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Fungal Bioprocessing to Improve Quality of Pennycress Meal as a Potential Feeding Ingredient for Monogastric Animals

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Abstract: Pennycress, as an annual cover crop in North America, has around 30–36% of oil and 20–25% of crude protein. Pennycress oil can be converted into biodiesel, while pennycress meal (PM) has limited use in animal nutrition, mainly due to the high content of glucosinolates and indigestible fiber. The nutrition of PM can be improved by processing with edible fungi. This study used *Pleurotus ostreatus* (PO), *Rhizopus oryzae* (RO), *Aspergillus oryzae* (AO), and *Mucor circinelloides* (MC) to ferment PM (60% moisture content) at 28 °C for 6 to 12 days. Compared to non-fermented PM, essential amino acids such as threonine (Thr) in PO and AO and tryptophan (Trp) and lysine (Lys) in all fungal treatments were enriched. PM fermented by all fungi resulted in concentrated digestible fiber (cellulose) at 12–46%. RO, AO, and MC-fermented PM had degraded sinigrin by 81, 33, and 12% and phytate by 47%, 37%, and 33%, with a corresponding increase in free P by 44%, 1.17-fold, and 89%, respectively. In addition, zearalenone was reduced by 97%, 50%, 39.3%, and 32% in PO, RO, AO, and MC-fermented PM, respectively. This study demonstrated the feasibility of fungi to improve the feeding value of PM, potentially promoting the economic return of pennycress plantations.

Keywords: amino acids; anti-nutritional compounds; animal feed; fungi; oilseed cover crop; pennycress meal

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1. Introduction

Soil erosion, fertilizer runoff, water contamination, and an intense carbon footprint are troubling impacts of some unstainable agricultural practices, leading to growing concerns for human beings over food security, ecosystem health, and climate change [1]. This could become a unique challenge in the Northern region of the U.S. and many other places around the world where the soil in the crop field is not covered with vegetation due to harsh winter conditions. The development of new crops is required to mitigate these problems. Traditional cover crops such as cereal rye and winter legume can limit soil nutrient loss and reduce soil erosion and water contamination; however, the economic return is low and costly to establish [2].

Pennycress (*Thlaspi arvense* L.) is an oilseed plant that has been advocated to serve as a new type of cover crop because of the high oil content in its seeds (up to 36%) compared to soybean (18–22%) [3]. The oil from the pennycress seed can be used for the production of biodiesel (fatty acid methyl esters) [4] and renewable jet fuel [5]. Pennycress can produce as much as 840 L/ha of oils and 1470 kg/ha of oil cake annually on 16 million hectares of farmland in the U.S. Midwest Corn Belt [6]. However, oil cakes or the meal of pennycress after oil extraction can have low economic value. Improving the value of pennycress meal (PM) could significantly increase the revenues of farmers and promote the increased cultivation of pennycress. PM is rich in protein and fiber, with up to 31% of crude protein and 19% of crude fiber [7]. This high protein content gives PM potential as a feeding ingredient for livestock, fish, and pets. However, wild-type PM has a very limited inclusion

rate in animal feed due to the high content of erucic acid, glucosinolates, and indigestible polysaccharides that have detrimental effects on monogastric animals [7]. The presence of mycotoxins in pennycress seeds, similar to other oilseed crops, can also have negative impacts on meal value and its uses.

Biological treatments of low-quality PM could have many advantages, including increasing the protein content [8], improving the amino acid profile [9], reducing indigestible fiber [10], reducing glucosinolates [11] and mycotoxins [12] simultaneously. While there are not many reports on the biological processing of oilseed meal for pennycress, biological processing using microorganisms and enzymes have been investigated in many studies to improve the nutritional values of other, similar oilseed meals, like canola meal and soybean meal [11]. Solid-state fermentation is a bioprocessing technique that grows microorganisms on solid materials in the absence or near absence of free water [13], and it is commonly used for feed fermentation. The most commonly used fermented feed is fermented soybean meal, which has low anti-nutritional factors and allergic compounds and is used mainly for young pigs [14]. Piglets fed a fermented soybean meal experienced an increase in their average daily gain (ADG) and an improvement in their gain-to-feed ratio (G:F) compared with the control due to the increased activities of total protease and trypsin in the duodenum and jejunum [15]. The solid-state fermentation of canola meal using *Lactobacillus salivarius* decreased glucosinolates and crude fiber by 38% and 16%, respectively [16]. Thermal pretreatment combined with the fungal fermentation of canola meal showed a decrease in the glucosinolate content by 99% and increased protein content by 23% [17]. Fermentation using inoculants with mixed fungal strains (co-culture) could have combined effects on the substrate that cannot be achieved with a single strain. Examples include using the co-culture of *Aureobasidium pullulans* and *Trichoderm reesei,* which synergistically reduced glucosinolates in canola meal by 92%, which is higher than using each of them separately [18].

Due to the great advantages of bioprocessing, fermented soybean meal by *Aspergillus* sp. (*A. oryzae*, *A. niger*, *A. usamii*), *Bacillus* sp., and lactic acid bacteria has been commercialized to serve as essential feed ingredients for young animals [14]. In addition, the bioprocessing of canola meal for valued feeding materials is moving toward commercialization [18]. Compared to canola meal and soybean meal, the bioprocessing of PM is new, and its commercialization could significantly increase an economic return to promote the plantation of pennycress for soil and water protection. The fermentation of PM could improve its nutritional value and increase its inclusion ratio in monogastric animal diet and, therefore, bring a greater economic return for the plantation of this oilseed cover crop.

The overarching goal of this study was to evaluate the monoculture of four generally regarded-as-safe (GRAS) fungal strains to process PM under solid-state fermentation for the improvement of nutritional value, including the amino acid profile, available phosphorous, other minerals, and in vitro digestibility, while reducing the indigestible fiber component, phytate, glucosinolate (sinigrin), and mycotoxins. This study highlights the potential of changing PM into a highly nutritional monogastric animal feed ingredient for the animal production market.

2. Materials and Methods

2.1. Fungal Strains Preparation

The four fungal strains used in this study were *Pleurotus ostreatus*, *Rhizopus oryzae*, *Aspergillus oryzae*, and *Mucor circinelloides*. *P. ostreatus* and *R. oryzae,* which were initially screened and maintained in the Department of Bioproducts and Biosystems Engineering at the University of Minnesota. *A. oryzae* (ATCC 1011) and *M. circinelloides* (ATCC 24905) (previously reported as *Mucor indicus*) were purchased from American Type Culture Collection (ATCC). The fungal strains *R. oryzae*, *A. oryzae*, and *M. circinelloides* were maintained and propagated in a potato dextrose agar (PDA) medium in a Petri dish (100 × 15 mm^2) for 5–10 days with *P. ostreatus* for 10–14 days at 28 °C to fully colonize the dish. Seed cultures for fermentation using *R. oryzae*, *A. oryzae*, and *M. circinelloides* were prepared as

follows. The colonized mycelium in each Petri dish was washed with sterilized deionized (DI) water to collect the spores. The spores in the solution of each species were counted with a cellometer (Auto X4, Nexcelom Bioscience, LLC., Lawrence, MA, USA) and diluted to a level between 1×10^6 and 1×10^7 spores per mL. A volume of 1 mL of the spore solution for each species was inoculated to 100 mL of sterilized (121 °C for 15 min) potato dextrose broth (PDB) in a 250 mL Erlenmeyer flask and was incubated shaken at 150 rpm for 2 days at 28 °C to form a pelletized mycelium, which was ready to inoculate a solid-state fermentation substrate. The inoculation for *P. ostreatus* was directly performed by cutting pieces (1 × 1 cm) of mycelium in a PDA Petri dish to the substrate.

2.2. Solid-State Fermentation

Pennycress meal was provided by the Agricultural Utilization Research Institute (Waseca, MN, USA). The PM used in this study had 82.5% of the total solid, 17.5% of the moisture content, 22.7% of the total amino acids (dry matter, DM, basis), and 15.1% of structural carbohydrates (DM basis). An amount of 10 g of PM was weighed into each 250 mL Erlenmeyer flask and moisturized with DI water to reach a moisture content of 60% (*v*/*w*). The flask with the substrate was plugged with a foam stopper (JAECE Industries, Inc., North Tonawanda, NY, USA) to prevent airborne microbial contaminants while allowing air exchange and was covered with alumina foil to prevent moisture loss during the autoclave, followed by sterilization at 121 °C for 30 min to eliminate any germs in the meal that could cause microbial contamination. The inoculation of *R. oryzae*, *A. oryzae,* and *M. circinelloides* to the PM was performed aseptically by transferring 5 mL of the inoculation seed culture to each flask. The inoculation of *P. ostreatus* was performed by transferring to each flask 6 pieces (each size of 10 × 10 mm^2) of mycelium from a fully colonized PDA Petri dish. For comparison, a control flask with a substrate without inoculation (Ctrl) was performed. The inoculated flasks were plugged with foam stoppers, hand mixed, and incubated statically at 28 °C for 6 days for *R. oryzae*, *A. oryzae*, and *M. circinelloides*, and 12 days for *P. ostreatus* (white-rot fungus grows slowly). After fermentation, the substrate with fungal biomass was homogenized by mixing and was dried at 60 °C for 48 h. Each flask was weighed before adding the substrate and after drying with fermented materials to calculate the dry matter (DM). The loss of DM in each fermentation flask was calculated as the percentage of the DM differences between the fermented substrate and the non-fermented one (Ctrl) over the non-fermented one. The dried material was ground with a coffee grinder to pass through a 2 mm sieve and was stored at −20 °C for further analysis.

2.3. Analytical Methods

Structural carbohydrates (SC, consists of glucan, xylan, arabinan, galactan, and mannan) for fermented and non-fermented PM were determined based on a two-step acid hydrolysis method as described by the National Renewable Energy Laboratory (NREL) [19] followed by sugar analysis using high-performance liquid chromatography (HPLC, 1200 Infinity Series, Agilent Technologies, Inc., Santa Clara, CA, USA) [9]. Cellulose concentration was expressed as the glucan concentration. Hemicellulose concentration was calculated as the total concentrations of xylan, arabinan, galactan, and mannan.

Soluble sugar in each sample was suspended with DI water at a ratio of 1:10 (*w*/*v*), and the liquid filtrate (through 0.22 μm PTFE membrane) was analyzed with HPLC for the concentration of glucose, xylose, galactose, arabinose, and mannose corresponding to the SC [10]. The total Kjeldahl nitrogen (TKN) and total ammonium nitrogen (TAN) were determined based on the Kjeldahl method, as described in a previous study [8]. Crude protein was calculated using organic N at the protein conversion factor of 6.25. Organic N was obtained by subtracting TAN from TKN. The amino acid (AA) hydrolysis of each sample was performed using 6 N HCl at 110 °C for 24 h based on the AOAC method [20]. The hydrolyzed sample was diluted and filtered through a 0.22 μm PTFE filter and the filtrate was used for HPLC analysis [21].

Phytic acid and free phosphorous (P) were analyzed using the Phytic acid assay kit (Neogen, Lansing, MI, USA) based on the published protocol [22]. Sinigrin in each PM sample was first extracted with 70% methanol and then analyzed with HPLC. The HPLC analysis of sinigrin was initially pumped with 100% of 50 mM KH_2PO_4 (pH 2.5) and diluted with pure acetonitrile for up to 75% within 15 min with a total flow rate of 1 mL/min. The separation column and detector were the same as for the AA analysis. The wavelength for the detector was 210 nm with a reference wavelength of 350 nm. The concentrations of total aflatoxins (AFT), deoxynivalenol (DON), and zearalenone (ZEN) for each ground dry sample were determined using ELISA assay kits (BioVision Inc., Milpitas, CA, USA). The Microplate Bio-kinetics reader (EL340, Bio-Tek Instruments, Winooski, VT, USA) was used to determine the absorbance at a 450 or 630 nm wavelength for each well in the microplate after reactions.

Soluble anions and cations were determined with an ion chromatography system (ICS) (Dionex Thermo Fisher Scientific, Waltham, MA, USA). Briefly, each sample was mixed with DI water at a ratio of 1:25 (*w*/*v*) in an 80 °C water bath for 1 h. The mixture was filtered through a 0.22 µm Nylon filter before analysis. Cations were analyzed using ICS-AQUION equipped with an IonPac CS12A analytical column and CDRS 600 suppressor (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 1 min/mL using 20 mM methanesulfonic acid as the mobile phase. Anions were analyzed using ICS-2100 equipped with the IonPac AS19 analytical column and AERS 500e suppressor (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 0.25 mL/min using 20 mM KOH as the mobile phase.

The in vitro dry matter digestibility (IVDMD) analysis for each sample was performed via sequential enzymatic hydrolysis using pepsin (P7000, 421 pepsin units per mg solids, Sigma-Aldrich, St. Louis, MO, USA) and pancreatin (P1750, four times the specifications of the United States Pharmacopeia, Sigma-Aldrich) to simulate the gastric and small intestine in monogastric animals [23]. IVDMD and the in vitro digestibility of AA (IVDAA) were analyzed and calculated as reported in a previous study [10].

2.4. Statistical Analysis

The statistical analysis was performed with Tukey's multiple comparisons with means at a 95% confidence interval (p-value < 0.05) using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA). The statistical analysis was used to determine the pairwise statistical differences ($p < 0.05$) of SC, soluble sugars, CP, AA, soluble ions, phytic acid, sinigrin, free P, mycotoxins (total AFT, DON, ZEN), IVDMD and IVDAA between each treatment.

3. Results and Discussion

3.1. PM as Monogastric Animal Feed Ingredient and Its Limitations

The pennycress meal was generated after the removal of oil by the cold pressing of pennycress seeds. The PM had a brown color similar to that of the seeds. The composition of PM is shown in Table 1. PM has three times the amount of glucosinolates (mainly sinigrin) compared to the canola meal (9–12 µmol/g) [24,25]. The CP, total AA, and phytate contents in PM were similar to those in the corn distiller grain and soluble (DGS), which contained 30.8%, 23.7%, and 1.5%, respectively [26]. The total SC in PM was only half that of corn DGS (36.7%) and the canola meal (30.7%). Phosphorous plant-based seeds are mainly stored in the form of phytate, which has a low availability for monogastric animals due to a lack of endogenous mucosal phytase [27]. Because of this, exogenous microbial phytase is commonly added to the monogastric of animal diets to assist the utilization of P in plant-based feed ingredients [28]. The high CP (31.1%) and low SC (15.1%) in PM could make it a potentially rich protein and low-fiber feed ingredient for monogastric animals. However, in a previous study, the inclusion rate of PM in chicken diets was limited to below 10% due to poor animal performance, which was mainly due to the effects of anti-nutritional factors such as indigestible fiber and glucosinolates [7]. Therefore, reducing anti-nutritional compounds such as indigestible fiber, glucosinolates, and phytate in PM could improve its inclusion ratio in the monogastric animal diet.

Table 1. Chemical composition of pennycress meal (PM) in this study.

Parameter [a]	PM (This Study)
TS, %	82.5
MC, %	17.5
CP, % DM basis	31.1
Total SC, % DM basis	15.1
Total AA, % DM basis	22.7
Phytate, % DM basis	1.6
Glucosinolates, μmol/g, DM basis	30 (sinigrin)

[a] TS, total solid; MC, moisture content; CP, crude protein; SC, structural carbohydrates (including glucan, xylan, arabinan, galactan and mannan); AA, amino acids; DM, dry matter.

3.2. Change in Structural Carbohydrates in PM by Fermentation

The solid-state fermentation of PM by fungal strains changed the appearance of PM due to a significant accumulation of fungal biomass. The chemical composition of PM after fermentation also changed. As shown in Table 2, there was around 15% (DM basis) of structural carbohydrates (cellulose and hemicellulose), with 8% of cellulose and 7% of hemicellulose. With lignin (not measured in this study due to protein interference), the total fiber concentration should be similar to between 17 and 19%, as reported elsewhere [7]. PM fermented by *P. ostreatus*, *R. oryzae*, *A. oryzae*, and *M. circinelloides* resulted in DM loss of 1.4, 8.8, 7.3, and 7.2%, respectively. The cellulose concentration in PM fermented by *P. ostreatus* increased by 34% while hemicellulose was reduced by 46% compared to non-fermented PM (Ctrl). The increased cellulose concentration in *P. ostreatus* fermented PM indicated the selective degradation of lignin and hemicellulose by *P. ostreatus*, which reduced 35% of lignin and 60% of xylan, while only 6% of glucan was reduced during the fermentation of wheat straw for 60 days [29]. Similarly, fermented PM by *R. oryzae* and *A. oryzae* showed an increased cellulose concentration of 23% and 46%, respectively, and reduced the hemicellulose concentration by 35% and 38%, respectively, compared to Ctrl. This could be due to the utilization of hemicellulose and the reduction in other components, such as protein and phytate, by *R. oryzae* and *A. oryzae* [9]. Fermented PM by *M. circinelloides*, however, did not experience a significant change in the concentrations of cellulose and hemicellulose compared to Ctrl, although it was reported to produce cellulolytic enzymes [30].

Table 2. Structural carbohydrates and residual sugars in non-fermented (Ctrl) and fungal-fermented PM samples.

	Ctrl	PO	RO	AO	MC
Solid recovery, %	100.00	98.56	91.24	92.71	92.82
			mg/g DM basis		
Cellulose	82.70 ± 0.01 ^{a}	111.40 ± 0.50 ^{cd}	101.99 ± 0.24 ^{bc}	120.99 ± 0.22 ^{d}	92.94 ± 0.04 ^{ab}
Hemicellulose	68.00 ± 0.37 ^{ab}	41.71 ± 0.14 ^{c}	49.71 ± 0.22 ^{c}	54.11 ± 0.03 ^{bc}	73.37 ± 0.71 ^{a}
Glucose	7.15 ± 0.49 ^{ab}	3.95 ± 0.49 ^{c}	4.10 ± 0.28 ^{bc}	8.15 ± 1.48 ^{a}	6.75 ± 0.64 ^{abc}
Xylose	0.95 ± 0.64 ^{a}	0.75 ± 1.06 ^{a}	0.00 ± 0.00 ^{a}	0.50 ± 0.71 ^{a}	1.00 ± 0.57 ^{a}
Galactose	2.45 ± 0.92 ^{a}	0.00 ± 0.00 ^{a}	0.85 ± 0.21 ^{a}	0.70 ± 0.99 ^{a}	1.05 ± 0.35 ^{a}
Mannose	0.55 ± 0.78 ^{b}	1.05 ± 0.21 ^{ab}	0.55 ± 0.07 ^{b}	1.05 ± 0.07 ^{ab}	2.15 ± 0.07 ^{a}

Ctrl: non-fermented PM; DM: dry matter; PO: *Pleurotus ostreatus*; RO: *Rhizopus oryzae*; AO: *Aspergillus oryzae*; MC: *Mucor circinelloides.* Different superscript letter in each row represents significant difference ($p < 0.05$); otherwise, there was no significant difference ($p > 0.05$).

There were 7.15 and 2.45 mg/g glucose and galactose in non-fermented PM (Table 2). The glucose concentration was reduced by 45% and 43% in *P. ostreatus* and *R. oryzae*-fermented PM but increased by 14% in *A. oryzae*-fermented PM. The galactose concentration was reduced by over 50% to 100% in all fermented PM. The remaining soluble sugars in fermented PM could be the combined results of sugar consumption and the hydrolysis of cellulose and hemicellulose during fermentation. Unlike lignin, cellulose is a microbial

digestible fiber component whose increased concentration in the feeding materials could provide extra energy and benefit the gut health, and immune functions of monogastric animals [31]. The fiber component escapes digestion by endogenous enzymes in the stomach and proximal small intestine but is used by residing microbes in the distal small intestine and large intestine, where it is converted to short-chain fatty acids (SCFA). SCFA promotes the growth of beneficial microbes, intestinal integrity and immune function. *P. ostreatus*, *R. oryzae*, and *A. oryzae*, therefore, could be used to improve digestible fiber in PM.

3.3. Change in Protein and Amino Acids Profile in PM by Fermentation

The CP and AA profiles of the non-fermented (Ctrl) and fermented PM are shown in Table 3. The total AA concentration in PM did not significantly change ($p > 0.05$) after fermentation with either of the fungal strains, although a higher total AA was observed in *P. ostreatus* when fermented PM (increased by 3.9%). However, *A. oryzae*, *R. oryzae*- and *M. circinelloides*-fermented PM had a reduced total AA concentration compared to the control. When considering the DM loss of the substrate during fermentation, a significant ($p < 0.05$) loss of total AA was found in *R. oryzae* and *M. circinelloides*-fermented PM, while no significant change ($p > 0.05$) was found in *P. ostreatus* and *A. oryzae*-fermented PM compared to the control. Fungal strains could utilize the protein in the substrates as their nitrogen source for metabolisms and biomass accumulation, and the protease produced during fermentation could break down the protein into a peptide and amino acids [32]. *R. oryzae*, *A. oryzae*, and *M. circinelloides* were reported to utilize AA in corn-distiller grains (containing around 30% CP) without an exogenous supply of the nitrogen source but used less substrate than AA when urea was supplied [9]. The metabolized product from AA, mainly ammonia or ammonium, was generated by all fungal treatments in this study compared to the control (Figure 1B). A similar study was also reported with the fermentation of corn distillers' grains by *R. oryzae,* where the ammonium produced was proportional to the AA loss [26]. Crude protein represents organic nitrogen (N) which includes AA-N, nucleic acid-N, chitin-N, etc. The CP content (considering substrate DM loss) showed a similar trend as the total AA content (considering DM loss), indicating that the loss of CP during fermentation was mainly due to the loss of AA (Table 3).

Based on the AA profile of each fermentation treatment, some AAs were reduced while other AAs increased compared to the control. The AA in the substrate during fermentation could have three destinations: directly used for fungal protein synthesis, converted to non-protein nitrogenous compounds, or oxidized for energy with the release of the amine group [33]. Glutamate (Glu) deamination is the process of releasing energy and ammonia. It was noted that the concentration of Glu was reduced in *R. oryzae* and *M. circinelloides* fermented PM by 19.6% ($p < 0.05$) and 27.6% ($p < 0.05$) compared to the Ctrl, which was proportional to the production of ammonium (NH_4^+) (Figure 1B). All other AAs except for lysine (Lys) and threonine (Thr) could progress through transamination to form aspartate (Asp), alanine (Ala), and Glu, which could also be formed via the transamination of Asp and Ala [33]. Asp and Ala both showed some increase in fermented PM (Table 3), indicating an active transamination of the AA during fermentation. There was an improvement in Lys ($p < 0.05$) and Thr ($p > 0.05$) in *P. ostreatus* and *A. oryzae*-fermented PM compared to the Ctrl. Branched-chain AA, such as isoleucine (Ile), was improved ($p < 0.05$) in *P. ostreatus*. These fungal fermented PM had an AA profile with an improved key AA such as Lys, Ile, and Thr, which have been considered as limiting AA for monogastric animals [34]. *P. ostreatus* and *A. oryzae* due to their relatively lower downgrade of AA (e.g., production of NH_4^+) and improved essential AA, are more preferable than *R. oryzae* and *M. circinelloides* in PM fermentation.

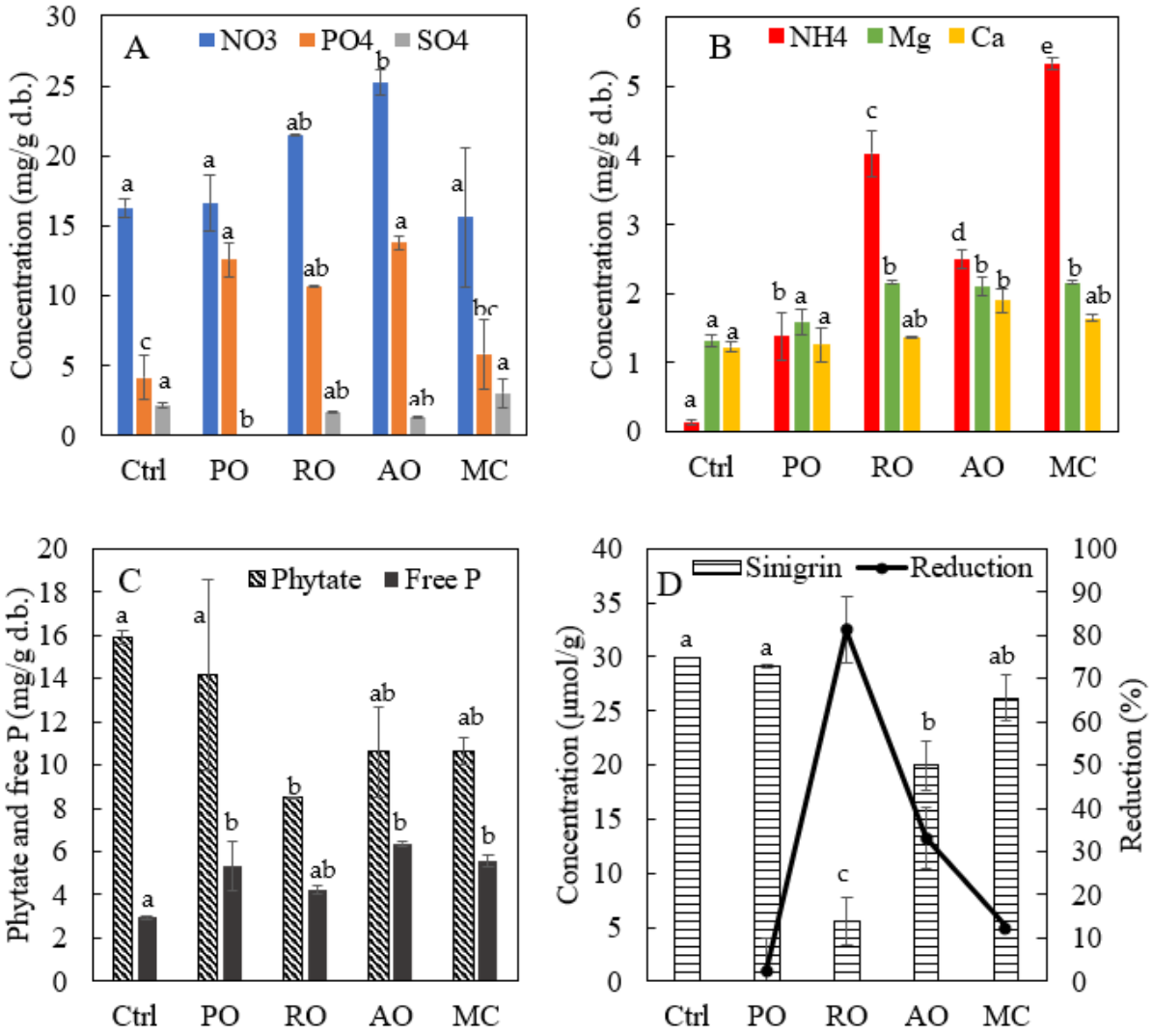

Figure 1. Concentration of selected anions (**A**), cations (**B**), phytate/free phosphorous (**C**) and sinigrin (**D**) in treatments without (Ctrl) and with fungal fermentation by *P. ostreatus* (PO), *R. oryzae* (RO), *A. oryzae* (AO), and *M. circinelloides* (MC). Value in each treatment was represented as the mean, and the error bar is represented as the standard deviation of two replicated fermentations. Different lower-case letters for the same category represents significant difference ($p < 0.05$).

Table 3. Crude protein (CP) and amino acids (AA) profile with essential AA (EAA) and non-essential AA (NEAA) in treatments without (Ctrl) and with fungal fermentation by *Pleurotus ostreatus* (PO), *Rhizopus oryzae* (RO), *Mucor circinelloides* (MC), and *Aspergillus oryzae* (AO).

	Ctrl	PO	RO	AO	MC
EAA			mg/g d.b.		
Arginine	19.20 ± 1.54 ^{a}	20.38 ± 0.48 ^{a}	14.94 ± 0.21 ^{b}	13.12 ± 0.43 ^{b}	12.60 ± 0.74 ^{b}
Histidine	6.09 ± 0.39 ^{a}	5.49 ± 0.06 ^{ab}	5.29 ± 0.25 ^{ab}	5.52 ± 0.08 ^{ab}	4.92 ± 0.21 ^{b}
Isoleucine	10.82 ± 0.43 ^{b}	12.12 ± 0.03 ^{a}	11.02 ± 0.33 ^{b}	11.54 ± 0.19 ^{ab}	10.68 ± 0.05 ^{b}
Leucine	18.69 ± 0.85 ^{a}	18.88 ± 0.09 ^{a}	17.11 ± 0.50 ^{a}	18.00 ± 0.35 ^{a}	17.13 ± 0.65 ^{a}
Lysine	9.41 ± 0.61 ^{b}	10.85 ± 0.09 ^{a}	10.21 ± 0.27 ^{ab}	11.35 ± 0.15 ^{a}	10.03 ± 0.35 ^{ab}
Methionine	3.96 ± 0.06 ^{a}	3.51 ± 0.10 ^{ab}	3.26 ± 0.21 ^{ab}	2.97 ± 0.25 ^{b}	3.65 ± 0.24 ^{ab}
Phenylalanine	11.61 ± 0.46 ^{ab}	11.98 ± 0.09 ^{a}	10.69 ± 0.32 ^{b}	10.85 ± 0.24 ^{ab}	10.46 ± 0.26 ^{b}
Threonine	11.87 ± 0.44 ^{a}	12.35 ± 0.06 ^{a}	11.28 ± 0.33 ^{a}	12.46 ± 0.19 ^{a}	11.73 ± 0.39 ^{a}
Valine	14.58 ± 0.66 ^{ab}	15.09 ± 0.36 ^{a}	13.04 ± 0.38 ^{b}	14.11 ± 0.20 ^{ab}	13.05 ± 0.43 ^{b}
Tryptophan	1.42 ± 0.32 ^{a}	1.46 ± 0.01 ^{a}	1.50 ± 0.00 ^{a}	1.56 ± 0.08 ^{a}	1.58 ± 0.11 ^{a}

Table 3. *Cont.*

	Ctrl	PO	RO	AO	MC
NEAA			mg/g d.b.		
Aspartate	16.99 ± 0.78 ^{a}	17.75 ± 0.09 ^{a}	17.15 ± 0.68 ^{a}	17.02 ± 0.52 ^{a}	16.85 ± 0.38 ^{a}
Glutamate	35.27 ± 1.46 ^{a}	32.99 ± 0.94 ^{a}	26.86 ± 0.95 ^{b}	31.96 ± 0.47 ^{a}	26.32 ± 0.77 ^{b}
Serine	10.63 ± 0.39 ^{c}	11.85 ± 0.08 ^{ab}	10.57 ± 0.26 ^{c}	12.22 ± 0.24 ^{a}	10.92 ± 0.41 ^{bc}
Glycine	15.95 ± 0.77 ^{a}	15.56 ± 0.51 ^{ab}	13.16 ± 0.31 ^{c}	13.94 ± 0.22 ^{bc}	12.96 ± 0.38 ^{c}
Alanine	13.35 ± 0.61 ^{b}	16.18 ± 1.57 ^{ab}	16.09 ± 0.82 ^{ab}	18.83 ± 0.28 ^{a}	16.50 ± 0.63 ^{ab}
Tyrosine	5.38 ± 0.30 ^{a}	4.34 ± 0.08 ^{a}	4.67 ± 0.78 ^{a}	5.27 ± 0.38 ^{a}	4.18 ± 0.30 ^{a}
Cystine	0.40 ± 0.01 ^{b}	0.48 ± 0.01 ^{a}	0.27 ± 0.03 ^{c}	0.44 ± 0.00 ^{ab}	0.23 ± 0.01 ^{c}
Hydroxyproline	21.33 ± 2.08 ^{a}	24.56 ± 0.48 ^{a}	25.96 ± 1.10 ^{a}	26.40 ± 0.61 ^{a}	23.31 ± 1.67 ^{a}
Glutamine	0.45 ± 0.00 ^{a}	0.42 ± 0.00 ^{ab}	0.41 ± 0.00 ^{abc}	0.37 ± 0.00 ^{c}	0.39 ± 0.02 ^{bc}
Total AA (mg/g d.b.)	227.39 ± 12.17 ^{a}	236.24 ± 4.38 ^{a}	213.50 ± 7.73 ^{a}	227.92 ± 3.35 ^{a}	207.47 ± 7.94 ^{a}
Total AA, with DM loss (mg/g)	218.78 ± 12.17 ^{ab}	232.89 ± 8.72 ^{a}	194.81 ± 8.29 ^{bc}	211.29 ± 2.73 ^{abc}	192.59 ± 7.72 ^{c}
Total CP, with DM loss (mg/g)	311.13 ± 2.56 ^{b}	340.06 ± 8.25 ^{a}	286.35 ± 7.10 ^{c}	306.82 ± 0.47 ^{b}	260.44 ± 0.39 ^{d}

Different superscript letter in each row represents significant differences ($p < 0.05$); otherwise, there was no significant difference ($p > 0.05$).

3.4. Change in Minerals, Phytate and Sinigrin in PM by Fermentation

Around 16 mg/g of phytate was present in PM, similar to corn DDGS (15 mg/g), which was lower than canola meal and cottonseed meal (28 mg/g) [26]. Phytate was degraded ($p < 0.05$) by 47% in PM fermented by *R. oryzae* and 33% in PM fermented by *A. oryzae* and *M. circinelloides*, compared to the Ctrl (Figure 1C). Different fungal species experienced a different degradability of phytate. *R. oryzae* was found to be an excellent phytase producer in solid-state fermentation [35]. *P. ostreatus*, *A. oryzae*, and *M. circinelloides* also showed a capacity for producing phytase [36–38]. The same strain might show different phytate degradation in different substrates. The phytate in corn DDGS was degraded by over 50% with *R. oryzae,* but only up to 10% phytate degradation was found in *R. oryzae* when fermented with canola meal and cottonseed meal [9,26]. By comparison, the free P (Figure 1C) or soluble phosphate (Figure 1A) was increased in fermented PM by *P. ostreatus*, *R. oryzae*, *A. oryzae,* and *M. circinelloides* compared to the control, which provides highly available P as a feed ingredient for monogastric animals. In addition to the direct release of P, the degradation of phytate also relieved its chelating with other mineral compounds, especially calcium (Ca), magnesium (Mg), and trace metals such as ferric (Fe) and zinc (Zn) [27]. It is shown in Figure 1B that the concentration of Mg increased in fungal-fermented PM, especially in *R. oryzae*, *A. oryzae*, and *M. circinelloides* ($p < 0.05$), while a significantly higher Ca concentration was noticed in *A. oryzae*-fermented PM compared to the control. NH_4^+ was produced by all fungal strains during fermentation (Figure 1B), but *R. oryzae* and *M. circinelloides* generated much higher NH_4^+ ($p < 0.05$) than the other fungi used in this study, which was mainly due to the deamination of AA (Table 3). The fungal strain *A. oryzae*, therefore, would be preferable for use considering the higher release of available minerals (P, Mg, Ca) and the lower generation of NH_4^+ compared to other fungal strains.

Sinigrin represents the majority of glucosinolates in PM. The concentration of PM in non-fermented and fermented PM is shown in Figure 1D. There was around 30 μmol/g of sinigrin in the non-fermented PM, which was similar to the genetically developed low-glucosinolate pennycress meal (up to 30 μmol/g) [39]. Wild-type pennycress seeds usually have 50 to 150 μmol/g of sinigrin, depending on their location and harvesting [40]. Pressing for oil extraction could degrade part of the glucosinolates, resulting in a PM with lower sinigrin. In addition, the autoclaving process of PM could degrade more glucosinolates, resulting in lower sinigrin in the meal before fermentation. PM was fermented for 6 days with *R. oryzae*, *A. oryzae*, and *M. circinelloides,* which resulted in the degradation of sinigrin by 81 ($p < 0.05$), 33 ($p < 0.05$) and 12% compared to the control (Figure 1D). However, *P. ostreatus* showed no degradation of sinigrin. It was reported that

the degradation of sinigrin mainly occurred intracellularly by a cytosolic enzyme called myrosinase [41]. Although the hydrolyzed products of sinigrin, allylisothiocyanate, and allylthiocyanate, could be generated after the direct myrosinase hydrolysis of sinigrin in penncyress, their presence was not detected in solid-state fermentation [41]. Sinigrin was reported to be degraded completely after 2 days by *Aspergillus clavatus* and *Fusarium oxysporum* in the solid-state fermentation of yellow mustard seed meal [41]. Bacteria species such as *Bifidobacterium pseudocatenulatum*, *B. adolescentis*, and *B. longum* could degrade sinigrin by 80% after 48 h [42]. Sinigrin has been considered a key antinutritional factor that prevents the use of PM as a valued feed ingredient. The toxicity of sinigrin for animals has been related primarily to the interference of iodine uptake and the synthesis of thyroid hormones, which could cause retard growth, impaired reproductivity, and liver and kidney functions [39]. The fungal strain *R. oryzae* was shown to have the highest degradability of sinigrin in this current study compared to other fungi used. *R. oryzae* was also reported to reduce aliphatic glucosinolates (including sinigrin) in three Brassicaseae species plants, *Sinapis alba*, *Brassica napus*, and *B. juncea* [43]. The degradation of sinigrin remarkably by *R. oryzae* and *A. oryzae* in PM proved a way for PM detoxification by GRAS fungi to increase the feeding value of PM to monogastric animals.

3.5. Change in Mycotoxins in PM by Fermentation

The total AFT, DON, and ZEN have been considered the most prevalent mycotoxins present in feedstuff, such as grains, seeds, and their co-products, and have significantly negative effects on the performance of animals, especially monogastric animals [12]. It was shown that (Figure 2A) the total AFT level in PM was below 0.25 ppb, which was lower than 20 ppb when regulated by the U.S. Food Drug Administration (FDA), indicating a lower risk of AFT in PM. A similar low level of total AFT was also found in corn DDGS (0.4–0.6 ppb) [9]. The fungal strains *P. ostreatus*, *R. oryzae*, and *M. circinelloides* had a slight reduction in the total AFT in PM during fermentation. However, *A. oryzae* did not show a reduction in the total AFT, which could be due to its close relation to *Aspergillus flavus*, which is known to produce aflatoxins [44]. A degradation in the total AFT by other *Aspergillus species* (e.g., *A. nigar*), *Rizopus species* (e.g., *R. oryzae*), and *Mucor* species (e.g., *M. alternans*, *M. griseocyanus*) have been reported [12].

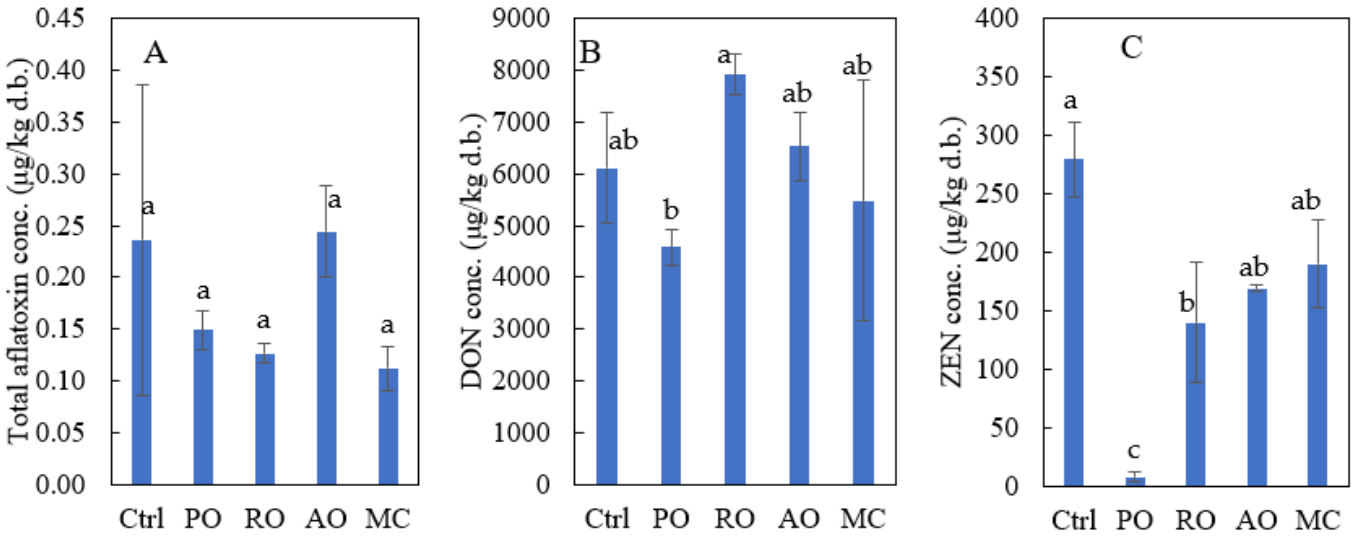

Figure 2. Total aflatoxin (**A**) deoxynivalenol, DON (**B**), and zearalenone, ZEN (**C**) in treatments without (control) and with fungal fermentation by *P. ostreatus* (PO), *R. oryzae* (RO), *A. oryzae* (AO), and *M. circinelloides* (MC). Different lower-case letters for the same category represent a significant difference ($p < 0.05$).

Compared to AFT, DON, and ZEN are the most commonly found mycotoxins with relatively higher levels [45]. According to FDA, the maximum allowable limit of DON in animal feed was 1000 ppb [46]. The European Union (EU) has regulated ZEN in animal feed to be less than 100 ppb for young pigs and 250 ppb for finishing pigs [47]. Both DON (Figure 2B) and ZEN (Figure 2C) in the PM of the current study exceeded the regulation limit by FDA and EU. Although the DON and ZEN levels could be diluted when supplemented in animal diets, their high levels could still raise many concerns for PM to be used as a feed ingredient by animal producers. The fermentation of PM in this study did not have significant effects ($p > 0.05$) on the reduction in DON by *R. oryzae*, *A. oryzae*, and *M. circinelloides*, although a 25% reduction in DON ($p > 0.05$) was observed in fermentation with *P. ostreatus*. The fungal strains that were found to degrade DON were limited [12]. However, ZEN was significantly reduced by 97% ($p < 0.05$) with *P. ostreatus*, 50% ($p < 0.05$) with *R. oryzae*, 40% with *A. oryzae*, and 32% with *M. circinelloides* in this study (Figure 2C). Other fungal strains reported to degrade ZEN included *Thamnidium elegans*, *Gliocladium roseum*, *Trichosporon mycotoxinivorans*, etc. [12]. The reduction in ZEN after fungal fermentation in the current study could make PM less of a risk in ZEN content according to the EU regulation of ZEN for finishing pigs. Therefore, *P. ostreatus* as a white-rot fungus showed the superior degradability of mycotoxins compared to other fungi used in this study.

3.6. In Vitro Digestibility of Dry Matter and Amino Acids

The in vitro digestibility of non-fermented PM and fungal-fermented PM was evaluated by simulating enzymatic hydrolysis in the stomach and small intestine of monogastric animals. The IVDMD evaluated the total DM loss of each sample during hydrolysis. Generally, no significant ($p > 0.05$) difference in IVDMD was observed between each treatment (Table 4), although *A. oryzae* fermented PM showed higher IVDMD than other treatments. The pepsin (endopeptidase) and pancreatin (mixture of amylase, lipase, and protease) in the digestibility analysis mainly digested protein, starch, and lipid in the solid sample. Unlike *R. oryzae* and *M. circinelloides*, *A. oryzae* produced both amylase and protease [48], which could serve for the pre-digestion of the PM via fermentation, resulting in more soluble components in the fermented PM such as soluble sugars (Table 2), soluble minerals such as NO_3^-, PO_4^{3-}, $Mg^{2\pm}$ and $Ca^{2\pm}$ (Figure 1A,B). *A. oryzae* fermented PM also resulted in the lower generation of ammonium compared to *R. oryzae* and *M. circinelloides*, which potentially retained proteins in the fermented PM for in vitro digestion.

The IVDAA showed no significant difference ($p > 0.05$) in all treatments except for a lower IVDAA in *P. ostreatus*-fermented PM than in the control (Table 4). Although a higher concentration of CP was observed in *P. ostreatus*-fermented PM (Table 3), which could be due to concentrating effects from the degradation of indigestible fiber (e.g., lignin) and the lower production of ammonium, its protein showed less digestibility than other fungal fermented and non-fermented PM (Table 4). This could be due to less protein solubilization by *P. ostreatus,* which was traditionally used for degrading lignin in lignocellulosic biomass to improve fiber utilization in ruminant animals [49]. Compared with *R. oryzae* and *M. circinelloides*, *A. oryzae* had the IVDAA most close to the control, with the digestibility of Lys and Trp higher ($p > 0.05$) than the control. Overall, the IVDMD and IVDAA of the fermented PM by all fungal strains were proved to have no difference ($p > 0.05$) from that of PM. However, the real digestibility and physiological effects of the fermented PM depend on its inclusion ratio when formulated with other feed ingredients in the diets of monogastric animals, which is worth further investigation.

The use of pennycress as both cover, oil crops and the efficient use of its co-products (pennycress seed meal) could have a large impact on the local environment and economy. Planting pennycress in the field in between corn and soybean plantations during winter and early spring could retain P and N in the soil, preventing their running off in early spring with melted snow to the ground and surface water systems. This could eventually reduce P and N fertilizer use in the ground while reducing the water pollution of concentrated P

and N. The pennycress seeds harvested contained a higher concentration of oil than canola and soybean, with erucic acid making up the largest fraction (31–39%). The pennycress oil is suitable for conversion to biodiesel and jet fuel [3]. PM is generated in large quantities after oil has been extracted from seeds. Genetic tools enabled the development of PM with low fiber and low glucosinolates, which could be served as an alternative protein feedstuff in the animal feed market [39,40].

Table 4. In vitro dry matter digestibility (IVDMD) and in vitro digestibility of amino acids (IVDAA) in treatments without (control) and with fungal fermentation by *Pleurotus ostreatus* (PO), *Rhizopus oryzae* (RO), *Mucor circinelloides* (MC), and *Aspergillus oryzae* (AO).

	Ctrl	PO	RO	AO	MC
IVDMD, %	55.39 ± 9.39 ^{a}	45.36 ± 2.39 ^{a}	52.93 ± 4.02 ^{a}	61.09 ± 2.13 ^{a}	53.33 ± 3.99 ^{a}
IVDAA			%		
EAA					
Arginine	77.28 ± 5.46 ^{a}	60.45 ± 2.17 ^{b}	56.48 ± 5.01 ^{b}	63.47 ± 2.25 ^{ab}	54.00 ± 4.52 ^{b}
Histidine	77.93 ± 5.77 ^{a}	54.54 ± 0.83 ^{c}	61.76 ± 4.14 ^{bc}	73.23 ± 0.25 ^{ab}	61.72 ± 4.00 ^{bc}
Isoleucine	74.40 ± 8.92 ^{a}	55.52 ± 0.63 ^{a}	59.41 ± 4.03 ^{a}	73.20 ± 0.63 ^{a}	60.70 ± 7.48 ^{a}
Leucine	76.49 ± 8.11 ^{a}	54.06 ± 0.46 ^{b}	58.21 ± 3.44 ^{ab}	72.41 ± 0.46 ^{ab}	60.40 ± 6.19 ^{ab}
Lysine	74.27 ± 7.36 ^{a}	61.27 ± 1.16 ^{a}	63.25 ± 4.15 ^{a}	77.14 ± 0.83 ^{a}	65.24 ± 2.94 ^{a}
Methionine	85.23 ± 5.31 ^{a}	61.71 ± 0.64 ^{b}	70.89 ± 3.23 ^{ab}	81.84 ± 5.97 ^{a}	70.64 ± 0.57 ^{ab}
Phenylalanine	76.05 ± 7.55 ^{a}	55.16 ± 0.84 ^{b}	60.99 ± 3.23 ^{ab}	72.45 ± 0.74 ^{ab}	62.23 ± 6.50 ^{ab}
Threonine	76.96 ± 7.26 ^{a}	56.98 ± 0.60 ^{b}	62.43 ± 3.36 ^{ab}	75.05 ± 0.87 ^{a}	65.45 ± 5.44 ^{ab}
Valine	76.66 ± 8.00 ^{a}	55.76 ± 1.32 ^{b}	59.70 ± 3.26 ^{ab}	73.49 ± 0.84 ^{ab}	61.55 ± 5.73 ^{ab}
Tryptophan	77.94 ± 8.65 ^{a}	69.16 ± 0.51 ^{a}	70.13 ± 0.99 ^{a}	79.29 ± 0.44 ^{a}	71.76 ± 5.14 ^{a}
NEAA					
Aspartate	82.58 ± 5.27 ^{a}	64.52 ± 1.92 ^{b}	71.62 ± 4.74 ^{ab}	80.27 ± 2.30 ^{ab}	73.96 ± 4.58 ^{ab}
Glutamate	81.48 ± 4.68 ^{a}	61.26 ± 2.06 ^{c}	65.95 ± 2.98 ^{c}	78.99 ± 0.59 ^{ab}	67.32 ± 3.88 ^{bc}
Serine	75.23 ± 7.48 ^{a}	56.69 ± 0.89 ^{b}	59.97 ± 3.31 ^{ab}	74.21 ± 0.98 ^{ab}	63.66 ± 5.80 ^{ab}
Glycine	73.43 ± 6.61 ^{a}	49.14 ± 3.26 ^{b}	51.48 ± 3.81 ^{b}	66.00 ± 1.31 ^{ab}	53.15 ± 4.42 ^{b}
Alanine	76.77 ± 7.54 ^{a}	56.51 ± 4.03 ^{a}	58.70 ± 5.69 ^{a}	76.86 ± 0.74 ^{a}	63.55 ± 6.03 ^{a}
Tyrosine	82.94 ± 6.35 ^{a}	62.60 ± 2.98 ^{a}	72.73 ± 8.53 ^{a}	83.86 ± 1.41 ^{a}	72.49 ± 5.65 ^{a}
Cystine	79.37 ± 5.47 ^{a}	55.15 ± 0.56 ^{ab}	49.53 ± 7.69 ^{b}	78.62 ± 0.64 ^{a}	52.36 ± 10.14 ^{b}
Hydroxyproline	72.43 ± 7.73 ^{a}	61.23 ± 4.03 ^{a}	65.71 ± 4.49 ^{a}	76.56 ± 0.75 ^{a}	63.45 ± 4.81 ^{a}
Glutamine	86.38 ± 19.27 ^{a}	48.47 ± 1.09 ^{a}	61.63 ± 1.09 ^{a}	63.08 ± 4.01 ^{a}	83.15 ± 23.82 ^{a}
Total	77.35 ± 6.63 ^{a}	58.23 ± 1.81 ^{b}	62.20 ± 4.11 ^{ab}	74.90 ± 0.91 ^{ab}	63.58 ± 4.99 ^{ab}

Different superscript letters in each row represent significant differences ($p < 0.05$); otherwise, there was no significant difference ($p > 0.05$).

This study demonstrated the feasibility and biological processing of PM to reduce important anti-nutritional factors, including indigestible fiber, phytate, major glucosinolate (sinigrin), and critical mycotoxins (AFT, DON, and ZEN) for monogastric animals, which could substantially improve its acceptance in the feed market to support local farm animal production. In addition, fungal treatment could also be used as a cost-efficient method to detoxify PM, making it possible for the PM to enter biorefinery, where its protein, carbohydrates, and fiber could be refined for human consumption. The application of pennycress products could eventually support the overall sustainability of the food-energy-water (FEW) nexus with a low carbon footprint.

4. Conclusions

Pennycress meal after solid-state fermentation with fungi had concentrated digestible fiber, reduced phytate content, and increased available phosphorus, magnesium, and calcium. One of the major glucosinolates, sinigrin, was greatly degraded by over 80% with *R. oryzae* and over 30% with *A. oryzae*. Zearalenone was reduced by over 90% with *P. ostreatus*. Fermented and non-fermented PM had no difference in the in vitro digestibility of dry matter and total amino acids, although *P. ostreatus*-fermented PM showed the

lower digestibility of total amino acids. The overall digestibility also depended on other ingredients, which were used to formulate animal diets and animal feeding tests. The current study proved the feasibility of fungal fermentation in improving the feeding value and detoxification of PM. As the chemical composition of pennycress seeds is rapidly being improved via genetic tools, future varieties might have a better response to the fermentation process, leading to more valuable monogastric feed ingredients and a possible direction toward human consumption.

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