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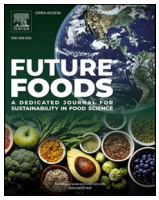

Impact of oat pretreatment on okara composition and its upcycling using a filamentous fungus for food applications: A biorefinery approach

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ABSTRACT

Oat milk production generates a substantial amount of a byproduct, named oat okara, which holds promise in advancing circular economy practices in the food industry when upcycled into food products or ingredients. In this study, filamentous fungus, *Rhizopus oligosporus* was employed to enhance nutritional properties, including a reduction of antinutritional factors. The fermented oat okara food product showed a modest increase in protein content, improvement in amino acid profiles, and reduction in phytic acid and saturated fatty acids relative to raw okara. In the second phase of the study, oat okara was produced through various enzymatic (α-amylase) and acidic (HCl, H_3PO_4) pretreatments to assess their impact on okara composition. The results demonstrated substantial starch reduction, especially under high enzyme load, and notable increase in protein and fiber contents. Overall, the results underscored that controlling processing parameters—particularly enzyme dosage and acid type—can significantly alter oat okara's nutritional and functional properties, making it more suitable for incorporation into functional food products.

1. Introduction

With increasing global awareness of environmental sustainability, there has been a significant shift in consumer preferences toward plant-based alternatives due to their reduced carbon footprint and alignment with sustainable development goals. Among plant-derived food sources, oats (Avena sativa) have garnered considerable attention due to their rich nutritional profile and versatility in various food applications. This is partly because oats are abundant in essential macronutrients, including carbohydrates, proteins, and the bioactive soluble fiber β-glucan, which has been extensively studied for its cholesterol-lowering effects and cardiovascular health benefits (Yang et al., 2023). In recent years, oat milk has emerged as a leading plant-based alternative to conventional dairy milk, characterized by a favorable nutritional composition and a lower environmental impact. Consumer preference for oat milk is driven by factors such as health benefits, mild sensory attributes, dietary restrictions, allergen considerations, and ethical concerns related to animal welfare (Yu et al., 2023). Consequently, the demand for oat milk is projected to increase substantially in the coming years.

A major byproduct of oat milk production is oat okara, a solid residue generated during the extraction process. Prior research indicated that for every kilogram of oat milk produced, approximately 0.45 kg of oat okara was generated (Helstad et al., 2023). On the other hand, sustainable management of agro-industrial residues, such as oat okara, has become a critical area of focus, and biorefineries have emerged as a promising approach to valorizing these byproducts. Guided by principles of green chemistry, waste valorization, and industrial symbiosis, biorefineries aim to transform agricultural residues into high-value products including biofuels, bioplastics, animal feed, and functional food ingredients. This integrated approach offers environmental benefits by minimizing waste generation and economic advantages by creating novel value chains and reducing reliance on nonrenewable raw materials. Current utilization strategies for oat okara include its application as animal feed, as well as its conversion into biogas or fertilizer (Wang et al., 2023). The valorization of oat okara within the framework of sustainable food systems presents an untapped opportunity for enhancing resource efficiency in an oat-based biorefinery.

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Oat okara is rich in proteins and dietary fibers, making it a suitable candidate for functional food applications. It has been reported to contain up to 32 % protein and 35 % fiber on a dry weight basis (dwb) (Helstad et al., 2023). Additionally, recent studies have identified oat okara as a valuable source of phenolic compounds, particularly phenolic acids and low-molecular-weight phenolics (Meanti et al., 2024). Presently, only a few food products or ingredients have been developed using oat okara. In one study, oat okara powders replaced wheat flour (5 % and 10 % w/w) in bread formulations with freeze-drying employed to preserve color, improve solubility, and moisture sorption. Higher okara substitution reduced baking loss, increased crumb density, and firmness (Nachtigall et al., 2025). Another study involved development of high-protein biscuits using oat okara as well as assessing the effects of chickpea flour fortification. Pure oat okara biscuits exhibited high protein, fiber, β-glucan, polyphenols, and antioxidant capacity. Chickpea flour enrichment was found to enhance protein, carbohydrates, and antioxidant properties while improving appearance (Wang et al., 2023). Furthermore, protein and dietary fiber from oat okara can be extracted using techniques such as air classification and ultrafiltration, respectively (Le et al., 2025).

Although these applications demonstrate oat okara's potential, they are limited in scope and do not fully exploit its functional and nutritional attributes. A promising alternative is microbial fermentation, which can enhance the digestibility, bioavailability, and sensory characteristics of plant-based ingredients (Hadj Saadoun et al., 2021; Ichikawa et al., 2022; Queiroz Santos et al., 2018). One particularly promising avenue is the fermentation of oat okara with the filamentous fungus *Rhizopus oligosporus*. A recent study showed that a fermentation process employing *R. oligosporus* enhanced the nutritional composition and flavor profile of sweet potato residue (Yin et al., 2023). In particular, the levels of soluble dietary fiber, protein, phenolic compounds, and flavonoids increased markedly, accompanied by a significant elevation in antioxidant activity. To our knowledge, however, to date there are no scientific reports on oat okara upcycling by fermentation using *R. oligosporus*.

The physicochemical characteristics of oat okara undoubtedly influence its upcycling potential. Besides the source of the raw oats, another key factor to consider are unit operations during oat milk extraction, such as milling, soaking, straining, as well as heat, enzymatic and chemical pretreatments (Yu et al., 2023). Of the mentioned operations, enzymatic and chemical pretreatments are of particular interest due to their extensive impact on a material's structure. Enzymatic hydrolysis using α-amylase, for instance, is performed to release glucose and maltose from oat starch. The β-glucan present in oats would also affect oat milk's viscosity, due to formation of high molecular weight (Mw) semisolid structures. Enzymatic depolymerization reduces Mw, improving solubility and sensory properties. Low Mw β-glucan also enhances the bioavailability of phenolic compounds. Pretreatment of oats with β-glucanase reduces Mw and improves water-holding capacity. In summary, enzymatic pretreatments are needed to refine oat milk texture, flavor, and nutritional profile, breaking down rough oat particles into smoother forms and releasing smaller molecular flavor substances, yielding sensory qualities comparable to traditional milk (Yu et al., 2023). Regarding chemical pretreatment, acids can play a role in starch modification (Sanika Bhokarikar, 2024). In addition, enhanced protein extraction from the oats can be achieved as previously demonstrated using H_3PO_4 (Babolanimogadam et al., 2023). Until now, literature has mostly focused on the impact of such pretreatments on characteristics of oat milk (Ibrahim et al., 2024; Ren et al., 2023; Sanika Bhokarikar, 2024) while assessments of their impacts on the properties of solid residue fraction are scarce (Aiello et al., 2021).

In light of these insights, this study aimed to develop a novel fermentation-based approach to enhance the nutritional and functional properties of oat okara. To upcycle oat okara into a food product, it should meet certain quality thresholds. Therefore, another key objective of this study was to investigate how upstream processing methods—such as enzymatic and chemical pretreatments—impact the nutritional composition of oat okara. It was hypothesized that fermentation with *R. oligosporus* would enhance the nutritional value of oat okara while enzymatic and chemical pretreatments during oat milk production modifies oat okara's structure, making it more suitable for food applications.

2. Materials and methods

In the first phase of this study, industrial okara from Elajo Technology Solutions (Oskarshamn, Sweden) was used, whereas in the second phase, non-industrial okara was produced in the laboratory.

2.1. Phase I: impact of fermentation on nutritional profile of oat okara

2.1.1. Substrate preparation

Dry industrial oat okara was kept in a dry place at room temperature until use. The industrial okara was first milled using a mill (Mockmill Lino 200, Iowa, USA) to achieve a uniform particle size. The milled okara was then combined with water and approximately 2 % *R. oligosporus* inoculant. The *R. oligosporus* spores were prepared according to methods described by Sandoval et al. (2024). Mixing of the wet substrate and inoculum was performed to ensure even dispersion and homogenization of the substrate prior to fermentation.

2.1.2. Pilot solid-state fermentation and final product preparation

To demonstrate oat okara upcycling into a familiar edible product, the fermented oat okara was transformed into meatball prototypes. The prepared substrate was fermented in a controlled fermentation plant at Millow AB, at a scale of 10 kg (on wet basis) per batch. This fermentation was carried out at a temperature of 32 °C and under optimal humidity levels of 80 – 90 % to support microbial activity for 24 h. Following fermentation, the biomass obtained was thoroughly mixed using a food processor (Kenwood, Tokyo, Japan). The mixture was shaped into uniform meatballs and subjected to a thermal pretreatment to ensure structural integrity. The formation of the prototypes was performed by hand as described by Penchalaraju et al. (2023) with some modifications. To maintain resource efficiency, the product development process was done without addition of any binders. The average diameter was 31 mm. The final product was stored frozen until subsequent nutritional evaluation.

2.2. Phase 2 - impact of enzymatic and acidic pretreatments during oat milk production on oat okara composition

2.2.1. Material

Whole grain oats were sourced from a local supermarket, ICA Nära, in Borås, Sweden. Enzymatic hydrolysis was conducted using heat-stable α-amylase (A3306; Merck KGaA, Darmstadt, Germany). Acidic pretreatment was performed using HCl and H_3PO_4. All chemicals were of analytical grade and bought from Merck KGaA, Darmstadt, Germany.

2.2.2. Oat milk preparation and collection of oat okara

Raw oats were milled to a fine consistency (0.5 mm) using a mill (Mockmill Lino 200, Iowa, USA). Oat slurry was prepared by mixing the oat flour with distilled water to achieve a dry matter content of 10 %. The acid pretreatments (Ac) involved adding 5 mL of 85 % phosphoric acid (AC-P) and 12 mL of 2 M hydrochloric acid (AC—H) to the slurry, followed by heating. After heating, the pH of each acid-treated sample was adjusted to 7.0 using 2 M sodium hydroxide (equivalent to 80 g/L, i.e., 8 g NaOH per 100 mL solution, w/v). For the enzymatic pretreatment (En), heat-stable α-amylase was first dissolved in phosphate buffer (pH 6.8) to prepare a working enzyme solution. From this buffered solution, 0.5 mL, 1.0 mL, and 2.0 mL were added to separate oat slurries for hydrolysis, corresponding to 30, 60, and 120 U/mL slurry in En-1, En-2, and En-3, respectively. The slurry was then heated to ensure

homogenization. The control pretreatment (C) was prepared by heating the slurry at 80 °C for 15 min. For acid pretreatments (AC—H, AC-P) the slurry was heated at 80 °C for 60 min. For enzymatic pretreatments (En-1, En-2, En-3) the slurry was heated at 80 °C for 60 min in the presence of heat-stable α-amylase.

The control, enzymatic, and acid-treated samples were processed using a Nutra Milk food processor (Nutra Milk, USA), followed by filtration through a cheesecloth to separate the oat milk from the solid residue (okara). The collected oat okara was used for compositional analysis of moisture, ash, protein, fat, and fiber content. A summary of the sample pretreatments is provided in Table 1.

2.3. Analytical methods

All samples were freeze-dried and ground using a ball mill (RETSCH GmbH, Haan, Germany) prior to analysis. Moisture content was determined using a KERN DBS 60–3 moisture analyzer (KERN & SOHN GmbH, Balingen-Frommern, Germany). Selected analyses, including mineral composition, total dietary fiber, amino acid profile, and fatty acid composition, were outsourced to Eurofins (Lidköping, Sweden), an accredited third-party laboratory.

2.3.1. Determination of crude protein and amino acid composition

The protein content of samples was quantified using the Kjeldahl method, with a nitrogen-to-protein conversion factor of 6.25 (Hayes, 2020). Nitrogen determination was carried out using an InKjel P digestion system and a behrotest® S1 distillation unit (Behr Labor-Technik, Düsseldorf, Germany). For each analysis, approximately 0.5 g of sample was digested with 20 mL of concentrated sulfuric acid (98 %) in the presence of an antifoaming agent and a KT1 catalyst tablet (Thompson & Capper Ltd, Runcorn, UK). The digestion was conducted at maximum power for 100 min, following a 10-minute preheating phase of the digestion block. After digestion, the acidic mixture was neutralized using 32 % sodium hydroxide and subjected to steam distillation for 5 min. The liberated ammonia was captured in 50 mL of a 4 % boric acid solution and subsequently quantified by titration with 0.1 M HCl until reaching a pH endpoint of 4.6.

The amino acids were determined using the ISO 13,903:2005 (ISO, 2005) with reference to EC Regulation 152/2009 (EC, 2009) and AOAC 994.12 (AOAC, 2023a). Samples (0.1–1.0 g) were oxidized with cold performic acid (prepared from formic acid, hydrogen peroxide, water, and phenol) at 0 °C for 16 h, followed by neutralization with sodium metabisulfite. Hydrolysis was performed using 6 N HCl containing 0.1 % phenol at 110 °C for 24 h.

After cooling and pH adjustment to 3–4, hydrolysates were diluted and spiked with isotopically labeled internal standards. Samples were derivatized using the AccQ-Fluor reagent kit (Waters Corporation, Milford, MA, USA) and analyzed by UHPLC: Vanquish (Thermo Fisher Scientific, Waltham, MA, USA); MS/MS: TSQ Altis (Thermo Fisher Scientific, San Jose, CA, USA) with multiple reaction monitoring (MRM). Quantification was based on external calibration using derivatized amino acid standards (Latimer, 2023).

2.3.2. Determination of total fat and fatty acid profile composition

Crude fat content was determined using the Labtec Line Digestion System (FOSS, DK-3400 Hilleroed, Denmark) with petroleum ether extraction. Freeze-dried samples (1.00 g) were placed in cellulose thimbles, and pre-weighed metal collection cups were filled with 45 mL petroleum ether (boiling point 40–60 °C).

Extraction was carried out by magnetic suction with a water flow rate of 2L/min. The system was operated at 85–90 °C with a rinsing phase for 15 min, followed by boiling for 30 min, and a final rinsing step for 10 min. After extraction, the cups were oven-dried at 103 °C for 30 min, cooled in a desiccator, and reweighed. Fat content was calculated gravimetrically based on the weight difference. Determination of fatty acids was performed using an internal method developed by Eurofins.

Table 1
Pretreatment conditions for each sample.

Sample label	Description
Control	Untreated oat slurry heated at 80 °C
En-1	Enzymatic pretreatment, 0.5 ml α-amylase, heated at 80 °C
En-2	Enzymatic pretreatment, 1 ml α-amylase, heated at 80 °C
En-3	Enzymatic pretreatment, 2 ml α-amylase, heated at 80 °C
AC-H	Acid pretreatment, 12 ml 2 M HCl, heated at 80 °C
AC-P	Acid pretreatment, 5 ml 85 % H3PO4, heated at 80 °C

2.3.3. Determination of dietary fiber

Total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF) in phase 1 were determined according to AOAC modified method (AOAC, 2023b). Approximately 1.00 g of dried and milled oat okara was enzymatically digested with heat-stable α-amylase at ~95 °C to gelatinize and hydrolyze starch, followed by protease digestion at 60 °C to remove proteins, and amyloglucosidase pretreatment to hydrolyze residual starch. The residue was filtered to separate IDF, which was washed, dried at 105 °C, and corrected for protein and ash content. The SDF in the filtrate was precipitated with 95 % ethanol, filtered, dried, and corrected similarly. TDF was calculated as the sum of IDF and SDF, and results were expressed as a percentage of the dry weight. In phase 2, Neutral detergent fiber (NDF) was measured based on the operator's manual using ANKOM200 Fiber Analyzer (ANKOM Technology, New York, USA), using the Van Soest method with heat-stable α-amylase and sodium sulfite. Approximately 1.00 g of dried, milled okara was placed in filter bags and digested in a neutral detergent solution at boiling temperature for 60 min. The residues were rinsed thoroughly with hot water and acetone, then dried at 103 °C to constant weight. NDF was calculated gravimetrically and expressed as a percentage of the sample's dry weight, representing the insoluble dietary fiber.

2.3.4. Determination of phytic acid

Phytic acid was quantified using the K-PHYT kit (Megazyme, Bray, Ireland) for total phosphorus, with some modifications to the kit protocol. This analysis was only performed on Phase I samples, that is, industrial oat okara and the fermented okara-based product.

2.3.5. Determination of starch and free sugar

Starch content was determined using the K-TSHK kit (Megazyme, Bray, Ireland), optimized for samples containing D-glucose and maltodextrins. Samples were first milled to pass through a 0.5 mm screen. A 100-mg portion of each sample was extracted with 80 % (v/v) aqueous ethanol, involving sequential heating, vortexing, and centrifugation steps to remove free sugars. The remaining pellet was treated with thermostable α-amylase (diluted 1:30 in 100 mM sodium acetate buffer, pH 5.0) and incubated in a boiling water bath for 6 min, with intermittent mixing. This was followed by hydrolysis with amyloglucosidase (20 U) at 50 °C for 30 min. The hydrolysate was transferred to a 100 mL volumetric flask, diluted to volume with distilled water, and centrifuged. An aliquot of the supernatant was analyzed enzymatically using a spectrophotometric method at 340 nm, where NADP+/ATP and hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) were used to quantify D-glucose. Absorbance was recorded before and after enzyme addition, and starch content was calculated based on the glucose released. Lactose, glucose, sucrose, fructose, maltose and galactose were analyzed using a modified AOAC method [18].

2.3.6. Determination of minerals

The mineral content of the samples was analyzed by using inductively coupled plasma mass spectrometry (NexION® 5000, PerkinElmer, Connecticut, USA), following the ISO 17,294–2:2023 standard (ISO). Samples were subjected to acid digestion prior to multi-element

analysis. The elements quantified included sodium (Na), potassium (K), iron (Fe), magnesium (Mg), calcium (Ca), and zinc (Zn). Results were reported in milligrams per kilogram (mg/kg) on a dry weight basis.

2.3.7. Determination of ash

Ash content was measured by incinerating approximately 200 mg of oven-dried sample in a muffle furnace at 550 °C for 3 h, following the guidelines of the American Association of Cereal Chemists . Prior to combustion, samples were dried overnight at 105 °C to remove moisture. The ash content was calculated and reported as a percentage of the sample's dry weight.

2.3.8. Statistical analysis

The experiments and analyses were performed in triplicate with data presented as mean ± 2 standard deviations. Data were analyzed using student's *t*-test on Office Excel 2024. A confidence interval of 95 % was implemented with the corresponding *p*-value <0.05 considered statistically significant.

3. Results

This study aimed at developing a novel fermentation-based strategy to improve the nutritional properties of oat okara. To facilitate its upcycling into viable food products, the material must achieve defined quality criteria with regards to its nutritional composition (Thorsen et al., 2022). Accordingly, the effects of upstream processing techniques—specifically enzymatic and chemical pretreatments—on the nutritional composition of oat okara should be elucidated. Experimental trials were conducted in two phases. During the initial phase, dry industrial oat okara was first prepared and underwent fungal fermentation in SSF after which the fungal biomass was converted to meat ball substitutes. The main goal of this phase was to evaluate the shift in nutritional properties and applicability of the final product as upcycled food. The comparison between oat okara and the fermented okara meatball highlights some modifications in the nutritional composition due to fermentation process. During the second phase, laboratory experiments were carried out with non-industrial oat okara prepared using either enzymatic or acidic pretreatment methods. The main objective of this phase was to evaluate the role of oat milk processing and applicability of the final product as upcycled food. The pretreatments included three enzymatic conditions (En-1, En-2, and En-3), as well as two acidic conditions (AC—H and AC-P), alongside a control group with raw oats. A comparative analysis of the pretreatments and their implications on oat composition was performed.

3.1. Phase I: impact of fermentation on the nutritional profile of oat okara

Table 2 shows the results of the proximate analysis performed on the industrial oat okara and the oat okara-based meat ball. There was also a decline in crude fat from 13.05 to 10.90 *g*/100 g, suggesting that lipid metabolism leads to potential conversion into secondary metabolites. One of the potential benefits of SSF with filamentous fungi is the augmentation of protein content in the substrate. Protein content increased slightly from 39.89 to 42.50 *g*/100 g. Though not significantly different ($p > 0.05$), the change in the present study indicated protein enrichment due to enzymatic activities and microbial growth.

The amino acid profile of the fermented okara-based meatball compared to oat okara revealed some nutritional improvements as illustrated in Table 3. Essential amino acids (EAA), such as lysine (1.53 to 1.87 *g*/100 g), alanine (1.81 to 2.23 *g*/100 g), and threonine (1.27 to 1.39 *g*/100 g), increased post-fermentation, enhancing the overall protein quality. However, slight decreases were observed in arginine (2.63 to 2.31 *g*/100 g) and glutamic acid (7.17 to 6.75 *g*/100 g), which may result from microbial metabolism. Methionine (0.65 to 0.50 *g*/100 g) and cysteine (1.06 to 0.62 *g*/100 g) decreased, potentially due to their involvement in sulfur metabolism during fermentation. Despite these reductions, the overall essential amino acid composition showed slight improvements from 11.02 to 11.54 *g*/100 g.

As shown in Table 4, fermentation led to considerable changes in the fatty acid profile. Saturated fatty acids generally decreased which is beneficial for health, as lower saturated fat intake is associated with reduced cardiovascular risk. A notable reduction in palmitic acid (C16:0) from 17.70 to 14.00 % supports this observation. Meanwhile, an increase in oleic acid (C18:1 n-9) from 36.50 to 41.70 % was noted, which enhances the nutritional value of the product by promoting heart health. Polyunsaturated fatty acids (PUFAs) slightly declined primarily due to the reduction in linoleic acid (C18:2 n-6) from 38.80 to 34.10 %.

As presented in Table 5, sodium was reduced from 488.06 to 250.00mg/100 g, potassium increased from 4668.44 to 6041.67mg/kg, and other minerals (Fe, Mg, Ca, Zn) showed either minor decline or increase. A reduction in phytic acid was recorded—from 3.1 % in unfermented oat okara to 1.7 % in the fermented product (approximately 45 % decrease).

3.2. Phase 2 - impact of enzymatic and acidic pretreatments during oat milk production on non-industrial oat okara composition

The enzymatic pretreatments indicate (Table 6) that a higher amount of enzyme resulted in either lower yield (En-3) or a yield comparable to the control (for En-2). This suggested that increasing the enzyme

Table 2
Characteristics of industrial oat okara compared with the fermented okara-based meat balls on dry weight basis (dwb). Values given as mean ± 2 standard deviations.

Parameter	Oat okara Value	Okara meat ball Value	Unit
Dry matter	94.25 ± 9.43	48.00 ± 4.80	*g*/100 *g*
Ash total	5.53 ± 0.55	5.92 ± 0.59	*g*/100 *g*
Crude Protein Kjeldahl (Nx6.25)	39.89 ± 3.99	42.50 ± 4.25	*g*/100 *g*
Crude fat	13.05 ± 1.31	10.90 ± 1.09	*g*/100 *g*
Dietary Fiber	29.07 ± 4.36	24.58 ± 2.69	*g*/100 *g*
Fructose	<0.04	<0.04	*g*/100 *g*
Glucose	0.11 ± 0.03	1.46 ± 0.22	*g*/100 *g*
Lactose	<0.04	<0.04	*g*/100 *g*
Maltose	8.60 ± 1.29	<0.05	*g*/100 *g*
Sucrose	0.30 ± 0.09	<0.06	*g*/100 *g*
Galactose (calculated)	<0.04	<0.07	*g*/100 *g*

Table 3
Amino acid profile of industrial oat okara compared with the fermented okara-based meat balls on dry weight basis (dwb). Values given as mean ± 2 standard deviations.

Parameter	Oat okara Value	Okara meat ball Value
Tryptophan	0.52 ± 0.05	0.51 ± 0.05
Alanine	1.81 ± 0.25	2.23 ± 0.31
Arginine	2.63 ± 0.37	2.31 ± 0.32
Aspartic acid	2.97 ± 0.42	3.29 ± 0.46
Glutamic acid	7.17 ± 1.00	6.75 ± 0.95
Glycine	1.90 ± 0.27	1.86 ± 0.26
Histidine	0.83 ± 0.12	0.88 ± 0.12
Hydroxyproline	<0.20	<0.20
Isoleucine	1.41 ± 0.20	1.50 ± 0.21
Leucine	2.83 ± 0.40	2.88 ± 0.40
Lysine	1.53 ± 0.21	1.87 ± 0.26
Ornithine	<0.05	<0.05
Phenylalanine	1.91 ± 0.27	1.91 ± 0.27
Proline	1.94 ± 0.27	1.88 ± 0.26
Serine	1.81 ± 0.25	1.79 ± 0.25
Threonine	1.27 ± 0.18	1.39 ± 0.19
Tyrosine	1.36 ± 0.19	1.18 ± 0.17
Valine	1.97 ± 0.28	2.01 ± 0.28
Cysteine +Cystine	1.06 ± 0.09	0.62 ± 0.09
Methionine	0.65 ± 0.09	0.50 ± 0.07

Table 4
A partial fatty acid profile of industrial oat okara compared with the fermented okara-based meat balls as % of fatty acids on dry weight basis (dwb). Values given as mean ± 2 standard deviations.

Parameter	Oat okara Value	Okara meat ball Value
C 14:0 (Myristic acid)	0.20 ± 0.04	0.20 ± 0.04
C 16:0 (Palmitic acid)	17.70 ± 1.77	14.00 ± 1.40
C 16:1 n-7 (Palmitoleic acid)	0.30 ± 0.06	0.30 ± 0.06
C 18:0 (Stearic acid)	1.20 ± 0.24	1.70 ± 0.34
C 18:1 n-9 (Oleic acid)	36.50 ± 3.65	41.70 ± 4.17
C 18:2 n-6 (Linoleic acid)	38.80 ± 3.88	34.10 ± 3.41
C 18:3 n-3 (α-Linolenic acid)	1.40 ± 0.28	1.40 ± 0.28
C 20:1 n-9 (Gondoic acid)	0.80 ± 0.16	0.80 ± 0.16
C 22:1	0.70 ± 0.14	0.20 ± 0.04

Table 5
Contents of phytic acid, selected mineral of industrial oat okara compared with the okara-based meat balls on dry weight basis (dwb). Values given as mean ± 2 standard deviations.

Parameter	Oat okara Value	Okara meat ball Value	Unit
Na	488.06 ± 122.02	250.00 ± 62.50	*g*/100 *g*
K	4668.44 ± 1167.11	6041.67 ± 1510.42	mg/kg
Fe	201.59 ± 50.40	191.67 ± 47.92	mg/kg
Mg	5411.14 ± 1352.79	4166.67 ± 1041.67	mg/kg
Ca	2970.82 ± 891.25	3958.33 ± 1187.50	mg/kg
Zn	137.93 ± 34.48	129.17 ± 32.29	mg/kg
Phytic acid	3.1 ± 0.45	1.7 ± 0.32	*g*/100 *g*

concentration does not necessarily enhance the yield of dried okara. For En-1, En-2, and En-3, the yields were 10.18 %, 8.28 %, and 7.01 % on a dry basis, respectively. Interestingly, the control sample, which was only heated, also had a yield of 8.28 %, similar to En-2. The differences in okara yield can be explained by the varying amounts of enzyme used. Lower enzyme concentrations (En-1) yielded the highest amount of okara, while higher enzyme concentrations (En-3) yielded the lowest. This trend suggested that appropriate enzyme concentrations might facilitate starch degradation, reducing the solid residue. However, the similar yields observed in the control and En-2 pretreatments indicate that other factors, such as the solubilization effect of heat or incomplete enzymatic hydrolysis, might also play a role.

The okara yield for samples AC—H and AC-P was 11.53 % and 11.60 %, respectively, indicating a similar yield between the two acidic pretreatments. Furthermore, when compared to the enzymatic pretreatments, the acid-treated samples exhibited a higher yield of okara. This difference in yield could potentially be attributed to the lower degree of starch degradation in the acid-treated samples, resulting in a higher residue content. The acidic pretreatments might have caused less extensive hydrolysis of starch molecules compared to enzymatic pretreatments, thereby leaving behind a greater proportion of the original oat material in the residue. Regarding the moisture content, the highest amount was 88.73 % in the okara under En-2 pretreatment. On the other hand, AC-P pretreatment resulted in okara samples with the lowest moisture concentration of 80.27 %.

The observed reduction in starch concentration upon the addition of the enzyme solution to the oat slurry highlights the enzyme's starch-degrading capability. En-1 resulted in 66.6 % reduction in starch concentration, indicating that the enzyme effectively hydrolyzes starch molecules into smaller oligosaccharides. Increasing the enzyme concentration to En-2 further decreased the starch concentration by 71.9 %, while En-3 caused 80.7 % starch reduction. This dose-dependent response suggested that higher enzyme concentrations enhanced the catalytic activity, leading to more extensive hydrolysis of starch. The enzyme likely acted by breaking the α−1,4 glycosidic bonds in the starch molecules, progressively reducing the starch content as the enzyme concentration increased (de Souza and de Oliveira Magalhães, 2010). In the control samples, which were heated for 15 min at 75 °C without enzymatic pretreatment, the starch decreased by 62.4 %, close to the starch reduction which was observed in the En-1 pretreatment. This similarity suggested that heating alone can solubilize starch molecules, therefore decreasing the starch content. The above findings underscore the enzyme's efficacy in starch degradation, with higher doses resulting in more breakdown of starch molecules. The results also highlight the role of heating in starch solubilization, suggesting a combined effect of heat and enzymatic pretreatment in reducing starch content.

The enzymatic pretreatments showed an apparent increase in protein content in the residue compared to raw oats ($p < 0.05$) which was probably due to the breaking down of starch resulting in freeing previously entrapped proteins. Aiello et al. (2021) similarly reported a significant improvement in the protein content of oat okara due to enzymatic pretreatment. In their study, oat okara was treated with different food-grade enzymes and the samples treated only with alpha amylase showed a marked release of bound carbohydrates, reflecting the enzyme's specificity for starch. As starch was degraded, more protein became accessible for extraction, leading to higher protein yields compared to the untreated control.

Acid hydrolysis at low water content was also found to significantly enrich the protein concentration in the solid (insoluble) residue derived from oat bran (Sibakov et al., 2013). In the referenced study, oat bran initially contained around 23 % protein, but after acid pretreatment with phosphoric acid at 100–130 °C, the hot-water extraction step removed much of the solubilized carbohydrates—particularly beta--glucan—leaving behind a more protein-dense fraction. Specifically, when oat bran was hydrolyzed at 100 °C, the remaining residue contained about 30.2 % protein; raising the temperature to 120 °C increased it to 34.5 %, and further increasing to 130 °C elevated it to 35.0 %. It is also worth mentioning that in the present study, HCl pretreatment had no significant difference in protein enrichment compared to H_3PO_4.

The results indicate that raw oats differ markedly ($p < 0.05$) from all other pretreatments in terms of fiber content. Comparisons among processed samples reveal that En-3 was significantly different from En-1 and En-2 ($p < 0.01$), but did not differ from AC—H or AC-P, suggesting that the chosen enzymatic conditions for En-3 produced fiber values comparable to acid-hydrolyzed versions. Meanwhile, differences among the other pretreatments, such as control, En-1, and AC-P, do not reach statistical significance under the current conditions. The fat content analysis showed that En-1 had the highest and AC—H had the lowest fat

Table 6
Characterization of non-industrial oat okara prepared using enzymatic and acidic pretreatments (unit: g/100 g on dry weight basis (dwb) except for moisture content. Values given as mean ± 2 standard deviations.

Sample	Okara yield	Protein content	Starch content	Fat content	Fiber content	Moisture content	Ash content
Raw oats	–	13.56±1.56	57.6 ± 1.40	6.50±0.60	14.22±1.76	8.20±0.52-	2.60±0.04
Control	8.28±0.55	22.60±2.90	21.40±0.56	10.00±0.40	40.00±1.87	85.22±0.24	4.60±0.02
En-1	10.18±0.60	22.24±0.78	19.25±0.10	7.80±0.20	39.00±1.00	88.06±0.24	4.60±0.02
En-2	8.28±0.53	22.10±5.12	16.34±3.00	7.52±0.20	39.00±0.80	86.73±0.10	3.95±0.00
En-3	7.01±0.50	24.03±3.60	11.78±0.66	7.65±0.60	43.00±0.80	83.80±1.10	5.23±0.02
AC-H	11.53±0.45	25.8 ± 3.00	22.34±0.32	7.10 ± 0.40	41.00±.60	88.55±0.62	0.60±0.02
AC-P	11.60±0.55	23.9 ± 0.12	21.86±1.04	7.50±0.20	41.00±1.20	80.27±0.12	4.20±0.00

content, despite control and raw oats.

4. Discussion

4.1. Phase I: impact of fermentation on the nutritional profile of oat okara

Similar to this study, Hellwig et al. (20222) observed a protein increase within the same range using bread as a substrate. In a separate study focusing on fermentation of several legumes including chickpeas, pigeon pea and soybean, the protein enrichment was in the range of 5 - 14 % (Toor et al., 2022). Higher protein enrichment has however been reported in other studies such as that by Canedo, et al. (2016) where an increase of more than 100 % was reported with brewery spent grain (BSG) as a substrate after 7 days. It can therefore be concluded that changes in the protein content are dependent on factors such as the substrate composition and process parameters applied.

Improvement in amino acid composition is beneficial for developing protein-rich food products with balanced amino acid profiles, catering to dietary needs and combating protein-energy malnutrition. The amino acid profile of a food product is crucial for its nutritional quality, especially concerning EAA that cannot be synthesized by the human body and must be obtained through the diet. SSF with filamentous fungi can enrich substrates with these vital amino acids. Similar to many filamentous fungi, *R. oligosporus* can produce proteases that cleave protein, thereby amending the profile of amino acids and increasing the concentration of EAA (Sandoval et al., 2024).

There is evidence suggesting that fermentation can also influence the lipid composition of substrates, including the fatty acid profile (Meanti et al., 2024) . During fermentation, the metabolic activities of fungi can lead to the degradation of certain fatty acids and the synthesis of others, thereby altering the overall fatty acid composition. For example, fungal fermentation may reduce the content of saturated fatty acids while increasing unsaturated fatty acids (Nachtigall et al., 2025) which are considered more beneficial for cardiovascular health. However, the extent and nature of these changes are highly dependent on the specific substrate and fungal species used. Further research is needed to elucidate the precise mechanisms and outcomes of fatty acid profile modification during SSF.

The effect of *R. oligosporus* fermentation on sodium and potassium contents is rarely studied. It is however known that proper maintenance of intracellular potassium and sodium concentrations is vital for cell growth (Yenush, 2016). With regards to the iron (Fe) content, a minor decline from 201.59 to 191.67mg/kg was observed. This decline was likely due to microbial utilization as iron is essential for numerous functions, including respiration as well as during synthesis of numerous molecules such as amino acids and lipids (Philpott, 2006). In the human diet, iron is indispensable for the production of oxygen-binding proteins (especially hemoglobin and myoglobin) and for assembling heme-containing as well as other iron-dependent enzymes that mediate vital electron transfer and redox reactions (Abbaspour et al., 2014). Magnesium (Mg) also decreased from 5411.14mg/kg to 4166.67mg/kg, possibly reflecting metabolic changes during SSF. This mineral is an important enzyme cofactor vital for numerous biochemical reactions, including cell division (Walker and White, 2017). Some studies have reported an increase in magnesium levels (Okorokov et al., 1975), which could be due to the mineralization processes during fungal growth.

Phytic acid is an antinutritional factor commonly found in plant-based foods. It chelates essential minerals such as iron, zinc, calcium, and magnesium, rendering them unavailable for absorption in the human digestive tract (Salim et al., 2023). The presence of phytic acid thus diminishes the nutritional value of foods. Fermentation has been shown to effectively reduce phytic acid content in the present study. The degradation of phytic acid during fermentation is primarily attributed to the production of phytase enzymes by the fermenting fungi (Rani and Ghosh, 2011). These enzymes hydrolyze phytic acid into inositol and free phosphate, thereby mitigating its antinutritional effects and enhancing mineral bioavailability. The observation in this work agrees with a previous study in which fermentation of flaxseed oil cake with *R. oligosporus* resulted in a decrease in phytic acid levels of up to 48 % (Duliński et al., 2017).

The effect of SSF on oat okara in this study was largely found to favor preservation of critical nutritional properties in the final food product while lowering a major antinutrient factor. Building on the discussion provided in this paper, a dedicated investigation on bioavailability of the minerals in the fermented product relative to the unfermented substrate could be valuable in subsequent studies. Furthermore, exploring benign techniques to boost the upcycled food's functionality is recommended for further investigations, for instance with regards to altering the concentration of bioactive compounds.

4.2. Phase 2 - impact of enzymatic and acidic pretreatments during oat milk production on oat okara composition

In the present work, increasing the starch concentration decreased while the protein, fiber and ash contents showed different trends when the enzyme concentration was raised from En-1 to En-3. En-3 okara showed the highest content in protein, fiber, and ash and contained 24.03 % protein, 43.00 % fiber, and 5.23 % ash. Pretreatment with HCl resulted in significant alterations in the oat composition. The HCl-treated okara showed decreased starch content from about 57 % in oat to about 22 % in okara and ash was found to be a minimal difference in oat okara, however fiber increased by only 1 %. The lowest ash content in the HCl-treated sample compared to all enzymatic pretreatment and phosphoric acid pretreatment suggested the strong solubilizing effect of HCl on mineral components. Phosphoric acid is a weaker triprotic acid and commonly used in the food and beverage industry for its buffering properties and ability to enhance flavors (Hansson et al., 2001). Both pretreatments using enzymes and acids effectively reduced the starch content in the okara. The enzymatic pretreatment using α-amylase (En-3) reduced the starch content by 11 %, the highest among the tested methods.

The yield of okara, which forms the solid fraction after oat milk extraction, was similar to Babolanimogadam et al. (2023) but half of what was reported by Deswal et al. (2014) after pretreatment with α-amylase. The conditions applied here could be beneficial oat milk facilities since recovery of the liquid fraction is their primary goal. Enzymatic pretreatments are widely used in oat milk production to boost the yield of the liquid fraction while reducing the solid residue. By breaking down β-glucans, starch, and proteins, enzymes such as amylases and proteases release more soluble solids and nutrients into the oat milk. As a result, the oat residue that remains after filtration was lower in total solids, as was the case presented here.

Sibakov et al. (2013) studied acid- and enzyme-catalyzed hydrolyses of an oat bran concentrate with high β-glucan content to evaluate changes in composition, solubility, and molecular weight. Before pretreatment, the bran had intact cell walls and significant amounts of β-glucan (33.9 %), protein (23 %), and starch (9.2 %). Under acidic conditions (ortho-phosphoric acid, 100–130 °C), 48–53 % of the bran was solubilized, with 62–69 % of β-glucan extracted. Average β-glucan molecular weight decreased from 780,000 to as low as 34,000 g/mol, and cell wall structures were mostly destroyed, producing sharper β-glucan distributions. The extracted fraction contained 42–46 % β-glucan, while insoluble residues retained about 6 % β-glucan. In contrast, enzymatic hydrolysis (Depol 740 L, 50 °C) required one to four hours and released 29–47 % of bran solids, extracting up to 78 % of β-glucan. The resulting extracted fraction held 52–59 % β-glucan. Both pretreatments improved the yield of soluble fractions.

In Table 6, enzymatic (En-3) and acidic (AC—H) pretreatments led to significant protein increases relative to raw oats. Specifically, En-3 displayed a 77 % increase (from 13.56 % to 24.03 % protein), while AC—H produced a 90 % increase (13.56 % to 25.80 %). This illustrates the effectiveness of both methods in enhancing the protein fraction of the

oat by-products—though the acidic pretreatment (AC—H) yielded a slightly higher gain. By contrast, Sibakov et al. (2013) observed more moderate increases in protein within the insoluble residues of hydrolyzed oat bran. Their enzyme-hydrolyzed residue showed around a 55 % increase (from 23.0 % to 35.6 %), and the acid-hydrolyzed residue, a 52 % increase (23.0 % to 35.0 %). Although the percentage increases are somewhat lower compared with Table 6, this difference likely arose from variations in the raw materials' overall composition, hydrolysis conditions (temperature, pH, residence time), and downstream separation processes. Overall, both datasets confirm the substantial potential of enzymatic or acidic modifications to concentrate protein in oat materials. Nevertheless, the higher protein increases in Table 6 could also reflect differences in methodology.

A comparison of starch concentrations from Table 6 and Sibakov et al. (2013) revealed differing impacts of enzymatic and acid pretreatments on oat-based materials. In Table 6, raw oats exhibited a high starch content (57.6 %), which undergoes significant reduction after processing. Enzymatic pretreatment (En-3) decreased starch to 11.78 %, amounting to a substantial overall reduction from the raw state. The acid-catalyzed counterpart (AC—H) retains more starch at 22.34 %, though it was still markedly lower than in the unprocessed oats. These decreases suggested that both hydrolytic strategies substantially break down or extract starch, with enzyme-based methods appearing more vigorous in Table 6, particularly at higher enzyme load. Previously, raw oat bran concentrate had a comparatively modest starch level (9.2 %) (Sibakov et al., 2013) Acid-hydrolyzed residues end up at 7.6–11.1 % starch, whereas enzymatically hydrolyzed residues range from 12.2 % to 14.2 %. Acid hydrolysis with mineral acids such as HCl can selectively disrupt the amorphous regions within starch granules. This attack shifts the relative proportion of crystalline domains, potentially increasing overall crystallinity and altering amylopectin chain organization (Wang and Copeland, 2015).

Collectively, the above findings underscore how starting material composition and processing parameters (time, temperature, enzyme dosage or acid type) critically influence starch depletion or retention, impacting the functional qualities of the resulting oat derivatives.

5. Conclusions

This work showed that oat okara can be successfully transformed into a valuable food ingredient via fungal fermentation and appropriate upstream processing. Fermentation modestly improved protein content, amino acid profile, and fatty acid composition, while also reducing phytic acid. Meanwhile, enzymatic pretreatments—particularly En-3—achieved dramatic starch reduction and elevated protein content, whereas acid hydrolysis (AC—H) conferred the highest overall protein gain but retained more starch. These variations highlight how specific methods align with different product goals. By transforming an underutilized oat milk byproduct into a nutrient-rich ingredient, this approach advances circular economy objectives and opens avenues for new plant-based food formulations with enhanced nutritional profiles.

Ethical statement – studies in humans and animals

The research reported in this manuscript focused exclusively on the physicochemical and microbial processing of plant-derived oat okara using the filamentous fungus *Rhizopus oligosporus*. It did **NOT** involve:

- Human participants, personal data, or human biological materials;
- Live vertebrate or higher invertebrate animals, animal tissue, or animal-derived primary cells.

Accordingly, no approval from an Institutional Review Board (IRB), Institutional Animal Care and Use Committee (IACUC), or equivalent ethics committee was required under national or institutional regulations. Because no human subjects were enrolled, informed consent and consent to publish were not applicable.

The study adheres to internationally recognized standards for research integrity, and where relevant, the principles of the Declaration of Helsinki, the ARRIVE guidelines, and the 3Rs (Replacement, Reduction, Refinement) for animal welfare. No deviations from these standards were necessary.

CRediT authorship contribution statement

Neda Rousta: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Steven Wainaina:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Formal analysis, Conceptualization. **Eva Wen Huey Lin:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis. **Nawal Iman:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis. **Coralie Hellwig:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Mohammad J. Taherzadeh:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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