high polyphenol content of *R. oryzae* comes with the challenge of lower protein digestibility when cultivated on grape marc. Therefore, further studies are needed to improve the protein digestibility of *R. oryzae*, especially when grown on grape marc.

3.5. Mineral accessibility of fungal biomass

Mineral accessibility describes the extent to which essential minerals in solid foods are released and solubilized after gastrointestinal digestion. In this study, the insoluble portion of the *in vitro* digested samples was separated from the soluble fraction using a 0.2 μm filter. The accessibility of iron (Fe) and zinc (Zn) was determined by measuring the percentage of each mineral released into the soluble fraction relative to the total content in the original solid sample (Figs. 3a and 4a).

Filamentous fungi grown on synthetic glucose media showed the highest mineral accessibility overall. Iron accessibility reached 100 % for NI and AO, while RO showed a lower value of around 52 %. When wine lees was used as the cultivation media, Fe accessibility decreased across all fungal species, ranging from 24 to 34 %. The most dramatic reduction in Fe accessibility was observed in fungi grown on grape marc, where values dropped below 9 % for NI and AO, and became negligible for RO. A similar pattern emerged with vinasse, where NI and AO retained moderate Fe accessibility (23–33 %), but RO again exhibited near-zero levels.

Zinc accessibility followed a similar trend. Three species of fungi

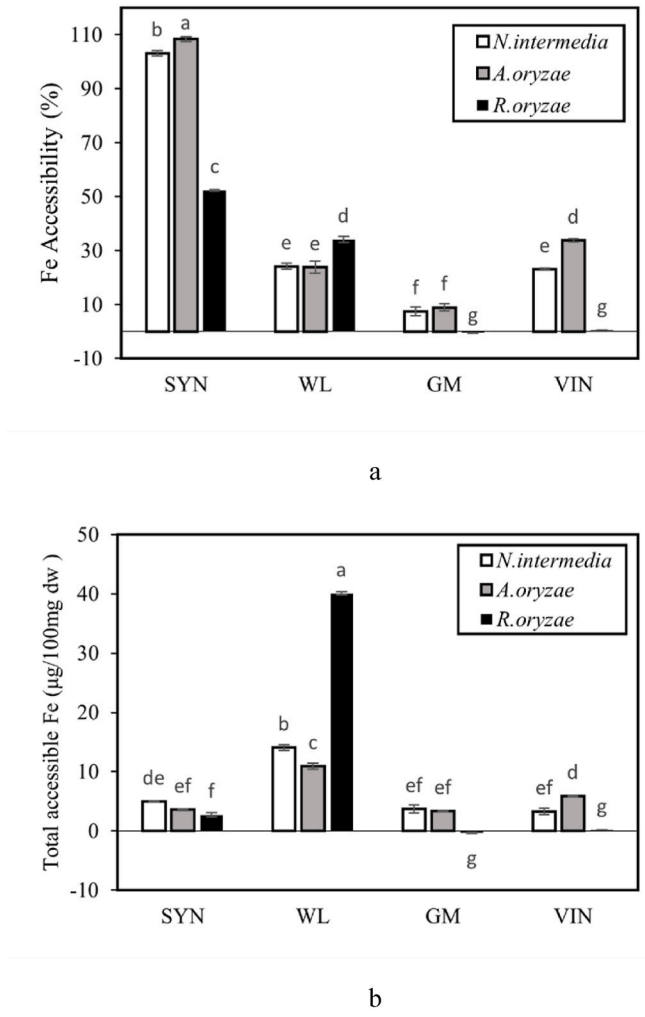


Fig. 3. (a) Iron accessibility (%), and (b) total accessible iron (µg per 100 mg dw) for the different filamentous fungi *N.intermedia* (NI), *A.oryzae* (AO), and *R. oryzae* (RO) cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN). Data are expressed as mean values ± standard deviation depicted as error bars. For all media n = 3,except wine lees, n = 5. Values that do not share the same subscripts denote statistically significant differences ($p < 0.05$) among samples.

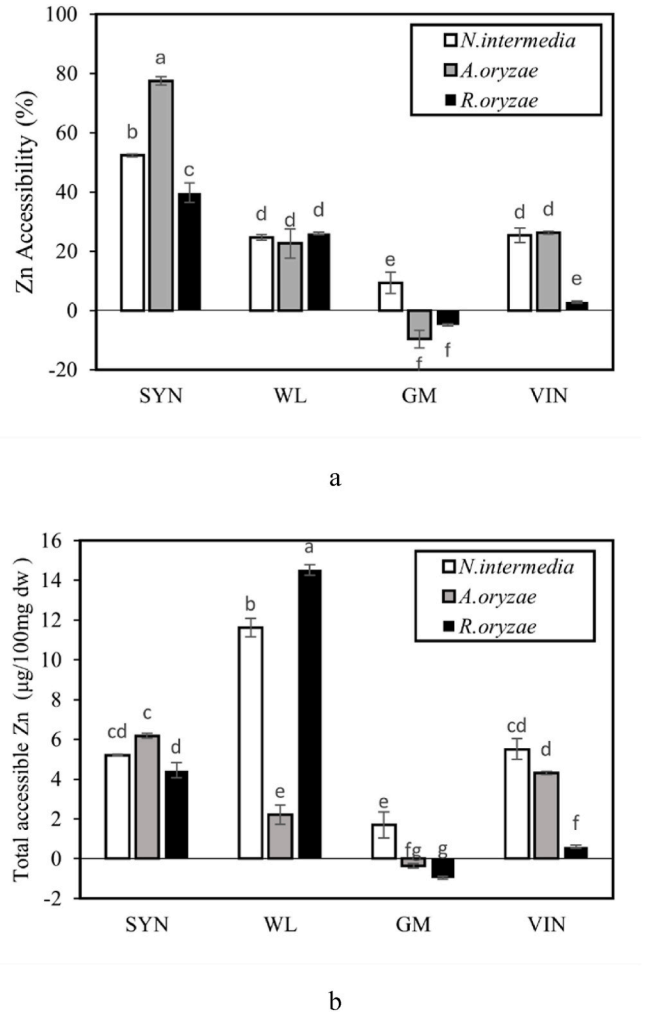


Fig. 4. (a) Zinc accessibility (%), and (b) total accessible zinc (µg per 100 mg dw) for the different filamentous fungi *N.intermedia* (NI), *A.oryzae* (AO), and *R. oryzae* (RO) cultivated on synthetic glucose media (SYN), pre-distillation wine lees (WL), grape marc (GM), and vinasse (VIN). Data are expressed as mean values ± standard deviation depicted as error bars. For all media n = 3,except wine lees, n = 5. Values that do not share the same subscripts denote statistically significant differences ($p < 0.05$) among samples.

cultivated on wine lees resulted in Zn accessibility around 22–26 %, a moderate reduction compared to synthetic media, ranging from 39 % to 77 %. Grape marc cultivation led to the lowest values, with NI under 10 %, while AO and RO even showed negative value, indicating anti-nutrient properties for zinc. In vinasse-grown fungal biomass, NI and AO maintained moderate Zn accessibility (25–27 %); however, RO remained low (approximately 2.85 %).

It is important to note that presenting mineral accessibility in relative terms (i.e., as a percentage of total mineral content in the biomass), can be misleading when comparing biomass with very different mineral content. The total accessible minerals after *in vitro* digestion of fungi are presented in Figs. 3b and 4b. Although fungi grown on synthetic media showed the highest relative iron accessibility, the absolute amount of iron released into the soluble phase from a 100 mg fungal sample was significantly lower, ranging from 2.7 to 4.9 µg Fe. In contrast, fungi cultivated on wine lees, released significantly higher total amount of iron (10–40 µg Fe). Despite of the significantly lower accessibility percentage value compared to synthetic media, both Ascomycetes fungi, NI and AO, cultivated on grape marc and vinasse revealed similar total iron release (3.3–3.6 and 3.3–5.9 µg Fe, respectively). However, RO showed negligible values when cultivated on grape marc and vinasse. The same pattern was also exhibited for zinc except for AO, which gave relatively low amounts of accessible zinc when cultivated both on wine lees and grape marc.

These results support our hypothesis that the accessibility of essential minerals from filamentous fungi could be affected by both the fungal species and the type of winery and distillery by-product; particularly the potential of the fungi to accumulate antinutrients from the substrate. Thus, that fungal biomass cultivated on synthetic glucose media exhibited the highest relative iron and zinc accessibility may be attributed both to the lower total mineral content in these samples and the absence of antinutrients. In contrast, filamentous fungi cultivated on winery and distillery by-products, such as wine lees and grape marc, accumulated substantially higher mineral levels, reflecting the levels present in these by-products and likely the fungi ability to store excess iron and zinc intracellularly (Robinson et al., 2021). We hypothesized that minerals stored in reserve forms are less bioaccessible than those incorporated into functional roles such as enzyme cofactors, however further research is needed to confirm this hypothesis.

The substantially reduced mineral accessibility observed in *R. oryzae* cultivated on grape marc supports the hypothesis that accumulated polyphenols in the fungal biomass negatively affect mineral solubility.

Phenolic compounds are well known to impair iron bioavailability by forming insoluble iron(III)-phenol complexes in the gastrointestinal tract (Saini et al., 2016). Additionally, phenolic compounds have been shown to interact with zinc, with the strength of binding varying depending on the specific phenolic structure (Clergeaud et al., 2016; Duan et al., 2023).

An interesting trend was observed with fungi cultivated on vinasse. While NI and AO maintained moderate levels of iron and zinc accessibility, RO showed a marked reduction. One plausible explanation is the mentioned presence of melanoidins—complex Maillard reaction products known to bind iron and potentially reduce its bioavailability (Pant & Adholeya, 2007). The lack of inhibitory effect in NI and AO is unclear, however, it might be due to that NI and AO accumulated less of the melanoidins compared to RO.

In this study, mineral accessibility was assessed by measuring the proportion of soluble minerals in the intestinal phase derived from *in vitro* digestion. Further work is required to evaluate the bioavailability of minerals from filamentous fungi grown on winery and distillery by-products that include the intestinal absorption, either through *in vitro* studies using Caco-2 intestinal cell models or ultimately in *in vivo* trials.

4. Conclusion

In this study, the content of protein, minerals and polyphenols was evaluated along with essential amino acid profile, protein digestibility and mineral accessibility of three edible filamentous fungi species, *Neurospora intermedia*, *Aspergillus oryzae*, and *Rhizopus oryzae*, grown on three winery and distillery by-products: grape marc, wine lees, and vinasse. It was demonstrated that the nutritional quality of edible filamentous fungi was strongly affected by the cultivation substrate and fungal species, reflecting differences in nutrient composition and uptake capacity. Fungi grown on wine lees exhibited higher protein content compared to those cultivated on grape marc and vinasse. The mineral content was strongly affected by the species and cultivation medium. Notably, *R. oryzae* accumulated more iron than the other species, and wine lees as a substrate gave the highest iron levels. Among the key factors, polyphenols emerged as particularly influential due to their potential to interact with proteins and minerals, thereby hampering protein digestibility and mineral accessibility. The highest polyphenol content was observed for *R. oryzae* cultivated on the grape marc. The fungi *N. intermedia* and *A. oryzae* displayed consistently high protein digestibility across all media, whereas *R. oryzae* had reduced digestibility when grown on grape marc, most likely due to accumulated polyphenols from the cultivation media. Moreover, all fungi cultivated in grape marc exhibited a notable reduction in Fe and Zn accessibility. These findings underscore the potential to optimize the nutritional value of fungal biomass through substrate selection, demonstrating that winery and distillery by-products are viable media for cultivating fungi as functional mycoprotein ingredients.

CRediT authorship contribution statement

Luziana Hoxha: Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ricky Wang:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Mohammad J. Taherzadeh:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ingrid Undeland:** Writing – review & editing, Conceptualization.

Ethical approval

Not applicable.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107711.

Data availability

Data will be made available on request.

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