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In vitro protein digestibility of edible filamentous fungi compared to common food protein sources

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ABSTRACT

Edible filamentous fungi, as a source of mycoprotein, is an emerging sustainable protein source as it can be cultivated on food-industry sidestreams, thus providing the food system with circularity. However, the digestibility of mycoprotein from different species of fungi is yet to be studied and compared to commonly consumed food proteins derived from muscle. Using the static INFOGEST *in vitro* gastrointestinal (GI) digestion protocol, but with less pancreatin than the recommended amount to omit high background from enzyme autolysis, this study investigated the protein degree of hydrolysis (DH%) and amino acid accessibility of five species of edible fungi in comparison with salmon fillet, chicken breast, beef tenderloin and casein. Three of the edible fungi species reached protein DH% between 58% ± 2.6% and 62% ± 5.6% during GI digestion compared to chicken, salmon, and beef reaching 62%–67% as well as casein at 55%. The amino acid accessibility of fungi (81%–92%), was comparable to that of salmon, chicken breast, and beef (90%–94%). This study thus indicated that edible fungi is a sustainable and nutritionally sound protein source.

1. Introduction

Feeding the growing world population, which is estimated to reach 9.7 billion by 2050, is clearly addressed as a challenge in the United Nations 2030 Agenda, particularly in Sustainable Development Goal (SDG) #2 aiming to archive "Zero Hunger". However, food protein, an essential macronutrient in the human diet, is currently sourced mainly from livestock meat which significantly contributes to total greenhouse gas emissions (IPCC, 2022). This is a dilemma when both ensuring food security and mitigating global climate change (SDG 13).

In order to tackle this conflict, numerous innovations have been made in recent decades to develop sustainable protein sources, including plant-based meat analogs, *in vitro* cultured meat, algae, edible insects, and edible fungi. Edible filamentous fungi have the benefit of being able to be grown on food-grade industrial side-streams, thus ensuring circularity and resource efficiency in the global food system (Gmoser et al., 2020; Sar et al., 2022). Besides this huge environmental benefit, edible filamentous fungi, as a source of mycoprotein, are recognized as a nutritious food raw material, having both high contents of essential amino acids and dietary fibers, such as β-glucan, chitin, and chitosan (Colosimo et al., 2020). The filamentous structure of the fungi resembles muscle fibers, thus providing a meat-like texture of benefit if it is to be applied in food (Gmoser et al., 2020).

The most recognized marketed fungi-based food product is Quorn®, *Fusarium venenatum*, which has been carefully studied for its nutritional profile and health benefits (Ahmad et al., 2022). There are, however, also numerous other species of edible filamentous fungi that are currently used by the food industry: *Aspergillus oryzae* in the production of koji, soy sauce, and sake in Japan; *Neurospora intermedia*, in the production of an Indonesian fermented food product called *oncom*, and *Rhizopus oligosporus* as well as *Rhizopus delemar* in the production of tempeh, an Indonesian fermented soybean food product. The mentioned edible filamentous fungi are from two different phyla, ascomycetes, including *Aspergillus oryzae*, *Neurospora intermedia*, and *Fusarium venenatum*, as well as zygomycetes, including *Rhizopus delemar* and *Rhizopus oligosporus*.

Having a high protein content (Karimi et al., 2021; Sar et al., 2022), edible filamentous fungi are expected to play a promising prospective role in future food production. However, apart from the protein content as such, the protein quality, i.e., the amino acid profile and the protein

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digestibility, is a defining factor for how promising an alternative protein product is. There are reasons to believe that the digestibility of fungi compared to e.g. muscle tissue could be slightly hampered by the chitin-enriched fungal cell walls (Colosimo, Warren, et al., 2021). The golden standards to measure protein digestibility for humans are based on *in vivo* trials, for example, Protein Digestibility Corrected Amino Acid Score (PDCAAS) and Digestible Indispensable Amino Acid Score (DIAAS) comprising trials with rats and swine, respectively. Udall et al. (1984a) indicated that the protein digestibility for edible filamentous fungi, *Fusarium graminearium,* was 78% compared to 95% of milk protein. Another study done using human ileostomy patients found that the PDCAAS ratio of edible filamentous fungi, *Fusarium venenatum*, was very close to that for egg white but lower than for chicken and beef (Finnigan et al., 2019).

In vivo methods to determine food digestibility are indeed more representative of the consumption of food, however, they are expensive and time-consuming (Egger et al., 2017). *In vitro* digestion models aim to counteract those limitations, and since 2014, a standardized *in vitro* protocol to simulate gastrointestinal (GI) digestion has been developed by an international network, termed the INFOGEST digestion protocol (Brodkorb et al., 2019; Minekus et al., 2014). The first *in vitro* study of protein digestibility from edible filamentous fungi was conducted by Colosimo et al. (2020), investigating the proteolysis mechanism in *Fusarium venenatum* by the INFOGEST static digestion protocol. After this, Ariëns et al. (2021) compared several emerging protein sources, including edible filamentous fungi (*Fusarium venenatum)*, by using the INFOGEST *in vitro* static digestion model.

To the author's knowledge, no study provides protein digestibility results on an extended range of different species of edible filamentous fungi.In addition, no earlier comparisons have been made to other commonly consumed protein sources such as meat and fish. This study aims to fill these knowledge gaps by evaluating the protein digestibility of five species of fungi biomass among *Zygomycetes* and *Ascomycetes*; *A. oryzae, N. intermedia, F. venenatum, R. delemar,* and *R. oligosporus,* using the INFOGEST *in vitro* digestion protocol. The *in vitro* protein digestibility was estimated by the protein degree of hydrolysis and amino acid accessibility. The digestibility of fungal protein was compared with proteins in salmon, chicken, and beef fillets as well as with milk protein (casein).

2. Material and method

2.1. Materials

Frozen salmon fillet, fresh chicken breast, and fresh beef tenderloin (entrecote) were purchased from the local grocery (ICA City, Borås, Sweden). All chemicals were analytical grade (Sigma Aldrich, Sweden). Pepsin from porcine pancreas (P6887) and human salivary amylase (A1031) were supplied from Sigma Aldrich, Sweden, while pancreatin from porcine pancreas (8x USP) was provided by MP Biomedicals, USA.

2.2. Cultivation of edible filamentous fungi

Five edible filamentous fungal strains (*R. microsporus* var. *oligosporus* CBS 112586, *R. oryzae* var. *delemar* CBS 145940, *A. oryzae* var. oryzae CBS 819.72, *N. intermedia* CBS 131.92, and *F. venenatum* ATCC 20334) were used. The fungi *A. oryzae* and *N. intermedia* were cultivated on 30 g/l glucose and 5 g/l yeast extract using a 4-L bubble column bioreactor (Belach Bioteknik, Sweden). Using the same cultivation media, *R. oligosporus* and *R. delemar* were cultivated using a 26-L bubble column bioreactor (Bioengineering, Switzerland). The fungi *F. venenatum* was cultivated using peptone media of 20 g/l glucose and 4 g/l peptone using a 4-L bubble column bioreactor. Shake flask pre-cultures using the same media were added as inoculum. Fungi cultivation was carried out at 35 °C (except for *F. venenatum* at 28 °C due to its optimum temperature) and 1.0 vvm aeration for 24 h. The fungi were then harvested using a sieve, washed, and hand-squeezed to obtain fresh wet fungi biomass.

2.3. Static in vitro gastrointestinal digestion

Fresh chicken breast, fresh beef entrecote, thawed frozen salmon, and the fresh wet fungi biomass were minced with a cast iron mincer (KitchenCraft, UK). The rest of the minced products were frozen at −20 °C before being freeze-dried. Freeze-dried samples were then milled using a ball mill (Retsch, Germany) and subsequently subjected to moisture analysis, amino acid analysis, and *in vitro* digestion.

A preliminary experiment was carried out to fine-tune the *in vitro* digestion protocol in terms of using either a fresh sample or a freeze-dried milled sample. Therefore, two fungi species, *A.oryzae* and *N. intermedia*, were also analyzed directly after mincing for their moisture content and were then subjected to *in vitro* digestion without freeze-drying and milling. Another factor investigated in the preliminary experiment was the addition of salivary α-amylase during the oral phase of *in vitro* digestion.

A schematic diagram of the *in vitro* digestion protocol used in this study is illustrated in Fig. 1. In essence, the INFOGEST 2.0 digestion protocol according to Brodkorb et al. (2019) was followed, but with small modifications; (i) reduction in the amount of the added salivary amylase and pancreatin (ii) omission of NH_4CO_3 in the simulated digestive fluid. Activities of the digestive enzymes were measured using INFOGEST recommended methods; the Anson method was used for pepsin activity and p-toluene-sulfonyl-L-arginine methyl ester (TAME) was used as the substrate in pancreatin activity analysis, resulting in 2661 U/mg pepsin and 6.1 U/mg pancreatin, respectively. Salivary amylase activity was provided by the supplier at 118 U/mg solid.

In the digestion of the different protein sources, an amount of each milled sample corresponding to 40 mg of protein, measured as total amino acids, was mixed with 1 ml of water in a 13 ml plastic test tube. For the digestion blank, only pure water at the same amount as in the samples was added. To simulate the oral phase, 1 ml of simulated salivary fluid containing salivary amylase (7.5 U/ml digest) was added to the sample and incubated for 1 h. The gastric phase was started by adding 2 ml of simulated gastric fluid containing pepsin (2000 U/ml digest) into the oral digests, adjusting the pH to 3 using 5 M HCL, followed by incubation for 2 h. The gastric digestion was terminated by increasing the pH to 7 using 1 M NaOH. For the intestinal phase, 4 ml of simulated intestinal fluid containing pancreatin (10 U/ml digests) were added to the gastric digests (Ariëns et al., 2021), adjusting to pH 7 using 1 M NaOH, followed by incubation for 2 h. The intestinal phase was then terminated by adding 800 μl of Bowman-Birk inhibitor (0.05 g/l). All the incubation was done at 37 °C with gentle mixing at five rpm (Stuart Rotator SB3, UK). The digests were then aliquoted and stored at −80 °C for a maximum of 4 weeks.

2.4. Degree of hydrolysis

The degree of hydrolysis (DH%) of the initial sample suspension, gastric digests, and intestinal digests was measured using an o-phthalaldehyde reagent (OPA) according to Nielsen et al. (2001). The OPA reagent was made up of 100 mM sodium tetraborate, 0.1% sodium dodecyl sulfate, 5.7 mM Dithiothreitol, 6.15 mM o-phthalaldehyde, and 2% ethanol. To determine the initial DH, an amount of freeze-dried milled sample corresponding to 40 mg protein was added to 8 ml of water and vortexed, while the aliquots of gastric and intestinal digests were thawed and vortexed. All samples were directly pipetted for dilution without any centrifugation. The diluted samples (120 μl) were then added to the OPA reagent (1000 μl), incubated at ambient temperature, 20 °C, for 10 min, and measured at 340 nm using a spectrophotometer. Sample absorbance readings were compared with the standard curve using L-serine as the standard.

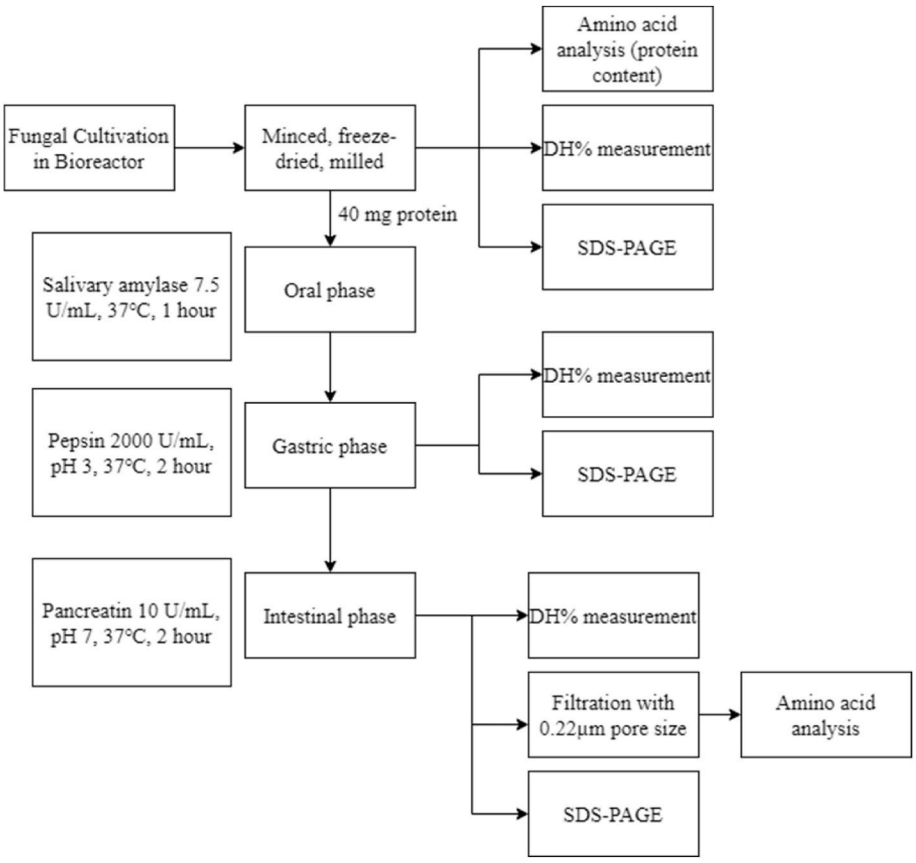

Fig. 1. Schematic diagram of the *in vitro* digestion protocol used in this study.

The degree of hydrolysis is calculated by equation (1).

$$DH\ (\%) = \frac{h(sample) - h\ (digestion\ blank)}{h_{tot}(sample)} x\ 100 \quad (1)$$

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

2.5. Gel electrophoresis

All the electrophoresis equipment, gels, and reagents were provided by Bio-Rad (Solna, Sweden). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the initial homogenates, gastric digests, and intestinal digests. Briefly, the sample was mixed with of Laemmli Sample Buffer, with the ratio to the sample of 3:1: sample, containing DTT as a reducing agent, and heated at 70 °C in a water bath for 10 min. The mixture at approximately 20 μg protein was then loaded into the pre-cast 12% polyacrylamide gel. Tris-glycine was used as the running buffer. Electrophoresis was run at 200 V for about 30 min. The gel was then stained using Coomassie blue R-250 and incubated on a platform shaker for at least 1 h. The gel was then unstained using an unstaining solution containing 40% methanol and 10% acetic acid.

2.6. Amino acid accessibility and analysis

To estimate how much of the amino acids in the digests accessible for uptake, intestinal digests were filtered with a 0.22 μm syringe filter, producing a filtrate that was analyzed for amino acids (Trigo et al., 2021). All the non-digested samples were also subjected to amino acid analysis, including casein, freeze-dried salmon, chicken breast, beef, and all species of edible filamentous fungi. For filtered intestinal digests, 0.5 ml of 12 M HCl was added to 0.5 ml of the sample in a glass tube. For non-digested samples, 4 ml of 12 M HCl was added to approximately 50 mg of samples, followed by the addition of 4 ml water.

The air inside the glass tubes was purged with nitrogen gas followed by a complete seal with the caps and heated at 110 °C for 24 h using a heat block. The whole content of the tubes was then 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 0.22 μm syringe filter prior to analysis with LC/MS.

Two microliters of all samples were run in an LC/MS system (Agilent 1100 HPLC and 6120B Single Quadrupole MS) as earlier described (Trigo et al., 2021). The chromatogram was analyzed using the Mass-Hunter Quantitive Analysis software (version B.09.00, Agilent Technologies). Due to the acid hydrolysis, the method was not suitable to quantify tryptophan. Further, asparagine and glutamine were co-determined with aspartic acid and glutamic acid, respectively.

Amino acid accessibility was calculated using equation (2).

$$Amino\ acid\ accessibility\ (\%) = \frac{Total\ amino\ acids\ of\ filtered\ digest - Digestion\ blank\ total\ amino\ acid}{Initial\ total\ amino\ acid\ of\ sample} x\ 100 \quad (2)$$

2.7. Data analysis

Each sample type was subjected to 3 replicates of amino acid analysis and three replicates of *in vitro* gastrointestinal digestion. Each digesta was then only subjected to a single analysis. Mean values from the analytical replicates were used in the statistical analysis to test whether there were significant differences between sample types. For this purpose, we used one-way ANOVA followed by Tukey's post hoc test for pairwise comparison. 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® 21.1.1 (©2022 Minitab, LLC). Error bars in the figures represent one time the standard deviation.

3. Results and discussion

In order to be used as sustainable food protein raw material, it is required to assess the protein digestibility of edible filamentous fungi in comparison with other common protein-rich food raw materials. Firstly, the proteins in casein, salmon, chicken breast, beef, and 5 species of fungi were characterized by their amino acid profile. Secondly, the protein digestibility of the fungi and the common food proteins were investigated by using INFOGEST static *in vitro* gastrointestinal model. The initial hypothesis was that the protein digestibility of edible filamentous fungi would be lower than common muscle-based protein sources such as meat or fish as the chitin-enriched fungal cell wall could hinder the enzymatic hydrolysis.

3.1. Characteristics of proteins in the fungi, muscle, and casein

Chicken breast had the highest protein measured as total amino acid content of all muscle food proteins tested in this study (78.2% on dry weight, dw, basis), followed by beef and salmon at about 59.9% and 49.0% dw, respectively (Table 1). The lower protein content of salmon and beef than chicken reflects their high lipid content. The total amino acid content of the five edible filamentous fungi species varied from 32% to 45% dw, with *N. intermedia* having the highest content. The non-protein-part of the fungi is primarily made up of dietary fiber such as β-glucan, chitin, and chitosan (Colosimo, Mulet-Cabero, et al., 2021; Svensson et al., 2022), with lipids contributing to around 6%–8% (Rousta et al., 2022).

The total amino acid of *Aspergillus oryzae*, *Rhizopus delemar*, and *Neurospora intermedia* cultivated on industrial side-stream has earlier been reported to range from 29%, 27%, and 31% dw, respectively (Karimi et al., 2021; Sar et al., 2022). The higher total amino acid obtained in this study could be due to cultivation in a synthetic media. Moreover, the fungi used were harvested at the beginning of the logarithmic growth phase, which has been shown to affect the filamentous fungi crude protein content (Sar et al., 2022). A relatively high crude

Table 1
Amino acid profile of casein, salmon, chicken breast, beef, and five species of edible filamentous fungi (*Aspergillus oryzae, Neurospora intermedia, Fusarium venenatum, Rhizopus delemar,* and *Rhizopus oligosporus*). Results are given on a dry matter basis and are expressed as mean values ± standard deviation (n = 3).

	Casein	Salmon	Chicken breast	Beef	*Aspergillus oryzae*	*Neurospora intermedia*	*Fusarium venenatum*	*Rhizopus delemar*	*Rhizopus oligosporus*
Total amino acid (g/100 g dry mass)	101.9 ± 1.05^{a}	49.03 ± 2.85^{d}	78.23 ± 3.02^{b}	59.98 ± 2.05^{c}	37.90 ± 0.48^{f}	45.13 ± 0.66^{e}	43.60 ± 0.57^{g}	32.10 ± 0.57^{g}	37.61 ± 0.59^{g}
Essential amino acid (% of total AA)									
Histidine	2.71 ± 0.17^{a}	3.38 ± 0.03^{d}	2.92 ± 0.10^{b}	3.8 ± 0.23^{c}	2.84 ± 0.35^{f}	3.29 ± 0.01^{e}	3.14 ± 0.10^{g}	2.99 ± 0.05^{g}	3.08 ± 0.03^{g}
Leucine	8.92 ± 0.27^{ab}	8.51 ± 0.09^{bc}	8.5 ± 0.33^{bc}	8.24 ± 0.11^{cd}	7.54 ± 0.18^{e}	7.23 ± 0.12^{e}	7.81 ± 0.27^{de}	8.4 ± 0.13^{bc}	9.12 ± 0.11^{a}
Isoleucine	4.99 ± 0.15^{c}	5.24 ± 0.14^{c}	5.32 ± 0.37^{c}	4.93 ± 0.07^{c}	4.92 ± 0.15^{c}	4.96 ± 0.16^{c}	5.12 ± 0.18^{c}	5.91 ± 0.01^{b}	6.63 ± 0.03^{a}
Lysine	7.91 ± 0.01^{d}	9.94 ± 0.55^{c}	10.56 ± 0.39^{c}	9.87 ± 0.22^{c}	9.82 ± 0.09^{c}	9.77 ± 0.01^{c}	13.09 ± 0.19^{a}	11.13 ± 0.15^{b}	10.18 ± 0.03^{bc}
Methionine	2.73 ± 0.01^{c}	2.9 ± 0.10^{bc}	2.54 ± 1.24^{ab}	2.38 ± 0.04^{a}	1.06 ± 0.26^{c}	1.75 ± 0.27^{abc}	0.82 ± 0.33^{bc}	1.3 ± 0.12^{bc}	1.54 ± 0.07^{bc}
Phenylalanine	4.9 ± 0.08^{ab}	4.24 ± 0.05^{abc}	4.31 ± 0.16^{a}	4.05 ± 0.08^{abcd}	4.08 ± 0.11^{d}	4.25 ± 0.01^{abcd}	4.11 ± 0.15^{cd}	4.74 ± 0.03^{bcd}	5.23 ± 0.03^{abcd}
Threonine	4.28 ± 0.05^{b}	4.86 ± 0.02^{c}	5.18 ± 0.15^{c}	5.03 ± 0.06^{c}	5.53 ± 0.15^{c}	5.19 ± 0.18^{c}	5.37 ± 0.22^{c}	5.66 ± 0.19^{b}	5.37 ± 0.03^{a}
Valine	5.71 ± 0.05^{e}	4.96 ± 0.16^{bcd}	5.31 ± 0.11^{d}	4.51 ± 0.10^{cd}	5.29 ± 0.12^{ab}	5.46 ± 0.15^{bcd}	5.96 ± 0.10^{abc}	6.51 ± 0.04^{a}	6.9 ± 0.20^{abc}
%EAA	42.15 ± 0.80^{ef}	44.62 ± 3.89^{c}	44.03 ± 1.14^{de}	42.82 ± 0.93^{de}	41.07 ± 1.43^{f}	41.90 ± 0.93^{ef}	45.41 ± 1.57^{bc}	46.65 ± 0.75^{b}	48.04 ± 0.58^{a}
Non-essential amino acids (% of total AA)									
Glycine	1.76 ± 0.03	4.88 ± 0.31	5.23 ± 0.36	4.98 ± 0.32	5.43 ± 0.28	5.38 ± 0.02	5.82 ± 0.08	5.38 ± 0.13	5.43 ± 0.07
Alanine	2.83 ± 0.03	5.88 ± 0.26	6.19 ± 0.37	5.85 ± 0.29	7.92 ± 0.36	7.79 ± 0.20	9.05 ± 0.08	6.9 ± 0.11	6.39 ± 0.19
Arginine	3.21 ± 0.37	6.34 ± 0.05	5.49 ± 0.33	6.23 ± 0.13	5.98 ± 0.22	6.96 ± 0.24	5.38 ± 0.12	5.06 ± 0.23	5.29 ± 0.14
Cysteine	0.11 ± 0.01	0.51 ± 0.17	0.39 ± 0.22	0.38 ± 0.01	0.44 ± 0.35	0.5 ± 0.35	0.43 ± 0.04	0.5 ± 0.03	0.48 ± 0.01
Glutamic acid	21 ± 0.28	15.8 ± 0.38	14.49 ± 0.12	16.75 ± 0.46	14.3 ± 0.45	12.72 ± 0.21	11.32 ± 0.24	11.53 ± 0.14	10.93 ± 0.05
Aspartic acid	6.95 ± 0.08	10.07 ± 0.35	10.46 ± 0.23	9.49 ± 0.11	9.75 ± 0.29	9.89 ± 0.06	8.58 ± 0.01	10.24 ± 0.14	9.72 ± 0.12
Proline	10.08 ± 0.58	3.99 ± 0.07	3.89 ± 0.21	4.32 ± 0.11	4.51 ± 0.18	4.4 ± 0.13	5.65 ± 0.14	4.45 ± 0.10	4.52 ± 0.06a
Serine	5.56 ± 0.01	4.13 ± 0.08	4.3 ± 0.11	4.18 ± 0.13	5.27 ± 0.09	4.96 ± 0.11	4.77 ± 0.10	5.13 ± 0.07	4.86 ± 0.05
Tyrosine	6.34 ± 0.09	4.36 ± 0.37	4.93 ± 0.39	4.99 ± 0.36	5.33 ± 0.29	5.49 ± 0.40	3.58 ± 0.29	4.16 ± 0.18	4.34 ± 0.11

protein content of edible filamentous fungi has been widely reported previously for commercial *F. venenatum* (Ahmad et al., 2022) and also for other species (Sar et al., 2022). Crude protein, however, measures also the total nitrogen present in as fungal cell wall, chitin, and chitosan, why nitrogen-to-protein conversion factors must be carefully determined.

The amino acid profile of all samples is shown in Table 1 together with the relative amount of essential amino acids (EAA). Generally, amino acid profiles for *A. oryzae* and *N. intermedia* are in correspondence with those reported in previous studies by Rousta et al. (2022), especially in relation to the percentage of EAA. Also, in agreement with our previous study (Gmoser et al. (2020), all five edible filamentous fungi contained high amounts of the important EAA lysine. Apart from lacking methionine, plant-derived protein is often also lacking lysine (Kim et al., 2011) which makes filamentous fungi important players in the ongoing dietary protein shift.

Chicken breast and salmon contained 44% EAA of the total amino acid, which was significantly higher than the two species of ascomycetes fungi, *A. oryzae, N. intermedia.* However, the other two species of zygomycetes fungi, *R. delemar* and *R. oligosporus,* had a significantly higher content of EAA compared to chicken breast and salmon; 46%, and 48%, respectively. The relative leucine content of the zygomycetes *R. delemar* and *R. oligosporus* was 8.4% and 9.1%, respectively, and is comparable to that of salmon (8.5%), chicken breast (8.5%), and beef (8.2%). However, the ascomycetes fungi had significantly lower levels ranging from 7.2 to 7.8% (Table 1). Leucine is the amino acid that stimulates muscle synthesis and an increase in the postprandial blood leucine level was observed after consuming edible filamentous fungi (Finnigan et al., 2019).

3.2. *In vitro protein digestibility and amino acid accessibility*

The *in vitro* method based on the static INFOGEST protocol was employed to simulate GI digestion. The recommended protocol, however, was recognized to have several shortcomings in studying food protein digestibility, thus, the protocol needed to be slightly modified and adapted with respect to the amount of sample. Once the protocol was settled, the estimation of protein digestibility of five species of edible filamentous fungi, salmon, chicken, beef, and casein was carried out by measuring the protein DH% and polypeptide/peptide molecular weight distribution. Further to this, the amino acid accessibility was followed.

3.2.1. *In vitro digestion protocol customization and modification*

The harmonized static INFOGEST 2.0 *in vitro* digestion protocol suggests which composition of electrolytes to use for the simulated digestive fluids and also the enzymatic activity of salivary α-amylase (75 U/ml), pepsin (2000 U/ml), and pancreatin (100 U/ml) (trypsin activity) to use in order to simulate the oral phase, gastric phase, and intestinal phase, respectively (Minekus et al., 2014). The method, however, faces a significant drawback in studying protein digestibility, as reported by several authors (Ariëns et al., 2021; Atallah et al., 2020) and also confirmed in our preliminary experiments. Pancreatin tends to autolyze, releasing peptides or amino acids that cannot be distinguished from the digestion product of the studied food protein. The high amount of pancreatin advised in the INFOGEST protocol thus caused a problem of high background hydrolysis level as revealed when only pure water was used as a digestion blank. Therefore, the amount of pancreatin in this study was reduced ten-fold to 10 U/ml tryptic activity as suggested by Ariëns et al. (2021). Moreover, the INFOGEST protocol includes ammonium carbonate as one of the electrolytes. The ammonium ion is however also a primary amine that reacts with OPA, interfering with the quantification of the total primary amines from amino acids. The ammonium ion was also presented in the human salivary alpha-amylase; thus in this study, the amount of salivary alpha-amylase was also reduced by ten-fold to 7.5 U/ml during the oral phase of *in vitro* digestion. Due to the reduction of salivary amylase activity by a factor of ten, as described previously, the duration of oral phase digestion was increased from the recommended 2 min to 1 h.

In the preliminary experiment, several other details of the protocol, including the amount of protein to be digested, the effect of milling, freeze-drying, and the inclusion of salivary amylase during the oral phase digestion, were also examined by a one-factor-at-a-time experiment. The importance of iso-protein measurement was examined by doubling the amount of sample and keeping the same amount of digestive enzymes. This approach, however, did not affect the protein DH% for the gastric phase as long as the original INFOGEST protocol was kept, having an amount of pepsin of 2000 U/ml, which could hydrolyze the protein to its maximum extent. On the other hand, for the modified intestinal phase protocol, the reduced amount of pancreatin (from 100 U/ml to 10 U/ml) could not fully hydrolyze the higher amount of protein in the sample in 2 h. Therefore, it is recommended to conduct this *in vitro* digestion protocol on an iso-protein basis.

It was hypothesized that the process of freeze-drying could affect the cell wall structure of the filamentous fungi. Thus, the DH% of freeze-dried milled fungi samples was compared with that obtained with freshly harvested samples. Fig. 2 shows there was a significant increase in the DH% of the freeze-dried milled sample compared to the fresh minced sample for both *A. oryzae* and *N. intermedia* after intestinal digestion. This was likely due to that freezing causes the expansion of water into ice and disrupts the structure of the cell walls. Freeze-thawing is a commonly used cell disruption technique (Miller et al., 1999). Assessing the protein digestibility of fresh edible filamentous fungi is more representative of its application as food, but it causes technical difficulties during fungal cultivation as the biomass needs to be assessed for digestibility the same day as it is harvested. On the other hand, although freeze-drying is mainly applied to very high-value products such as certain berries and outdoor meals, it allows the sample to be stable for a long time and can be a viable route to go as it also facilitates transporting. For these different reasons, all of the edible filamentous fungi and muscle protein sources were freeze-dried prior to the *in vitro* GI digestion.

Regarding milling of the freeze-dried samples, this provides sample homogeneity, yet it was suspected to affect the protein digestibility. It was however proven not to be a significant experimental factor, which was in line with the previous report by Colosimo et al. (2020), evaluating the link between several physical treatments of *F. venenatum* and the released peptides. Therefore, all the freeze-dried samples were milled to ensure sample homogeneity.

Based on this series of preliminary experiments, the final adjusted INFOGEST protocol for evaluating the *in vitro* protein digestibility of filamentous fungi and other common food proteins was established as

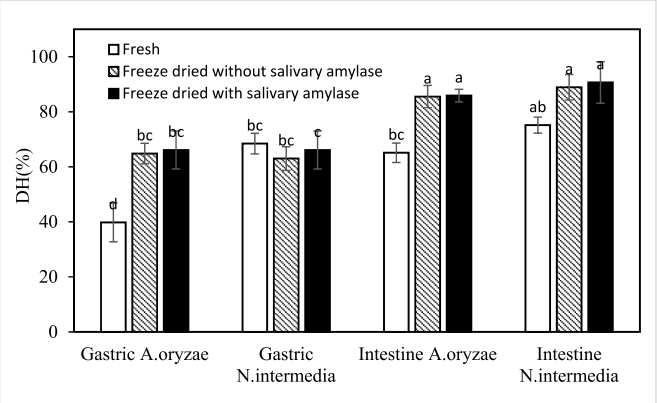

Fig. 2. Effect of the inclusion of salivary-amylase and freeze-drying plus milling on the protein degree of hydrolysis (DH%) of edible filamentous fungi *Aspergillus oryzae* and *Neurospora intermedia.*

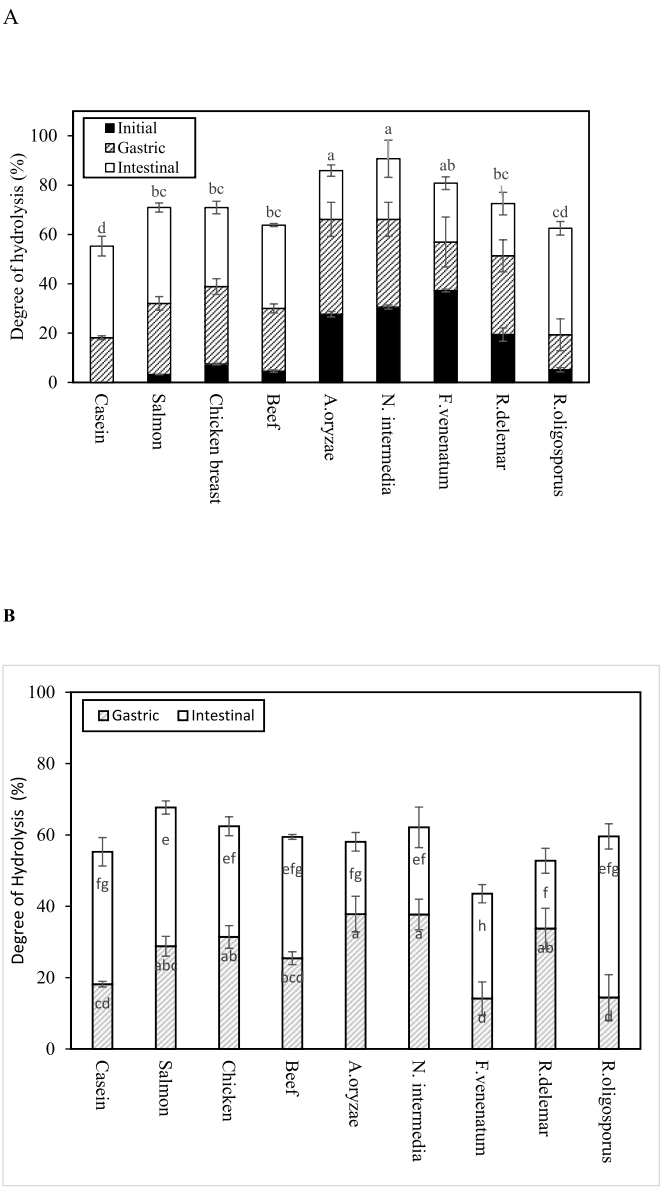

Fig. 3. Protein degree of hydrolysis (DH%) after *in vitro* digestion of casein, salmon, chicken breast, beef, and five species of edible filamentous fungi, (a) total, including initial DH%, (b) normalized towards initial DH%. "Initial" shows the DH% present in samples prior to the digestion. "Gastric" 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.

described in Fig. 1. In short, the oral, gastric and intestinal steps were executed with: 7.5 U/ml salivary amylase for 1 h, 2000 U/ml pepsin for 2 h, and 10 U/ml pancreatin, for 2 h. All samples were isoprotein adjusted at 40 mg of freeze-dried milled sample.

3.2.2. Degree of hydrolysis (DH%) of edible filamentous fungi and other common food proteins

DH% indicates the extent of the protein hydrolysis by comparing the number of primary amines of the digested sample to the initial total amino acids. Fig. 3a shows the total DH% after digestion of casein, three muscle food products (salmon fillet, chicken breast, and beef tenderloin), and 5 species of edible filamentous fungi. The initial sample prior to digestion, and the gastric as well as intestinal phases are depicted. The initial sample denoted the fractions of primary amines that were already released into the aqueous phase before the addition of digestive enzymes, while the gastric and intestinal phases indicated the released primary amine induced by pepsin and pancreatin, respectively. The salmon had an initial DH of 3.2%; after being hydrolyzed by pepsin in the gastric phase, the DH increases to 32%, followed by the intestinal phase where the DH reached 70%. The chicken breast and beef had comparable values with a final DH of 71% and 63%, respectively. All the food proteins reached significantly higher DH% than the control casein, which showed DH of 18% and 55% after the gastric and intestinal phases, respectively.

Among the species of edible filamentous fungi examined, protein from *N. intermedia* had the highest total measured DH with 90% after intestinal digestion, followed by *A. oryzae*, and *F. venenatum* at 85% and 80%, respectively. The high protein DH% of edible filamentous fungi in comparison with the common protein source such as chicken breast was however explained by a higher apparent initial DH% prior to subjecting it to digestion. *R. delemar* and *R. oligosporus* had the lowest total DH% after intestinal digestion among the edible filamentous fungi, 72% and 62%, respectively. This was explained by a lower initial DH% and a lower initial plus gastric DH%, respectively, and visualizes differences between different phyla of edible filamentous fungi. Ascomycetes fungi (*Aspergillus oryzae, Neurospora intermedia*, and *Fusarium venenatum*) cell walls contain β-glucan and chitin, while zygomycetes fungi (*Rhizopus delemar* and *Rhizopus oligosporus*) cell walls are constituted of chitin and chitosan. The mechanism by which different cell wall structures affect the protein digestibility in the gastric and intestinal steps requires further studies to be confirmed.

The high total protein DH% of fungi after completed GI digestion was mostly due to the high initial DH% of *A. oryzae*, *N. intermedia*, and *F. Venenatum;* ranging from 27% to 37% and thus corresponding to half of the total released of primary amines after the intestinal phase digestion. Also for *R. delemar*, the initial DH% was relatively high; 19.4%. One of the possible reasons is the presence of free amino acids in the cytoplasm of the fungi. Filamentous fungi cultivated in a liquid media could consist of soluble nitrogenous compounds and free amino acids up to 12% and 7% (w/w) of the total nitrogen, respectively (Bent & Morton, 1964). Another reason is the possible interference from non-protein amino compounds such as γ-aminobutyric acid, RNA, glucosamine, and several non-protein amino acids, when measuring the released primary amines. γ-Aminobutyric acid could make up 13% of total amino nitrogen (Bent & Morton, 1964), while RNA can make up 8%–10% of fungal dry-weight (Whittaker et al., 2020) or between 8 g and 25 g per 100 g protein (Kihlberg, 1972). Further, yeast extract, which was used in this study as the growth media, could stimulate the production of carnitine, which is also a non-protein amino-containing compound (Rousta et al., 2021). Altogether, the high initial degree of hydrolysis of some of the studied edible filamentous fungi requires further investigation to be confirmed.

In this study, casein was used as the control during the *in vitro* digestions. Its DH% after intestinal digestion (55%) was in good agreement with the study of Picariello et al. (2015) using casein as the protein substrate. The DH%, however, was higher in comparison to the study by Trigo et al. (2021) who reported 32.9% for casein. The reason could be the higher amount of pancreatin per gram protein substrate used during intestinal digestion; despite the ten-fold reduction compared to the INFOGEST protocol (10 U/ml); Trigo et al. (2021) only used 0.6 U/ml. In the same manner, the DH% of a raw chicken breast after intestinal digestion with a lower amount of pancreatin (approximately 4.8 U/ml) was at about 50% (Sangsawad et al., 2016), which is lower than our observed DH of 70%. On the other hand, Chen et al. (2020) used a higher amount of pancreatin (100 U/ml), as suggested by INFOGEST protocol, and reported a DH after *in vitro* intestinal digestion of cooked chicken breast of 89%. This is a strong indication that the amount of pancreatin in the intestinal phase affects the DH% of food proteins. The protein DH % for a salmon fillet in this study (70%) was however in correspondence with a previous study using the INFOGEST protocol with the full amount of pancreatin (Asensio-Grau et al., 2021), revealing a DH of 70%. Regarding beef, our result on DH (63%) agreed with the previous study

by Hernández-Olivas et al. (2022) at 70% in which they used 100 U/ml pancreatin.

To get a true estimate of the protein hydrolysis caused by the GI-digestion *per se*, the initial DH% can be subtracted from the final value obtained after gastric and intestinal phase digestion (Fig. 3b). This normalization revealed that the DH% of edible filamentous fungi induced by the GI-digestion (43–62%) was similar to, or lower than that for salmon, chicken and beef (59–67%) with significantly lower values being obtained for *Fusarium venenatum*. After normalization, the hydrolysis reached after the gastric and intestinal hydrolysis of *F. venenatum* was 14% and 43.5%, respectively. This result corresponds to a previous study on *in vitro* digestion of edible filamentous fungi by Colosimo et al. (2020), showing 20% released peptides in the gastric phase and 45% in the intestine phase. Another previous study showed that the *in vitro* digestion of edible filamentous fungi released primary amines at a similar amount as several other alternative protein sources, such as whey, yeast, and potato protein concentrate (Ariëns et al., 2021). That protein DH% of several species of edible filamentous fungi (*A. oryzae, N. intermedia, R.delemar,* and *R.oligosporus*) was comparable to that of common muscle food proteins, which do not contain cell wall structures, supports the study by Colosimo et al. (2020), suggesting that the main mechanism of proteolysis of fungal protein is driven by diffusion of digestive enzymes through fungal cell walls.

Casein, meat, and fish have been studied for the true ileal amino acid digestibility in pig and human, showing high digestibility values (>90%) (Bailey et al., 2020; Udall et al., 1984b). In this study, the common food proteins have a value for DH% ranging from 59%–67%. The DH% measurement has been validated as the proxy for the *in vivo* digestibility value (Sousa et al., 2023). However, true ileal amino acid digestibility includes absorption by intestinal cells. Moreover, the intestinal cell is known to secrete brush border enzyme, which can hydrolyze the oligopeptide into free amino acid. The low DH% value of common food protein compared to the *in vivo* method was due to the lack of the addition of brush border enzyme. Fungi has a comparable and slightly lower protein degree of hydrolysis compared to a common food protein. This is in line with the finding from the *in vivo* study in humans which showed filamentous fungi *Fusarium graminearium* having a protein digestibility value of 78% compared to milk protein at 95% (Udall et al., 1984b).

3.2.3. Polypeptide molecular weight of digested proteins before and after digestion

SDS-PAGE was used to visualize the molecular weight (MW) distribution of polypeptides from salmon, chicken breast, beef, and the five species of edible filamentous fungi prior digestion, after gastric digestion, and after intestinal digestion. The initial polypeptide profile was distinguishable for each sample (Fig. 4). Casein polypeptides are distributed between 25 kDa–37 kDa, consisting mostly of beta-casein. Salmon, chicken breast, and beef showed characteristic bands of muscle proteins such as myosin heavy chain (~205 kDa) and actin (~42 kDa). Salmon and chicken breast in general had a higher diversity of polypeptides compared to beef.

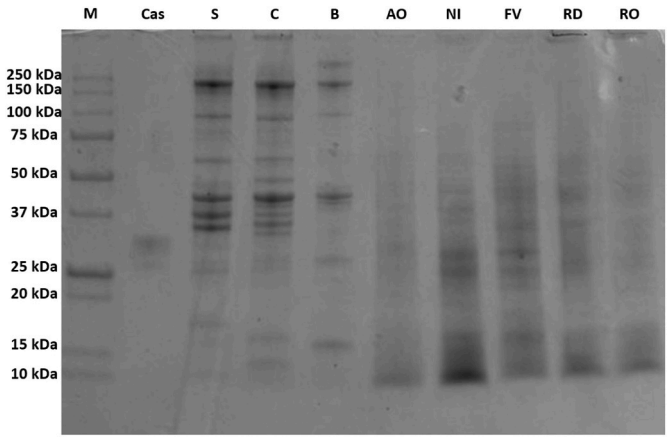

Fig. 4. SDS-PAGE gel of polypeptides in the initial sample prior gastrointestinal digestion. M: molecular weight marker; Cas: casein; S: salmon; C: chicken breast; B: beef; AO: *Aspergillus oryzae*; NI: *Neurospora intermedia*; FV: *Fusarium venenatum*; RD: *Rhizopus delemar*; RO: *Rhizopus oligosporus.*

All of the edible filamentous fungi had a noticeably different polypeptide profile compared to salmon, chicken breast, and beef in that they were enriched in polypeptides between 20 and 100 kDa and < 15 kDa. It was reported the average molecular mass of fungal protein is 50.96 kDa with only less than 10% of the protein in most fungi species having molecular weight of more than 100 kDa (Mohanta et al., 2021). The same pattern of polypeptides as we found for *F. venenatum*, was also observed by Colosimo et al. (2020).

During the gastric phase, most of the polypeptides were hydrolyzed into peptides with MW < 10 kDa, with only minor amounts of peptides/polypeptides > 20 kDa (Fig. S1). *R. oligosporus* was the only filamentous fungi species that responded differently to digestion by pepsin in the gastric phase, with significantly less digestion. This corresponds to the measurements of DH% (Fig. 3). During the intestinal phase, all polypeptides had been digested and could not be differentiated from the blank (Fig. S1).

3.2.4. Amino acid accessibility of edible filamentous fungi and common food proteins

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, solubilized and non-solubilized matter was separated by a 0.22 μm filter (Trigo et al., 2021). The digested water blank was used to normalize the released total amino acids in the intestinal digests against the background caused by autolysis.

As presented in Table 2, all amino acids from the control protein casein were accessible. In addition, the three muscle food protein sources also had a high amino acid accessibility, > 90% (Table 2). Casein and muscle sources could not be significantly differentiated ($p < 0.05$). The amino acid accessibility of edible filamentous fungi varied between different species. *A. oryzae* and *R. oligosporus* had an average amino acid accessibility comparable to muscle; at about 92%, and were also not significantly different from casein. The three other species of edible filamentous fungi, however, had significantly ($p < 0.05$) lower amino acid accessibility at about 85%. Accessibility of EAA from muscle was >95% and *A. oryzae* had the highest average EAA accessibility of all species of edible filamentous fungi at 96% followed by *N. intermedia* (92%). None of these samples significantly differed from casein ($p < 0.05$) while *R. oligosporus* (89%), *F. venenatum* (86%), and *R. delemar* (85%) had significantly lower EAA accessibility than casein. Most of the individual amino acids had accessibility > 80%, except histidine, at about 50% (Table 3).

The result agreed with the normalized DH% values (Fig. 3b), supporting the hypothesis that the presence of non-amino acid-derived primary amines contributed to the high initial values of DH% in the fungal samples (Fig. 3a). *A. oryzae* and *R. oligosporus,* which were the two edible filamentous fungi with the highest total protein accessibility, both formed pellets during the cultivation in the bioreactor, suggesting a potential effect of the fungal morphology. To the author's knowledge, no study has investigated this hypothesis, but it should be further studied.

The total amino acid and essential amino acid accessibility of filamentous fungi was thus comparable to that of common food protein, indicating the protein is readily hydrolyzed by gastrointestinal enzymes. However, it should be stressed that amino acid accessibility only gives an indication that the filtered digests are accessible for uptake by intestinal absorption. This result should be further investigated using e.g., the Caco-2 cell model and subsequently *in vivo*. Several factors such as post-cultivation heat inactivation of RNA, downstream fungi processing,

Table 2
Amino acid accessibility of casein, salmon fillet, chicken breast, beef tenderloin, and five species of edible filamentous fungi after *in vitro* digestion. Results are expressed as mean values ± standard deviation, n = 3, and show % of total amino acids in the digested samples that passed through a 0.22 μm filter.

	Initial		Filtered digest (mg)		Accessibility (%)	
	Total amino acid (mg)	Total essential amino acid (mg)	Total amino acid (mg)	Total essential amino acid (mg)	Accessible total amino acid (%)	Accessible essential amino acid (%)
Digestion blank	0	0	10.03 ± 0.194	3.88 ± 0.231	0	0
Casein	39.71 ± 0.612	16.73 ± 0.25	50.57 ± 0.792	20.94 ± 0.782	98.22 ± 6.3^{a}	101.97 ± 5.5^{e}
Salmon	40.07 ± 0.239	17.87 ± 0.10	42.08 ± 1.567	20.88 ± 0.141	90.90 ± 2.87^{ab}	95.13 ± 0.71^{efgh}
Chicken breast	39.23 ± 0.419	17.27 ± 0.18	42.9 ± 1.999	20.89 ± 0.479	94.13 ± 4.53^{ab}	98.5 ± 3.20^{ef}
Beef	39.84 ± 0.166	17.06 ± 0.07	43.14 ± 1.51	20.37 ± 0.721	92.02 ± 3.62^{ab}	96.61 ± 3.93^{efg}
Aspergillus oryzae	39.80 ± 0.141	16.35 ± 0.05	42.1 ± 0.444	19.62 ± 0.303	92.82 ± 0.46^{ab}	96.28 ± 1.54^{efg}
Neurospora intermedia	42.58 ± 0.170	16.88 ± 0.06	40.36 ± 2.086	19.45 ± 0.699	81.79 ± 5.33^{b}	92.21 ± 3.87^{efgh}
Fusarium venenatum	42.09 ± 0.08	18.08 ± 0.03	41.19 ± 0.716	19.58 ± 0.674	83.63 ± 5.39^{b}	86.79 ± 3.57^{gh}
Rhizopus delemar	41.27 ± 0.438	18.58 ± 0.197	41.01 ± 2.278	29.74 ± 0.886	84.7 ± 6.93^{b}	85.4 ± 5.68^{h}
Rhizopus oligosporus	39.95 ± 0.101	19.17 ± 0.04	42.73 ± 0.381	20.94 ± 0.399	92.25 ± 1.00^{ab}	89 ± 1.87^{fgh}

Table 3
Accessibility of each essential amino acid. AO: *Aspergillus oryzae*; NI: *Neurospora intermedia*; FV: *Fusarium venenatum*; RD: *Rhizopus delemar*; RO: *Rhizopus oligosporus*. The subscript is used to indicate the significant different between different sample for each amino acid.

	Histidine	Leucine	Isoleucine	Lysine	Phenylalanine	Threonine	Valine	Methionine
Casein	48.46 ± 6.63^{ab}	100.34 ± 8.15^{a}	105.9 ± 10.81^{a}	110.15 ± 2.4^{a}	103.7 ± 10.32^{a}	104.35 ± 5.51^{a}	109.14 ± 7.91^{ab}	88,48 ± 15,72^{ab}
Salmon	49.45 ± 9.35^{ab}	91.59 ± 3.62^{ab}	89.63 ± 7.02^{ab}	98.27 ± 5.07^{bcd}	96.8 ± 5.32^{a}	99.57 ± 4.11^{a}	101.41 ± 6.73^{abc}	112,18 ± 17,3^{ab}
Chicken	50.15 ± 2.45^{ab}	100.93 ± 4.57^{a}	103.79 ± 5.54^{ab}	106.26 ± 1.85^{ab}	98.38 ± 6.65^{a}	99.05 ± 4.3^{a}	110.67±4^{a}	71,77 ± 9,97^{b}
Beef	58.79 ± 8.42^{a}	101.19 ± 1.29^{a}	106.67 ± 2.78^{a}	96.12 ± 3.98^{cd}	106.3 ± 10.79^{a}	91.1 ± 7.32^{a}	116.0 ± 2.35^{a}	58,79 ± 11,77^{b}
AO	48.66 ± 1.73^{ab}	95.32 ± 0.81^{ab}	99.24 ± 1.98^{ab}	103.03 ± 4.31^{abc}	98.21 ± 1.67^{a}	91.72 ± 2.06^{a}	108.57 ± 2.05^{ab}	60,3 ± 18,98^{b}
NI	38.26 ± 4.51^{b}	89.59 ± 4.38^{abc}	90.83 ± 9.24^{ab}	109.79 ± 1.81^{a}	93.48 ± 6.22^{a}	96.55 ± 4.68^{a}	99.07 ± 7.08^{abc}	44,91 ± 14,51^{b}
FV	48.73 ± 6.58^{ab}	78.54 ± 4.03^{c}	83.05 ± 9.5^{b}	91.5 ± 2.63^{d}	90.11 ± 6.96^{a}	92.06 ± 4.48^{a}	86.8 ± 8.79^{c}	116,21 ± 5,13^{a}
RD	44.85 ± 3.37^{ab}	83.5 ± 6.72^{bc}	85.16 ± 11.14^{ab}	90.43 ± 2.58^{d}	91.97 ± 8.18^{a}	92.35 ± 4.59^{a}	86.36 ± 10.75^{c}	69,06 ± 26,66^{b}
RO	46.79 ± 1.87^{ab}	86.31 ± 1.37^{bc}	88.12 ± 6.34^{ab}	91.43 ± 0.92^{d}	98.41 ± 2.65^{a}	97.59 ± 3.42^{a}	90.83 ± 0.29^{bc}	97,61 ± 32,36^{b}

food formulation and final cooking may all affect protein digestibility and amino acid accessibility, and thus, also require investigation in future studies. For example, strong heat or oxidation may induce protein cross-linking, which prevents the access of digestive enzymes to the proteins (Lund et al., 2011).

4. Conclusion

This study aimed to assess the *in vitro* protein digestibility as well as amino acid accessibility of edible filamentous fungi and compare it with other common food proteins on the market, including casein, salmon, chicken breast, and beef. The food products were digested using a slightly modified version of the INFOGEST protocol, in which the amount of salivary and pancreatic enzymes was reduced ten-fold to mitigate the overwhelming background otherwise created in DH% and amino acid analyses. The *in vitro* digestions of edible filamentous fungi gave rise to DH% between 43% and 58, which was similar to (*A. oryzae, N.intermedia, R.oligosporus*), or lower (*F. venenatum, R.delemar*), than the DH% for salmon, chicken and beef (59%–67%); salmon which provided the highest value. *A. oryzae* and *R. oligosporus* also had an amino acid accessibility comparable to the muscle proteins, which also comprised the accessibility of EAA. An interesting notification was done in the pre-trials; that freeze-dried and milled *A. oryzae* provided higher DH% during digestion than fresh biomass. Altogether, our study pointed at a high protein DH% and amino acid accessibility of edible filamentous fungi, shedding light on its great potential as a sustainable food protein source in future diets.

CRediT authorship contribution statement

Ricky Wang: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Taner Sar:** Methodology. **Amir Mahboubi:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Rikard Fristedt:** Methodology. **Mohammad J. Taherzadeh:** Conceptualization, Writing – review & editing, Supervision. **Ingrid Undeland:** Conceptualization, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102862.

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