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NFS Journal 36 (2024) 100189

Contents lists available at ScienceDirect

NFS Journal

journal homepage: www.elsevier.com/locate/nfs

Original article

In vitro protein digestibility and mineral accessibility of edible filamentous Fungi cultivated in oat flour

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ARTICLE INFO

Keywords:
Oat
Filamentous fungi
Mineral accessibility
Amino acids
In vitro digestion

ABSTRACT

Edible filamentous fungi, a source of mycoprotein, are one of the sustainable alternative protein. This study compares protein digestibility (DH%) and amino acid and mineral accessibility in *Rhizopus oligosporus* cultivated in oat flour (OatRO) or glucose media (GluRO) by using the INFOGEST *in vitro* digestion protocol. Fungal total amino acids was higher in GluRO (39.0 ± 1.1 % dw) than OatRO (21.8 ± 1.3 % dw) which was also the case for calcium and magnesium content. After completed gastrointestinal digestion, there were no significant differences between GluRO and OatRO regarding DH% (27.21 ± 10.4 % and 29.4 ± 0.5 %), however, GluRO provided significantly higher amino acid accessibility compared to OatRO (64.3 ± 1.6 % and 55.1 ± 3.1 %). Mineral accessibility of GluRO was for Ca: 37.9 ± 1.8 %, Zn: 9.3 ± 0.4 %, Fe: 38.2 ± 1.9 %, Mg: 66.5 ± 1.4 % and Cu: 24.7 ± 1.3 % and for OatRO; Ca: −40.2 ± 2.4 %, Zn: −4.13 ± 0.15 %, Fe:14.6 ± 1.6 %, Mg: 74.5 ± 3.1 %, and Cu: 55.95 ± 0.8 %. Despite the low phytic acid content, OatRO thus showed antinutrient properties with respect to calcium, and zinc, suggesting that oat-derived fungi had antinutrients other than phytic acid. This study hereby revealed that the cultivation substrate affect amino acid and mineral accessibility of filamentous fungi and calls for deeper evaluations of antinutrients in oat-derived fungi.

1. Introduction

Edible filamentous fungi are emerging as a promising sustainable protein source, addressing the challenge of balancing two Sustainable Development Goals: achieving food security and improved nutrition (SDG 2), while urgently tackling climate change (SDG 13). Beyond its high nutritional content such as essential amino acids and dietary fiber beta-glucan [1], its meat-like texture, attributable to a filamentous structure, broadens its culinary appeal [2]. One of the edible filamentous fungi-based food in the market is Quorn®, derived from *Fusarium venenatum*. Several other species of edible filamentous fungi are *Aspergillus oryzae, Neurospora intermedia, Rhizopus oligosporus, Rhizopus oryzae,* and *Rhizopus delemar*. All these edible filamentous fungi have been successfully cultivated on food industry side-streams, thus, are a promising way towards circularity in the global food system [3,4,5].

For the application as food for human consumption, the fungi cultivation substrate not only should provide sufficient nutrients for good fungal growth, but also meet the safety requirements for human consumption. Oat flour is a promising filamentous fungi cultivation substrate for human food application due to its high nutritional value. Previous research have shown that filamentous fungi are capable to grow by submerged fermentation using only oat flour as substrate without any additional nutrient supplementation [6]. Oat flour contains significant amounts of essential minerals. However, oat flour contains phytate which could potentially carry over into the fungi as food, affecting the mineral bioavailability of fungi based food. Nevertheless, certain fungi species, such as *Rhizopus oligosporus*, are known to produce phytase, an enzyme that hydrolyses phytic acid [7].

Evaluation of protein quality and mineral digestibility is critical for how promising an alternative protein product is. The evaluation of nutrient digestibility can be approached *via* either *in vivo* or *in vitro* methods. While *in vivo* methods offer a more accurate representation of food consumption, they tend to be costly and time-consuming [8]. To circumvent these constraints, *in vitro* digestion models have been proposed. Since 2014, an international consortium has gradually developed a standardized *in vitro* procedure to mimic gastrointestinal (GI)

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https://doi.org/10.1016/j.nfs.2024.100189
Received 5 March 2024; Received in revised form 7 August 2024; Accepted 22 August 2024
Available online 25 August 2024

digestion, known as the INFOGEST digestion protocol [9,10]. Using this *in vitro* method, the protein digestibility of filamentous fungi has been previously evaluated for the mycoprotein Quorn® [11,12], and for four other different strains of edible filamentous fungi in comparison with chicken breast, salmon, and beef [13]. However, there have been very few studies on mineral accessibility of mycoprotein following *in vitro* digestion; in this case Quorn® [14].

As an emerging sustainable food for human consumption, it is critical to assess both the protein digestibility and mineral accessibility of edible filamentous fungi. To the author's knowledge, no study has assessed these features of edible filamentous fungi after cultivation on oat flour as the substrate. Since oat flour is made up of complex macromolecules with different nutrient composition compared to the defined glucose media, the protein quality and digestibility is expected to be affected when using this substrate. Due to the capability of the filamentous fungi to hydrolyze phytates, it is also hypothesized that filamentous fungi have a high mineral accessibility, despite being cultivated in oat flour, which contain high amount of phytates. Therefore, this study aims to investigate the protein digestibility as well as the accessibility of amino acids and minerals of *R. oligosporus* cultivated in oat flour or control (glucose) media. This is intended to enhance our understanding of the nutritional quality of edible filamentous fungi and thus, their potential as a sustainable food source.

2. Material and methods

2.1. Materials

Whole grain oat flour from AXA (Lantmännen Cerealia, Sweden) was used as the substrate for fungi cultivation. 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 pancreas (P6887) and human salivary amylase (A1031) was supplied from Sigma Aldrich, Sweden, while pancreatin from porcine pancreas (8× USP) was purchased from Fischer Scientific.

2.2. Cultivation of edible filamentous fungi

Edible filamentous fungal strain *R. microsporus* var. *oligosporus* CBS 112586 was used. The fungi were cultivated on either oat flour or glucose media using a 26-l bubble column bioreactor (Bioengineering, Switzerland). The composition of the defined glucose media was according to Rousta et al. [15]: 15 g/L glucose, 7.5 g/l $NH_4(SO_4)_2$, 3.5 g/l KH_2PO_4, 1 g/l $CaCl_2$, 0.8 g/l $MgSO_4.7H_2O$, 1 ml/l vitamin solution, and 10 ml/l trace element solution according to Sues et al. [16]. The oat media consisted of only 30 g/l oat flour and water [6]. Fungi inoculation was carried out by adding 1 l of inoculum previously grown in a shake flask for 24 h with the same medium as the final growth medium. The cultivation using 26-l bubble column bioreactor was conducted at 35 °C and 1 vvm aeration for 24 h. The fungi were then harvested using a sieve, washed with running tap water, and pressed to obtain wet fungi biomass. The wet fungi biomass was stored in the freezer at −20 °C until further analysis.

2.3. Static in vitro gastrointestinal digestion

The frozen fungal biomass was first thawed and homogenized with a hand blender (Bosch MS64M6170, Germany) to simulate oral mastication. The homogenized samples were measured for its moisture content using a moisture analyzer (Kern DBS 60–3, Germany). The *in vitro* gastrointestinal digestion protocol was conducted based on the INFOGEST 2.0 digestion protocol [9], with the electrolyte composition of simulated digestive fluids, pH of each digestive stage, and measurement of enzyme activity being based on the suggested protocol. Minor modification of the INFOGEST 2.0 protocol, as previously described in Wang et al. [13], were the reductions of salivary amylase and pancreatin enzymes.

In brief, the digestion was conducted using an amount of wet fungal biomass equal to 100 mg dry matter. This took into account the moisture content of the fungal biomasses at 74.66 ± 0.29 % and 76.34 ± 0.17 %, and oat flour at 10.54 ± 0.06 %. Before initiating the oral digestion, water was added to each fungal and oat flour sample, bringing the total volume to 1 ml. This approach ensured a consistent and comparable starting volume for all samples. In blank digestions, the sample was replaced by 1 ml of water. Oral digestion was simulated by the addition of 1 ml simulated salivary fluid containing salivary amylase (7.5 U/ml digest) and incubated for 1 h; followed by gastric digestion by the addition of 2 mL simulated gastric fluid containing pepsin 2000 U/ml at pH 3 and incubated for 2 h; followed by intestinal digestion by the addition of 4 ml simulated intestinal fluid containing pancreatin 10 U/ml and bile 1 mM at pH 7 and incubated for 2 h. The intestinal phase was then terminated by adding 800 µl of Bowman-Birk inhibitor (0.05 g/l). All the incubations were done at 37 °C with gentle mixing at 5 rpm (Stuart Rotator SB3, UK). The digests were then frozen stored at −80 °C until anaysis. All samples were digested with three replicates.

2.4. Degree of hydrolysis

The quantification of the Degree of protein Hydrolysis (DH%) for the sample prior to *in vitro* digestion, along with the gastric and intestinal digests, was accomplished utilizing the o-phthalaldehyde reagent (OPA) to measure the primary amines. The OPA reagent was prepared freshly by mixing 10 ml of OPA stock solution (0.05 M in ethanol), 10 ml of dithiothreitol (0.057 M in water), 5 ml SDS 20 %, and 75 ml 100 mM borate buffer [17]. All samples were centrifuged at 2000 rpm for 5 min. The supernatant was diluted and then added (120 µl) to the OPA reagent (1000 µl), incubated at room temperature for 10 min, and measured at 335 nm using a spectrophotometer. Sample absorbance readings were compared with the standard curve using L-serine as the standard.

The degree of hydrolysis is calculated by equation:

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

where h is the measured value of total primary amines (mmole 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.

2.5. Amino acid accessibility analysis

Amino acids content of undigested samples and intestinal digests were analyzed based on Trigo et al. [18]. Oat flour and the frozen fungi samples were freeze-dried and milled using a ball mill (Retsch MM400). All the solid samples were subjected to amino acid analysis according to the following: 8 ml of 6 M HCl was added to approximately 50 mg of samples followed by a complete seal with caps and hydrolysis at 110 °C for 24 h using a heat block. The whole content of the tube was transferred into a volumetric flask and topped up to 10 ml using water. The hydrolysate was then diluted twenty times with 0.2 M acetic acid and filtered using a 0.2 µm syringe filter prior to analysis with LC/MS.

Amino acid concentration in the intestinal digests after *in vitro* gastrointestinal digestion was measured before and after filtering the digests with 0.2 µm syringe filter. Four mL of HCL 12 M was added to 4 ml of the filtered intestinal digests, followed by the same aforementioned acid hydrolysis procedure and dilution.

Two microliters of all samples were run in an LC/MS system (Agilent 1100 HPLC and 6120B Single Quadrupole MS) as described by Trigo et al. [18]. The chromatogram was analyzed using the MassHunter Quantitive Analysis software (version B.09.00, Agilent Technologies).

Due to the acid hydrolysis, the method was not suitable to quantify tryptophan. Further, the amino acids methionine and cysteine are oxidized and thus not quantified. Asparagine and glutamine were co-determined with aspartic acid and glutamic acid, respectively.

Amino acid accessibility was calculated using:

$$\text{Amino acid accessibility (\%)} = \frac{\text{Total amino acids of filtered digest} - \text{Digestion blank total amino acid}}{\text{Initial total amino acid of sample}} \text{x } 100$$

2.6. Mineral accessibility analysis

All the freeze-dried and milled food samples were also subjected to mineral analysis according to [19]. All glasswares were acid washed with 30 % HNO_3 prior usage. Into 100 mg of samples, 2 mL of HNO_3 65 % was added and then heated at 110 °C for 2 h. The digestion tube was then cooled down to room temperature followed by the addition of 1 ml HNO_3 65 % and 1 ml of H_2O_2 30 % and continued to be heated up for another 2 h at 110 °C. The whole content of the digestion tube was then brought up to 10 mL in a volumetric flask with ultrapure water, followed by filtration with 0.2 μm syringe filter. The filtrate was then analyzed for the mineral concentration using Microwave Plasma Atomic Emission Spectroscopy (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA). The standard solution was prepared in 2 % HNO3.

Mineral accessibility after *in vitro* gastrointestinal digestion was measured by filtering the intestinal digests with 0.2 μm syringe filter. The filtrate was directly analyzed for the mineral concentration (Ca, Mg, Fe, Zn) using MP-AES as described above. The standard solution was prepared in water. The elements Ca, Mg, Fe, Zn, were measured at wavelength 643 nm, 518 nm, 371 nm, and 213 nm, respectively [20].

Mineral accessibility was calculated using equation.

$$\text{Mineral accessibility (\%)} = \frac{\text{Total mineral content in filtered digest} - \text{Digestion blank total mineral content}}{\text{Initial total mineral content of sample}} \text{x } 100$$

2.7. Phytic acid analysis

Phytic acid analysis was conducted using two methods. The first involved enzymatic dephosphorylation using a commercial kit (Megazyme, Ireland). In brief, about 1 g of the sample was dissolved in 0.66 M HCl and allowed to sit for at least 3 h. The extract was then centrifuged, and the supernatant neutralized with NaOH. Enzymatic dephosphorylation was performed in two ways: free phosphorus (without phytase) and total phosphorus (with phytase). Both were incubated in a 200 mM sodium acetate, pH 5.5 buffer at 40 °C for 10 min, followed by treatment with an assay buffer (400 mM glycine, 4 mM magnesium chloride, and 0.4 mM zinc sulfate, pH 10.4) and alkaline phosphatase at 40 °C for 15 min [21]. The reactions were stopped with 50 % trichloroacetic acid (TCA), centrifuged, and the supernatant was assessed for phosphorus concentration using a colorimetric method. The color reagent consisted of ascorbic acid (10 % *w/v* in 1 M sulfuric acid) and ammonium molybdate (5 % w/v in water). The supernatant was mixed with the reagent, incubated at 40 °C for 1 h, and then spectrophotometrically analyzed at 655 nm.

The second method involved analyzing phytic acid as inositol hexaphosphate (InsP6) by high-performance ion chromatography (HPIC), as described earlier [22].

2.8. Data analysis

Each sample type was subjected to 3 replicate digestions, and each digesta plus starting raw material was subjected to single replicate of analysis. Mean values from three digestion replicates were used in the statistical analysis to test whether there were significant differences between sample types. For this purpose, a one-way ANOVA followed by Tukey's *post hoc* test for pairwise comparison was used. Different groups of superscripts are used in tables to indicate the statistically significant pairwise comparisons at a threshold p-value <0.05. Statistical tests were conducted using Minitab 21. Error bars in figures represent one standard deviation.

3. Result and discussion

The aim of this study was to evaluate *in vitro* protein digestibility as well as amino acid and mineral accessibility of edible filamentous fungi *R.oligosporus* cultivated by submerged fermentation using oat flour (OatRO), or defined glucose media (GluRO) as control sample. Changes in the amino acid profile, essential mineral content, and phytic acid content were also investigated. The *in vitro* gastrointestinal digestions were carried out using the INFOGEST 2.0 protocol. The initial hypothesis was that filamentous fungi would have different protein quality and digestibility when it is cultivated in oat compared to glucose media since oat flour is made up of complex macromolecules with different nutrient composition compared to glucose media. Filamentous fungi cultivated in oat flour was also expected to have a low phytic acid content and therefore high mineral accessibility compared to the oat flour *per se*.

3.1. Protein, and mineral content of oat and fungi

The amino acids and essential mineral content of edible filamentous fungi *R.oligosporus* cultivated in oat flour were quantified. The total amino acids and amino acid profile of oat flour and filamentous fungi are presented in Table 1. Oat flour had a total amino acid content of 11.9 % on dry weight (dw) basis with the essential amino acid lysine forming 4.4 % of the total amino acids. These data are in line with the previous result analyzed for oat by Rousta et al. [6]. Protein from oat and other cereals are known for the low essential amino acid lysine [23]. The fungi *R. oligosporus,* when cultivated in glucose media (GluRO), exhibited a total amino acid content of 39 % dw. Lysine and leucine made up 8.4 % and 9.0 % of the total amino acids in GluRO, respectively, followed by 6.8 % isoleucine, 4.3 % phenylalanine, 7.9 % valine, 1.5 % histidine, and 5.3 % threonine. The total amino acid content and amino acid profile align well with our prior study that employed semi-synthetic glucose media for the cultivation of *R.oligosporus* [13]. The high lysine and leucine content of filamentous fungi has also been confirmed in other studies [4,24,6,5].

Table 1
Total and essential amino acids, minerals, and phytic acid content of oat flour and filamentous fungi cultivated in oat (OatRO) or glucose media (GluRO). Data are expressed as average values±standard deviation ($n = 3$).

	Oat flour	OatRO	GluRO
Total amino acid (% dw)	11.9 ± 0.62^{c}	21.8 ± 1.32^{b}	39.07 ± 1.12^{a}
Essential amino acids (% total AA)	38.83 ± 0.29^{c}	46.5 ± 0.32^{a}	43.45 ± 0.53^{b}
Lysine (% total AA)	4.5 ± 0.06^{b}	8.34 ± 0.23^{a}	8.4 ± 0.33^{a}
Leucine (% total AA)	8.77 ± 0.16^{b}	9.28 ± 0.1^{a}	9.09 ± 0.1^{a}
Isoleucine (% total AA)	5.42 ± 0.05^{b}	6.92 ± 0.05^{a}	6.83 ± 0.13^{a}
Phenylalanine (% total AA)	5.4 ± 0.18^{a}	4.92 ± 0.09^{b}	4.33 ± 0.14^{c}
Valine (% total AA)	6.71 ± 0.22^{b}	7.67 ± 0.25^{a}	7.89 ± 0.17^{a}
Histidine (% total AA)	2.1 ± 0^{a}	2.09 ± 0.17^{a}	1.57 ± 0.37^{a}
Threonine(% total AA)	4.33 ± 0.05^{c}	5.76 ± 0.15^{a}	5.36 ± 0.21^{b}
Mineral content (mg/100 g dw)			
Ca	52.3 ± 2.58^{c}	369.9 ± 9.67^{b}	612.5 ± 10.2^{a}
Mg	114.5 ± 4.38^{c}	179.2 ± 4.55^{b}	228.2 ± 19.2^{a}
Fe	4.47 ± 0.51^{b}	9.58 ± 0.27^{a}	2.95 ± 0.74^{c}
Zn	3.86 ± 0.7^{b}	16.11 ± 0.43^{a}	19.76 ± 3.42^{a}
Cu	0.57 ± 0.02^{b}	2.24 ± 0.13^{a}	2.46 ± 0.15^{a}

*Different letters in the same column denote statistically significant differences ($p < 0.05$) among samples for the same parameter.

Contrastingly, when oat flour was utilized as the fermentation substrate (OatRO), the total amino acid content of the fungi was significantly reduced from 39 % to about 21.8 %. This decrease might be attributed to the differences in the carbon-to-nitrogen (C/N) ratio between oat flour ang the glucose media. Previous research has demonstrated that the C/N ratio in cultivation media significantly influences the protein content of microbial protein; a higher C/N ratio correlates with lower protein content [25]. Additionally, a meta-analysis has shown that fungi carbon, nitrogen, and phosphorus stoichiometry is highly affected by abiotic nutrient composition [26]. In this experiment, the glucose synthetic media has C/N ratio of 7.54. On the other hand, oat flour - given the carbohydrate, protein, and fat content of 70 %, 11 %, and 5 % respectively [27], the C/N ratio is estimated at 23.2 [28].

Another potential explanation for the observed decline is the retention of oat flour particles in the fungal mycelium, even after meticulous washing post-harvest from the bioreactor. A previous study involving the cultivation of *Aspergillus oryzae* in oat media also noted an insignificant decline in total amino acid content compared to the fungus biomass grown in glucose media from 25.4 % to 23.5 % [6].

The protein quality of the oat flour, particularly in terms of essential amino acids, was significantly enhanced by fungi cultivation. For instance, the share of lysine increased from 4.5 % to 8.3 %, and the overall essential amino acid content increases from 38.8 % to 46.5 % of total amino acids. Lysine is of particular nutritional significance, as it is often the limiting amino acid in cereals [29]. In fact, the World Health Organization and Food and Agriculture Organization recommendation for lysine content of food is higher than 45 mg per g protein [30]. Both yeast and fungi are known to produce a high content of lysine through the α-Aminoadipate metabolism pathway, a mechanism that differs from the metabolism used by plants and bacteria [31]. Given these benefits, fungi have been proposed as an alternative protein source. Other essential amino acids (Table 1) surpasses the WHO/FAO recommendation value for histidine, isoleucine, leucine, phenylalanine, and threonine at 15, 30, 59, 38, and 23 mg/g protein, respectively [30]. However, the content of methionine, cysteine, and tryptophan must be further analyzed in order to perform a profound assessment on fungal potential as a complete amino acid source.

The essential mineral content of oat flour and the two edible fungi is presented in Table 2. Oat flour used in this study contained 52.3 mg Ca, 114 mg Mg, 4.4 mg Fe, 3.8 mg Zn, and 0.57 mg Cu per 100 g dry weight. These values agree with our previous study [6] and the values given by Swedish Nutritional Database for oat flour (Swedish National Food Agency, 2023). Oat is known to be particularly rich in minerals, however, it also contains a high amount of phytate which could hinder the mineral availability by the formation of insoluble complexes [7]. Fungi cultivated in glucose, GluRO, had a significantly higher calcium, magnesium, zinc, and copper content compared to oat flour: 612 mg Ca; 228 mg Mg; 19.76 mg Zn; and 2.46 mg Cu per 100 g dry weight. Iron content was however lower than for oat flour; 2.95 mg/100 g. High calcium content of filamentous fungi has also been reported by some previous studies, ranging from 0.1 % – 0.3 % of the fungi dry weight [24]. However, for OatRO, the calcium content was significantly lower, about half the of that of GluRO, at 369 mg Ca/100 g dry weight which was also true for magnesium at 179 mg/100 g. The reason could be that the glucose medium had a high calcium concentration at 274 mg/L and magnesium at 217 mg/L, whereas the 30 g/L oat flour media, only

Table 2
Phosphorous and phytic acid content of oat flour and filamentous fungi cultivated in oat flour (OatRO), and glucose media (GluRO). Data are expressed as mmol or gram (g)/100 g flour or wet filamentous fungi. Phytic acid was determined by two different methods; spechtrophotometry or HPIC.

Method 1: Spectrophotometric determination of Phytic Acid				
	Free phosphorous (mmol P/100 g)	Total phosphrous (mmol P/100 g)	mmol phytic acid/100 g	phytic acid g/100 g
Oat flour	1.16 ± 0.17	10.06 ± 0.99	1.49 ± 0.14	0.98 ± 0.1^{a}
OatRO	10.9 ± 0.68	11.49 ± 0.7	0.1 ± 0.01	0.07 ± 0.01^{b}
GluRO	79.69 ± 4.15	80.34 ± 4.2	0.11 ± 0.02	0.08 ± 0.01^{b}
Method 2: HPIC determination of Phytic Acid				
			mmol phytic acid/100 g	phytic acid g/100 g
Oat flour			1.66 ± 0.12	1.09 ± 0.08^{a}
OatRO			0.28 ± 0.04	0.19 ± 0.03^{b}
GluRO			0.16 ± 0.03	0.10 ± 0.02^{b}

*Different letters in the same column denote statistically significant differences ($p < 0.05$) among samples for the same parameter.

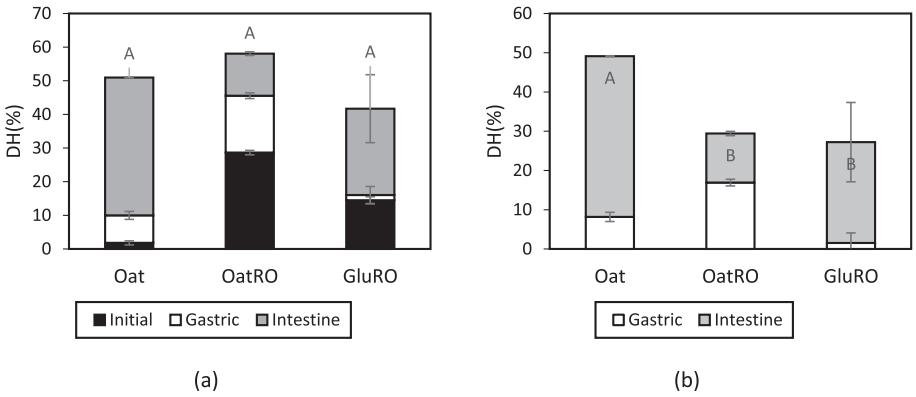

Fig. 1. DH% of oat flour as well as filamentous fungi *R.oligosporus* after each phase of gastrointestinal digestion. (a) total DH%, including initial DH%. (b) DH% normalized towards initial DH%. "Initial" shows the DH% present in samples prior to the digestion. "Gastric" shows the increase in DH% induced by the pepsin in the gastric phase. "Intestinal" shows the increase of DH% induced by pancreatin in the intestinal phase.

contained calcium and magnesium at 11.7 mg/L and 34.2 mg/L, respectively. The iron content of OatRO was however significantly higher than GluRO, at 9.58 mg/100 g dry weight as the oat flour provided higher iron content than the glucose synthetic media. These findings suggest that the essential mineral content of the fungi is largely influenced by the mineral content of the cultivation media. Regarding zinc content, there was no significant difference between GluRO and OatRO.

3.2. *Protein digestibility and amino acid accessibility after in vitro gastrointestinal digestion*

In this study, protein nutritional quality was further assessed by measuring the protein degree of hydrolysis and amino acid accessibility. 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. 1a depicts the total DH% after digestion of oat flour, OatRO and GluRO.

The initial DH% refers to the fraction of primary amines that were released into the aqueous phase prior to the addition of digestive enzymes in the oral phase, while the gastric and intestinal phases indicate the primary amine release induced by pepsin and pancreatin, respectively. The oat flour had a negligible initial DH of 1.8 %, however, after being hydrolyzed by pepsin in the gastric phase, DH increased to 9 %, followed by the intestinal phase providing DH 50.9 %. The result corresponds to a previous study on protein digestibility of oat bran protein concentrate having a gastric and intestinal DH of 20 % and 50 %, respectively [23]. The filamentous fungi showed a significantly higher initial DH% compared to oat at 14.4 % and 28.6 % for GluRO and OatRO, respectively. After intestinal digestion, the GluRO exhibited a not significant total DH, 41.7 %, compared to OatRO, 58.0 %.

The high initial DH% of filamentous fungi could be explained by the presence of non-amino acid molecules that contain primary amine functional groups, such as nucleic acids, γ-aminobutyric acid, and chitosan [13]. To estimate protein digestion induced by gastrointestinal digestion alone (Fig. 1B), the value of total DH% needs to be subtracted by the initial DH%. The normalized DH% showed that oat flour had DH % of 40.9 %, while OatRO and GluRO had 29.4 % and 27.2 %, respectively, the two latter not being significantly different. The lower gastrointestinal digestion of proteins in fungi compared to oat flour could be due to the chitinated cell wall of *R.oligosporus*. A previous study indicated that the fungi *R.oligosporus* achieved 60 % DH after *in vitro* gastrointestinal digestion, which could be due to the inclusion of yeast extract in the media [13].

It was seen that OatRO and oat flour proteins underwent partial hydrolysis during gastric digestion, whereas GluRO proteins exhibited

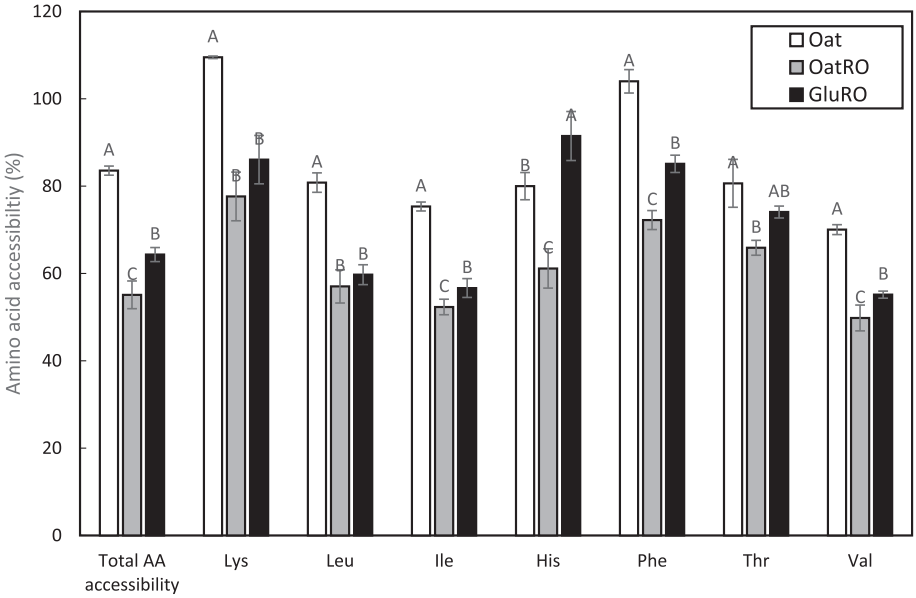

Fig. 2. Accessibility of total amino acids and each essential amino acid of oat flour as well as *R.oligosporus* cultivated in oat and glucose media.

minimal gastric digestion (Fig. 1B). Subsequently, both samples achieved similar digestion levels during intestinal digestion with pancreatin. This low gastric digestion of GluRO aligns with our previous study that digested *R.oligosporus* cultivated in glucose and yeast extract media [13]. Whether also the higher substrate to enzyme-ratio with low-protein fungi influenced the results remains to be proven.

Amino acid accessibility quantifies the fraction of amino acids which are released from the proteins into the aqueous fraction of the intestinal digests. In this study, the aqueous fraction and non-solubilized matter were separated by a 0.2 µm filter [18]. A digested water blank was used to normalize the released total amino acids in the intestinal digests against the background caused by pancreatic autolysis. The essential amino acid accessibility of oat flour as well as *R.oligosporus* cultivated in oat and glucose media, are illustrated in Fig. 2.

Oat flour had a total amino acid accessibility of about 83.5 % and lysine was completely accessible. Considering that the daily recommended amount of lysine is about 30 mg per kg body weight per day [30], oat flour is however still considered a poor source of this essential amino acid [23].

The *R.oligosporus* culivated in glucose media demonstrated a notably higher amino acid accessibility, with a value of 64.3 %, in contrast to fungi cultivated in oat-based media, which provided an accessibility of 55.1 %. A hypothesis is that the different nitrogen sources in the two cultivation media might influence protein accessibility within the fungi. Specifically, GluRO is cultivated utilizing the inorganic nitrogen source $(NH_4)_2SO_4$, while OatRO employs oat protein as its nitrogen source. A preceding study indicated that *R.oligosporus* cultivated in a medium with yeast extract achieved amino acid accessibility as high as 90 % [13]. Notably, yeast extract is a derivative of hydrolyzed yeast protein and encompasses simplistic amino acids and peptides. The presence of simpler amino acids and peptides may facilitate easier assimilation and metabolism by the fungi. The link between the nitrogen source in fungi cultivation media and the protein nutritional quality of filamentous fungi should be further studied.

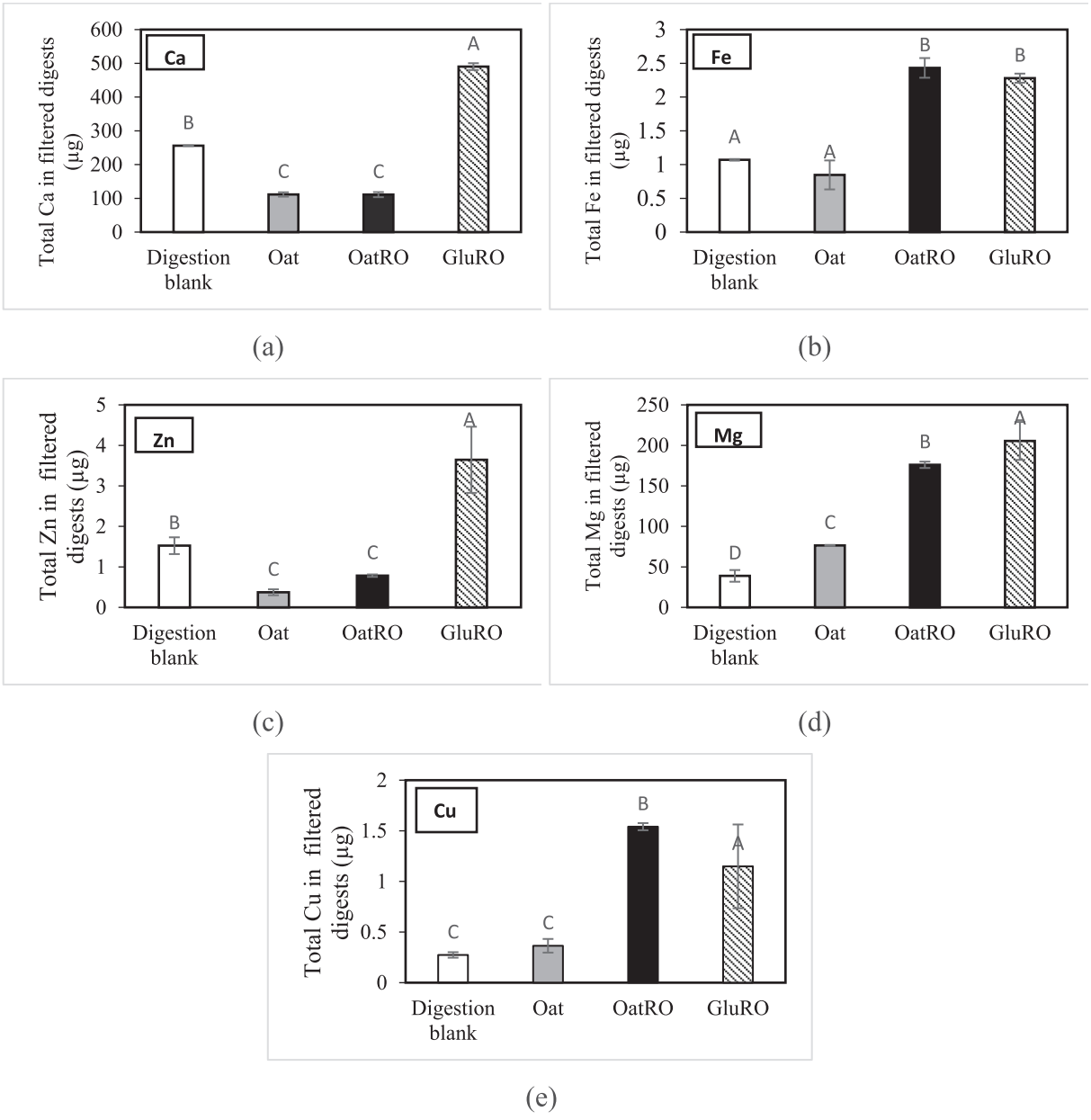

Fig. 3. Total mineral content in the filtered intestinal digests of oat flour and *R.oligosporus* cultivated in oat and glucose media after *in vitro* gastrointestinal digestion of 100 mg of the sample; (a) Ca; (b) Mg; (c) Zn; (d) Mg; (e) Cu.

Additionally, there was a significant difference in the accessibility of phenylalanine between GluRO and OatRO, recorded at 85.1 % and 72.2 %, respectively. A similar discrepancy was also evident in the accessibility of another aromatic amino acid, histidine, with values of 91 % for GluRO and 61 % for OatRO. The potential reason for these variations, especially considering both are aromatic amino acids, could be also due to the differences in proteins expression between fungi grown in oat and glucose media.

3.3. Effect of fungal cultivation on phytic acid content of oat

Phytic acid (inositol hexakisphosphoric acid; $InsP_6$) is known as the main anti-nutrient present in oat flour. It forms insoluble compounds with essential dietary minerals such as iron, calcium, copper, and zinc at physiological pH in the small intestine rendering them unavailable for intestinal absorption. Table 2 shows the phytic acid content of the oat flour and fungi samples analyzed by a enzymatic-spectroscopic method *vs* with HPIC.

The first method measured both free phosphorous and total phosphorous, which in the oat flour sample was 1.16 and 10.0 mmol P per 100 g dry weight, respectively. Phytic acid content can then be calculated as 0.98 g per 100 g dry mass, from the difference between total phosphrous and free phosphrous. Using the HPIC method, phytic acid specifically ($InsP_6$) is determined. Both of the methods estimated that oat flour has ~1 % phytic acid content per dry weight. This is in the range of commonly reported phytic acid content in oat flour (0.8–1.4 %) [32].

GluRO showed very low phytic acid content as assessed by both methods, around 0.1 %. Fungi and other single cell protein is expected to have no phytic acid because microorganisms such as fungi use polyphosphate instead of phytate as a phosphorous storage molecule [33]. Interestingly, using the enzymatic-spectroscopic method, high free phosphorous was observed for the GluRO at 79.6 mmol P per 100 g dry weight; this could be due to the presence of polyphosphates present in the cell wall of zygomycetes fungi [34]. For this reason, the phytic acid analysis of GluRO was also analyzed with the HPIC method to be able to detect phytic acid with a specific method, which not include other phosphorus containing compounds.

The OatRO showed a very low amount of phytic acid at 0.19 g/100 g dry weight. This shows the capability of *R.oligosporus* in producing phytase to degrade phytic acid during the submerged cultivation. The capability of *R.oligosporus* to produce phytate had been widely reported and employed as for solid-state fermentation of numerous plant-based raw materials, not least soy beans, to improve the mineral bioavailability [7]. This property makes it feasible to produce filamentous fungi-based food products using readily available cereals or grains, such as oat, without concerns regarding the persistence of phytic acid in the final product.

A theoretical bioavailability of Fe and Zn can be estimated by examining the molar ratio between phytic acid to Fe and Zn. For instance, in oat flour, OatRO, and GluRO, the molar ratios of phytic acid to iron are 20.6, 1.63, and 3.02, respectively, while the ratios of phytic acid to zinc are 28.1, 1.13, and 0.52, respectively. According to Hurrell and Egli [35], the ideal molar ratio for iron should be below 1, preferably below 0.4, whereas for zinc, it should be below 5, as suggested by European Food Safety Agency [36]. An molar ratio of phytic acid to Fe, exceeding 1 in GluRO and OatRO, may potentially hinder iron accessibility, while zinc accessibility is not anticipated to be adversely affected.

3.4. Mineral accessibility of oat flour and filamentous fungi

Mineral accessibility refers to the extent to which essential minerals in solid foods are released and dissolved into the liquid phase of the intestinal contents. Here, undissolved material of the *in vitro* digests was removed from the soluble phase using a 0.2 µm filter. The total amount of dissolved minerals in the recovered liquid phase of the intestinal digest is shown in Fig. 3. The essential minerals investigated included Ca, Fe, Zn, Mg and Cu and their accessibility was calculated as the ratio of the total mineral content of the original solid sample.

The filtered intestinal digests from 100 mg of oat flour indicate a total Fe content of 0.84 µg, which is slightly less than the 1.07 µg found in the filtered blank intestinal digests. The oat flour iron accessibility is calculated at −4.1 ± 4.7 % suggesting that oat flour exhibited anti-nutritional properties with regard to iron. On the other hand, filtered intestinal digests of OatRO and GluRO showed total Fe contents of 2.43 µg and 2.28 µg, respectively and their relative accessibilities were 14.59 ± 1.69 % and 38.2 ± 1.94 %, respectively. The lower Fe accessibility in OatRO is attributed to its higher initial iron content (9.58 mg/100 g) compared to that in GluRO (2.95 mg/100 g).

Regarding calcium, the filtered intestinal digests of oat flour showed a content of 111 µg, a value significantly lower than the 255 µg in the blank intestinal digests, suggesting it possesses anti-nutritional properties concerning calcium. A similar trend was noted for OatRO with a total 110 µg in the filtered intestinal digests (Ca accessibility −40.2 ± 2.4 %). On the other hand, GluRO displayed a substantial amount of calcium, 490.2 µg, in its filtered intestinal digests, equating to a calcium accessibility of 37.9 ± 1.80 %. Regarding zinc, the blank intestinal digests contained 1.52 µg while filtered digests of oat flour and OatRO had a lower value of 0.37 µg and 0.78 µg, having negative accessibility value of −37.03 ± 1.54 % and − 4.13 ± 0.15 %, respectively. In contrast, filtered GluRO digesta exhibited a higher content of 3.64 µg, with a zinc accessibility of 9.3 ± 0,46 %. Thus, OatRO possessed anti-nutritional properties concerning both calcium and zinc despite the very low amounts of phytic acid (Table 2).

For magnesium, the filtered intestinal digest values for oat flour, OatRO, and GluRO were 76.4 µg, 176.0 µg, and 205.4 µg, respectively. These values exceeded the 38.8 µg in the blank digestion, resulting in magnesium accessibilities of 44.3 % ± 0.15, 74.5 ± 3.12 %, and 66.47 ± 1.47 % for each sample, respectively. Regarding copper, levels in the filtered intestinal digests for the oat flour, OatRO, and GluRO were 0.36 µg, 1.54 µg, and 1.14 µg, respectively, while there was 0.27 µg in the filtered blank digest. Based on Cu-levels prior to digestion, this translated to copper accessibilities of 24.17 % ± 9.45, 55.95 ± 0.82 %, and 24.79 ± 1.36 % respectively.

The anti-nutritional properties observed in oat flour for iron, calcium and zinc are believed to be due to high levels of phytic acid. This finding aligns with a previous *in vitro* study by Sandberg and Svanberg [37] that reported an iron accessibility of 3 % in oat flour which was primarily attributed to the presence of phytic acid. Iron accessibility of 18 % was also observed for oat flour in another recent study [38].

Fungi cultivated using oat flour exhibited an anti-nutritional effect with respect to calcium despite the very low amount of phytic acid. Interestingly, the calcium anti-nutritional effect was not observed in

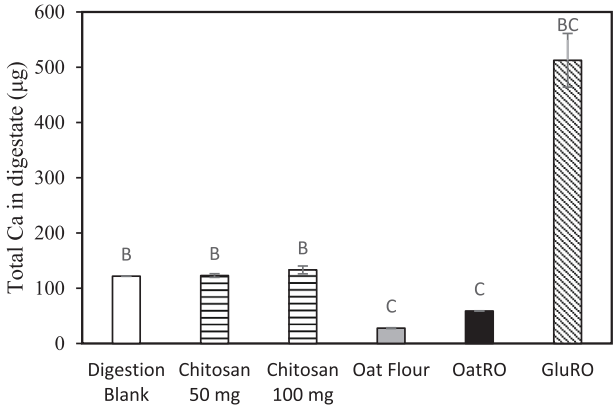

Fig. 4. Total mineral in the intestinal digestate on invitro digestion of chitosan, oat, OatRO, and GluRO.

fungi cultivated in glucose media. Moreover, the iron accessibility of GluRO and OatRO is higher compared to zinc accessibility, despite the molar ratio of phytic acid to iron being higher than one, while the molar ratio of phytic acid to zinc is lower than 5. There are several hypotheses to explain these observations. First, the presence of chitosan could contribute to antinutritional properties, considering its properties to adsorb divalent ions [39]. Since OatRO had a significantly lower Ca content compared to GluRO, it is hypothesized the calcium in the GluRO was partially released to the intestinal digests, which overshadowed the adsorbtion of calcium by chitosan. However, this does not explain the antinutritional properties with respect to zinc as OatRO and GluRO had a comparable amount of zinc. An experiment investigating the antinutrient impact of chitosan was conducted using pure shrimp-derived chitosan. The findings indicated that chitosan does not exhibit any antinutrient effects on calcium (Fig. 4).

Another possible reason is the presence of polyphosphates in the fungi cell wall. Cell walls of zygomycetes fungi is mainly made up both of copolymers of glucosamine and *N*-acetyl glucosamine, *i.e.*, chitin and chitosan (45–85 %), and of polyphosphates (4–20 %). The fungi *R.oligosporus* cultivated in glucose media displayed higher free phosphorus than OatRO (Table 2). A plausible hypothesis is that in the glucose media, which had a higher calcium content, the polyphosphates within the fungi (GluRO) became saturated with calcium ions, attributing to the observed higher calcium content (Table 1). In contrast, in the oat media (OatRO), where calcium levels are comparatively lower, the polyphosphates remain unsaturated, subsequently adsorbing calcium ions from the simulated digestive fluid during *in vitro* gastrointestinal digestion.

Additionally, an alternative antinutrient that needs to be considered is oxalic acid. This organic acid is known for its pronounced antinutritional effects on calcium and zinc, while its influence on iron is negligible [40]. It is noteworthy that filamentous fungi can produce oxalic acid. In fact, there is a potential in employing filamentous fungi, such as *Aspergillus niger*, for large-scale production of oxalic acid [41]. Moreover, fungi utilize oxalic acid as a metabolite to regulate calcium and other minerals in their environment [42]. Additionally, factors such as glucose concentration, pH, nitrogen source, and the C/N ratio play a significant role in influencing oxalic acid production by fungi [43]. In order to validate these hypotheses, further experiment should be conducted to investigate potential interactions of *R.oligosporus* cell wall component such as chitosan and polyphosphates as well as oxalic acid content with iron, calcium and zink to assess their impact on mineral accessibility in fungi.

Lastly, the lower value of total minerals calcium in the filtered intestinal digests compared to the filtered digestion blank as was observed in oat and OatRO samples, results in a negative calcium accessibility value. One possible explanation is the high concentration of calcium used in the electrolyte of the INFOGEST protocol, as calcium serves as a crucial cofactor for enzymes like amylase and trypsin *in vivo*. Excluding calcium during *in vitro* gastrointestinal digestion could answer this hypothesis, but such an approach would not accurately simulate *in vivo* conditions. One way to tackle this issue is to do the experiment at higher amount of initial sample.

Although filamentous fungi show potential as a food source based on their high mineral accessibility, cultivating filamentous fungi in oat media can impact not only the protein and mineral content, but also the protein digestibility as well as accessibility of amino acids and minerals in fungi-based foods. This study has been limited to measurements of soluble amino acids and essential minerals in filtrates of the intestinal digests, therefore, further investigations should be conducted monitoring mineral dialysability, bioaccessibility in Caco-2 cell model, and subsequently *in vivo* studies in order to obtain true nutrient bioavailability values.

4. Conclusion

In this study, protein digestibility as well as amino acid and mineral accessibility of edible filamentous fungi grown using oat flour or glucose media were investigated. Our findings revealed a notable reduction in total amino acid content of *R.oligosporus* when cultivated in oat media which could be due to different C/N ratio between oat flour and glucose media. The oat-cultivated fungi also showed a significantly higher protein degree of hydrolysis during simulated gastric digestion but not after simulated intestinal digestion. Paradoxically, this fungi however gave a lower amino acid accessibility compared to its glucose-cultivated counterpart. Interestingly, the use of oat as the growth medium also influenced the mineral content, with the calcium and iron content being significantly lower and higher, respectively, compared to fungi cultivated in glucose media. Both fungi types were nearly absent in phytic acid, however, the results suggest that fungi cultivated on glucose media might offer better accessibility of calcium, zinc and iron. This difference indicates the presence of other antinutrients in the fungi cultivated on oat flour, beyond just phytic acid. Our findings have implications for optimizations of both cultivation and composition of edible filamentous fungi, underscoring the need to select the appropriate medium based on desired nutritional properties.

Author statement

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Ethical statement

No studies in human or animal is conducted.

CRediT authorship contribution statement

Ricky Wang: Data curation, Formal analysis, Methodology, Writing – original draft, Conceptualization, Investigation. **Neda Rousta:** Methodology, Conceptualization. **Amir Mahboubi:** Supervision, Writing – review & editing. **Rikard Fristedt:** Methodology. **Ingrid Undeland:** Conceptualization, Supervision, Writing – review & editing. **Ann-Sofie Sandberg:** Writing – review & editing. **Mohammad J. Taherzadeh:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Investigation.

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.

Acknowledgement

This work was financially supported by FORMAS Swedish Research Council (Project number : 2023-02018).

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