Effect of solid-state fermentation on select antinutrients and protein digestibility of cold-pressed and hexane-extracted canola meals

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Abstract

In this study, the effects of solid-state fermentation (SSF), including strain (*Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590) and fermentation time (24, 48, and 72 h) on the nutritional value of cold-pressed (CP) and hexane-extracted (HE) canola meals were examined. SSF increased the protein content of both types of meals (from ˜36 to ˜40%) while reducing the oil content of CP meals (from ˜12 to 9%). There was a significant reduction (˜80%) in the phytic acid content of both types of meals after fermentation using either fungi. Overall, fermented samples showed a decrease in the total phenolic content from 2.7-3.1 to ˜1.0 mg gallic acid equivalents (GAE)/g DM (a ˜65% reduction), of which specifically the HE meal fermented with *A. niger* sample had the greatest decrease from 3.1 to 0.6 mg GAE/g DM (˜81% reduction). Seventy-two hours of fermentation decreased the *in vitro* protein digestibility (IVPD) of the meals. In contrast, a shorter fermentation time (24 h) increased the IVPD as compared to the controls (from ˜73% to 77-81%), with the exception of the CP meal fermented with *A. niger* which had decreased IVPD at all fermentation times. Overall, the changes indicate that SSF using *A. niger* or *A. oryzae* can be useful to positively modify the composition of different canola meals and improve their nutritional value by significantly increasing the protein content, decreasing the levels of antinutrients, while only slightly reducing IVPD.

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Short running title: Nutrition of fermented canola meals

Abstract

In this study, the effects of solid-state fermentation (SSF), including strain (*Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590) and fermentation time (24, 48, and 72 h) on the nutritional value of cold-pressed (CP) and hexane-extracted (HE) canola meals were examined. SSF increased the protein content of both types of meals (from ˜36 to ˜40%) while reducing the oil content of CP meals (from ˜12 to 9%). There was a significant reduction (˜80%) in the phytic acid content of both types of meals after fermentation using either fungi. Overall, fermented samples showed a decrease in the total phenolic content from 2.7-3.1 to ˜1.0 mg gallic acid equivalents (GAE)/g DM (a ˜65% reduction), of which specifically the HE meal fermented with *A. niger* sample had the greatest decrease from 3.1 to 0.6 mg GAE/g DM (˜81% reduction). Seventy-two hours of fermentation decreased the *in vitro* protein digestibility (IVPD) of the meals. In contrast, a shorter fermentation time (24 h) increased the IVPD as compared to the controls (from ˜73% to 77-81%), with the exception of the CP meal fermented with *A. niger* which had decreased IVPD at all fermentation times. Overall, the changes indicate that SSF using*A. niger* or *A. oryzae* can be useful to positively modify the composition of different canola meals and improve their nutritional value by significantly increasing the protein content, decreasing the levels of antinutrients, while only slightly reducing IVPD.

KEYWORDS

Antinutritional factors, *Aspergillus niger, Aspergillusoryzae* , canola meal, protein digestibility, solid-state fermentation

1 INTRODUCTION

Canola meal, leftover from oil extraction process is rich in protein (˜36-39%) and total dietary fibre (˜33%) and is typically used as animal feed. The meal byproduct is prepared using a conventional process involving solvent extraction (e.g., hexane extraction (HE), 60°C) and a desolventization step (110°C) (Kalaydzhiev et al., 2019), or a cold pressing (CP) process involving mechanical crushing of seeds at low temperatures (50-60°C) (Febrianto et al., 2011). The former achieves higher levels of oil extraction, however, leaves the remaining meal protein severely heat treated, whereas the latter achieves sub-optimal oil extraction but leaves proteins of higher quality in the meal. In addition to having different compositions and physicochemical properties, the two meal types have been reported to vary in their protein digestibility properties (Kasprzak et al., 2016).

In general, proteins of canola meal have a favorable pattern of essential amino acids and are considered a suitable feedstock for further processing for human consumption. However, food uses of canola meal are limited due to naturally present compounds such as phytic acid, sinapine, phenolic acids and polyphenols (Croat et al., 2017). These compounds can lead to poor protein digestibility, interfere with mineral absorption, and give undesirable colour and taste attributes (Wu & Muir, 2008). Phenolic compounds are the major antinutritional compounds found in canola meals with levels of ˜5 mg gallic acid equivalents (GAE)/g dry meal in common canola meals, which is almost 30-times higher than in soybean meals (Shahidi & Naczk, 1992). Thus, it is necessary to further process canola meals to minimize the levels of or eliminate these phytochemicals to make the meals more suitable for human consumption.

Solid-state fermentation (SSF) is a clean label process where there is nearly no free water in the solid substrate, making it acceptable for fungi growth. Recently, researchers have employed SSF of canola meals with various fungal strains to achieve significant reductions in glucosinolates (Shi et al., 2015), thiooxazolidones, phytic acid (El-Batal & Abdel, 2001), total phenolic compounds, and neutral detergent fibre (NDF) (Pal Vig & Walia, 2001) levels, with an enhancement in the protein content. Furthermore, Shi et al. (2015) found improved amino acid in-vitro digestibility after A. niger SSF on a composite substrate containing rapeseed cake and wheat bran. Hyphae can penetrate into structural matrices to loosen them and to allow higher utilization of nutrients entrapped in the matrices by fungi. Canola meals contain ligno-cellulosic materials and other hard-to-digest matrices that are hardened by the heating process during oil extraction of canola.

The direct comparison of the effect of SSF on the nutritional attributes of differently processed meals (CP and HE) has not yet been investigated. In this study, SSF with two types of fungal strains (Aspergillus niger NRRL 334 and Aspergillus oryzae NRRL 5590, both have generally recognized as safe (GRAS) status) was

used to ferment CP and HE extracted meals to different times to improve their nutritional value. It was hypothesized that SSF would induce protein hydrolysis and produce canola meals with increased protein content, decreased levels of antinutritional compounds, and improved protein digestibility with variations as a function of fungus and meal type.

2 MATERIALS AND METHODS

2.1 Materials

CP meal was obtained from Pleasant Valley Oil Mills (Clive, AB, Canada). Commercial HE meal was gifted by Bunge Canada (Harobe, MB, Canada). Fungal strains of *Aspergillus niger* NRRL 334 and*Aspergillus oryzae* NRRL 5590 were of GRAS status and obtained from Agricultural Research Service (ARS) Culture Collection (Northern Regional Research Laboratory (NRRL), Peoria, IL, USA). All media, reagents, and chemicals used were of analytical grade and obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

2.2 Sample preparation

2.2.1 Preparation of fungi spore suspensions

Both the *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 were incubated on potato dextrose agar (PDA) plates at 30 for 7 days. Spore suspensions were prepared using deionized water, followed by spore counting using direct microscopy (Leica, Model 97 S6E, Wetzlar, Germany) with a hemocytometer (Bright-Line, Horsham, PA, USA). Spore suspensions were prepared before the inoculation.

2.2.2 Solid-state fermentation

The HE and CP canola meals (200 g of each) were fermented with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 spore suspensions. Both spore suspensions were standardized to a spore concentration of 10^7 colony forming units (CFU) to apply on per gram of meal and used as the starter culture for fermentation as described by Croat et al. (2016; 2017) and Shi et al. (2015). The canola meal, spore suspension, and deionized water were mixed at speed 5 for 3 min using a commercial stand mixer (Pro 600, KitchenAid, Benton Harbor, MI, USA) before being spread out thinly (<1.5 cm) and evenly onto a stainless-steel sheet pan. The fermentation was performed at a 50% (w/w) moisture content at 30 over a 72-h period in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA). Mill-Q water was added based on the weight loss each day to maintain the 50% (w/v) moisture content. Samples (˜50 g) were collected on random spots from each batch at the time of initial inoculation (0), 24, 48, and 72 h, freeze-dried (Benchtop Pro 8L ZL-105, SP Scientific, Warminster, PA, USA) and saved for later analyses. All the meals were frozen at –20degC to terminate the fermentation until further freeze-drying. The dried powders were stored at 4 until used in testing.

2.3 Physicochemical analysis

2.3.1 pH determination

The pH value was determined for the fermented and unfermented meal slurries using a pH meter. The slurries were prepared by mixing 1 g of the dried meal and Milli-Q water at a 1:10 (w/v) ratio and allowed to stir for 30 min at room temperature (21-23). Measurements were made in triplicate on each of the replicate batches (n=3) and reported as mean +- one standard deviation.

2.3.2 Degree of protein hydrolysis

The degree of protein hydrolysis was measured as the released free amino groups using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method according to Adler-Nissen (1979) and Jung et al. (2005). In brief, a freeze-dried meal containing 1 g of protein (corrected by protein content) was added to distilled water, followed by stirring for 30 min at room temperature (21-23). The mixture was centrifuged (Sorvall RC-6 Plus centrifuge, Thermo Scientific, Asheville, NC, USA) at 6,000 xg for 15 min at room temperature, and the supernatant was collected. A 250-µL aliquot of the supernatant was added to 2 mL 20 mM sodium phosphate buffer (pH 7.8), followed by the addition of 2 mL 0.01% TNBS solution. After mixing using a

vortex for 15 s, the mixture was covered by aluminum foil and placed in a water bath at 50 for 1 h. The reaction was stopped by adding 4 mL 0.1 N HCl, and the sample was allowed to cool for 10 min. The absorbance of the reaction mixture was measured at 340 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, Madison, WI, USA). The total protein hydrolysis achieved by hydrolyzing proteins with acid was also measured as part of the degree of hydrolysis calculations. The total protein hydrolysis was determined by adding 24 mg of sample (corrected by protein content) to a screw cap Pyrex tube containing 15 mL of 6.0 N HCl. The tubes were incubated at room temperature for 20 h. The dispersion was adjusted to pH 7.0 with 2 M NaOH, followed by filtration through the Whatman Grade 3 filter paper (Whatman International Ltd., Maidstone, UK). A 250 µL aliquot of the above sample was then added to 2.0 mL 1% SDS solution in buffer. This was followed by the addition of 250 µL of the mixed solution to 2.0 mL 10 mM sodium phosphate buffer (pH 7.8). This mixed solution was performed in triplicate and analyzed according to the procedures above. The sample blank was prepared by adjusting a solution of 6.0 N NaOH and 6.0 N HCl to pH 7.0. A 1.5 mM glycine solution was used to create the standard curve to calculate the α-NH_2-glycine equivalents of each sample for use in the following equations to calculate the degree of protein hydrolysis:

$$h = (h_t - h_c) \times DF \quad (1)$$

$$DH = \left(\frac{h}{h_t}\right) \times 100\% \quad (2)$$

Where h is the net mM concentration ofα-NH_2-glycine equivalents, h_t is the mM concentration of α-NH_2-glycine equivalents at the time of testing, h_c is the mM concentration ofα-NH_2-glycine equivalent at the time of initial inoculation before the microorganism was added, h_{tot} is the mM concentration of α-NH_2-glycine equivalents after total acid hydrolysis, and DF is the dilution factor. Measurements were made in triplicate on each of the replicate batches (n=3) and reported as the mean ± one standard deviation.

2.3.3 Proximate composition

The crude protein content was determined according to AACC method 46-30.01 using a combustion nitrogen analyzer (FP-628 CAN, LECO Corp., Saint Joseph, MI, USA) with a nitrogen conversion factor of 6.25 (AACC, 2010). The content of ash was determined using 2 g of sample in a muffle furnace at 600 for a 2-h period according to AOAC method 943.05 (AOAC, 2005). The crude lipid was measured using the Goldfisch apparatus according to AOAC method 920.39 (AOAC, 2005). Measurements were made in triplicate on each of the replicate batches (n=3) and reported as the mean +- one standard deviation.

2.4. Antinutritional components: Phytic acid and total phenolic content

The phytic acid content (PAC) was determined using the phytic acid assay kit (Megazyme Inc., Sydney, Australia). All measurements were done in triplicate (n=3) and reported as the percentage of phytic acid on a dry weight basis (d.b.).

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method according to Olukomaiya et al. (2020) with some modifications. In brief, 0.5 g of sample was extracted using 5 mL 80% (v/v) methanol for 15 min (stirring at 500 rpm and room temperature). The supernatant was collected after centrifugation at 5000 x g for 10 min and filtered with the Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). The remaining pellet was extracted again using 80% (v/v) methanol, followed by centrifugation and filtration under the same conditions to obtain an aqueous phenolic extract for analysis. A 0.1 mL aliquot of the extract was mixed with 2.5 mL 10% (v/v) Folin-Ciocalteu reagent. After a 2-min reaction time, 2.5 mL 7.5% (w/w) sodium carbonate was immediately added to the sample mixture and allowed to incubate in the dark for 30 min at room temperature. The absorbance was measured at 750 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, Madison, WI, USA). Gallic acid (reagent grade) was used as the standard. All measurements were done with triplicate independent assays (n=3) and reported as milligrams of gallic acid equivalents (GAE) per gram of dry meal (mean +- standard deviation).

2.5 *In vitro* protein digestibility (IVPD)

The IVPD of control and fermented meals was determined using the pH-drop method according to Tinus et al. (2012). A three-enzyme assay containing 31 mg chymotrypsin (bovine pancreas [?] 40 units/mg protein), 16 mg trypsin (porcine pancreas 13,000–20,000 BAEE units/mg protein), and 13 mg protease (*Streptomyces griseus* [?] 15 units/mg solid) in 10 mL of water was prepared (fresh daily), kept in a 37 water bath, and adjusted to pH 8.0 +- 0.05 with 0.1 M NaOH and HCl. The sample suspensions were prepared by dispersing 62.5 +- 0.5 mg protein in 10 mL water. The samples were stirred at 37 for 1 h before pH adjustment to pH 8.0 +- 0.05 with 0.1 M NaOH and HCl. The protein digestibility measurements were done by adding 1 mL of the enzyme cocktail into the sample suspensions and recording changes in pH every 30 s for a total of 10 min. The equation below was followed to calculate the IVPD of each sample:

$$\text{IVPD } (\%) = 65.66 + 18.10 \times \text{pH}_{10\ min} \quad (3)$$

Where $\text{pH}_{10\ min}$ is the changes in pH since the addition of the enzyme solution to the end of the 10 min measuring period.

2.6 Statistical analysis

The fermentation was made in triplicate using separate plates and spore suspensions (n=3). A three-way analysis of variance (ANOVA) was used to study the statistical differences between samples as a function of meal type, fungal strains, and fermentation time with a significance level of$p$ =0.05. A post-hoc Tukey's test was used to detect statistical differences in fermentation time. All statistics were performed using the SPSS Version 28.0 software (IBM Corp. NY, IL, USA).

3 RESULTS AND DISCUSSION

3.1 pH, degree of protein hydrolysis, and proximate composition

The HE and CP canola meals were fermented with*A. niger* NRRL 334 and*A. oryzae* NRRL 5590 over a 72-h period. After 48 h of fermentation, significant growth of hyphae was observed, and spores started to form on the surface after 72 h of fermentation as shown in Figure 1. For pH upon fermentation (Table 1a), significant differences (p <0.001) between meal types (CP vs. HE) and among fermentation time groups were observed. The pH for all fermented samples increased with fermentation time, with the CP meals showing changes after 48 h while changes occurred earlier (24 h) for HE meals. The HE meals had an overall higher pH (˜8.2) than CP meals (˜7.6) at the end of the fermentation period (72 h), regardless of the strains used. In contrast to the result of the present study, Olukomaiya et al. (2020) found a reduction in the pH value from 6.26 to 6.13 after SSF for 7 days using *A. ficuum* , *A. sojae* , and co-culture *Lactobacillus salivarius* on autoclaved, solvent-extracted canola meal. The authors described this to be caused by an increase in organic acid content during SSF, especially when using*L. salivarius* . Aljubori et al. (2017) also reported relatively stable or decreased pH values when using *L. salivarius* . The differences in results may be explained by the variations in canola meals, fungi strains, and fermentation conditions used in these studies in comparison to the present study. In addition, the organic acids might have been used as nutrients by *A. niger* and *A. oryzae*during SSF, leading to increases in pH.

The protein hydrolysis, measured as the degree of hydrolysis (DH), increased throughout the fermentation period (Table 1b). Meal type, strain used, fermentation time, and their interactions were all significant factors (p <0.001) affecting DH. The DH of the meals increased from zero (0 h) to 15-30% (24 h), then to ˜40% (48 h), and finally to 45-50% after 72 h. For the first two days (0-48 h), the DH increased quite rapidly due to the large surface area that was exposed to oxygen, which helped with hyphae growth. The proteins were most likely hydrolyzed partially by the proteases synthesized by fungal cells, resulting in increased DH with the release of free amino acids. The relatively compact nature of the lower layers of the meal significantly lowered the speed of growth as fungi could not penetrate easily to the bottom of the solid substrate, hence the lower rate of DH increase at 72 h. The increase in DH was slower for HE meals (15.8-23.4%) as compared to CP meals (28.3-30.1%) on the first day (24 h) of fermentation, with the strain *A. oryzae* being less efficient at protein hydrolysis, particularly in the HE meal. At the end of the fermentation cycle, the DH was similar for CP meals and the HE meal fermented by *A. oryzae* (45.1-46.4%), while that of the *A. niger* HE meal

was higher (52.3%).

The changes to the proximate composition were similar for CP and HE meals fermented with both strains. For protein, lipid and ash contents, significant impacts (p <0.001) from meal type and fermentation time were observed. As shown in Table 2a, the crude protein level increased from ˜34 to ˜37% (a 9-11% increase) after 72 h of fermentation in the CP meals, regardless of the strain used. The protein levels were higher in the HE meals, ranging from 39.7-41.5%, and remained relatively constant upon fermentation (though still increased by *A. niger*). In contrast to protein, the crude lipid levels decreased from ˜12.5 to ˜9% for CP meals, whereas the contents in the HE meals decreased from ˜2.5 to ˜1% (Table 2b). There was a small increase in the ash content for CP meals from ˜6 to ˜7% after 72 h of fermentation, while that of HE meals increased from ˜8 to ˜10% (Table 2c). Compared to HE meals, CP meals had a lower initial crude protein content due to the higher proportion of oil; this trend remained even after a 72-h period of SSF. The high levels of residual oil in the CP meals can be inhibitory to fungal growth and lead to similar results found by Simon et al. (2017). They introduced and compared several strains (*Aureobasidium pullulans* NRRL-58522 3.0,*Aureobasidium pullulans* NRRL-Y-2311-1, *Trichoderma reesei*NRRL 3653, *Fusarium venenatum* NRRL 26139, *Paecilomyces variotti* NRRL 1115, *Rhizopus microspores* var. *oligosporus*NRRL 2710, *Neurospora crassa* NRRL 2332, *Mucor circinelloides* , and *Pichia kudriavzeii*) as SSF cultures on HE and CP meals and concluded that fungal strains preferred HE meals due to the lower level of residual oil.

The increase in the levels of crude protein and ash is attributed to the effect of concentration. The carbohydrates, including sucrose and fibre, acted as carbon sources for the fungi to grow, and carbon dioxide (CO_2) was produced as a result, concentrating the remaining compounds (protein and ash). Wang et al. (2012) reported a 27% increase in the crude protein content using composite strains with *Candida tropicalis* CICIM Y0079(T). The results agreed with those of Plaipetch and Yakupitiyage (2014), who found a 9% increase in the crude protein content in *Saccharomyces cerevisiae*yeast-fermented canola meal. Simon et al. (2017) reported an increase in crude protein from 42 to 44-50% (d.b.) in fermented HE meal and a ˜14% increase in fermented CP meal, depending on the strains. The increases in the nitrogen content of 11.6, 23.1, 34.2, 47.6, and 65.4% after 1, 2, 5, 8 and 10 days of incubation, respectively, were reported by Pal Vig and Walia (2001).

3.2 Contents of phytic acid and total phenolic compounds

The levels of PAC and TPC in the canola meals are presented in Table 3. All fermented samples showed decreases in PAC and TPC. Significant influences were observed only from fermentation time for PAC (p <0.001), whereas fermentation time, strain used, meal type x strain, meal type x time, strain x time, and the three-way interaction of meal type x strain x time were all statistical factors affecting TPC (p <0.05). The reduction of PAC from ˜5.9 to ˜1.3% (a 74-85% reduction) was achieved by phytase synthesized by *A. niger* and *A. oryzae*during SSF. In contrast, decreases in TPC upon fermentation as well as the corresponding increases have both been reported in the literature as the enzymatic breakdown of polyphenol complexes (e.g., lignins, tannins, flavonoids) leads to the accumulation of smaller units, and sometimes the formation of new bioactive compounds (Adebo & Medina-Meza, 2020). Depending on seed composition and structure (e.g., ease of cell wall breakdown), microbial strains employed, and the choices of the assay (and standard) for TPC determination, fermentation can bring different outcomes to the contents of ANF. All fermented samples showed a decrease in TPC from 2.7-3.1 to 1.0 mg GAE/g DM (a ˜65% reduction) except for the HE meal fermented by *A. niger* . This sample had an even greater decrease in TPC from 3.1 to 0.6 mg GAE/g DM (a ˜81% reduction). Overall, the reduction in PAC and TPC was correlated with fungal growth, indicating that the high residual oil in the CP meal was still suitable for *A. niger* NRRL 334 and*A. oryzae* NRRL 5590 to grow without affecting the reduction of ANF levels.

A lower reduction in PAC and TPC was reported by Olukomaiya et al. (2020) where a ˜20% reduction in PAC and a ˜17% reduction in TPC were achieved using *A. sojae* ATCC 9362 and *A. ficuum* ATCC 66876 on solvent-extracted (45% moisture, 30) canola meal, even though their fermentation period (7 days) was longer than in the current study. The initial values of PAC and TPC in their meals were 27.06 mg/g DM and 9.31 mg GAE/g DM, respectively, which were significantly lower and higher than those in samples from

the present study (˜59 mg/g DM and ˜2.9 mg GAE/g DM), respectively. Pal Vig and Walia (2001) also reported a similar result that a maximum decrease in PAC (a 42.4% reduction from the initial content of 29.3 mg/g DM) occurred after a 10-day fermentation using *Rhizopus oligosporus* with a water:rapeseed meal ratio of 1:3 at 25. The degradation of ANF in canola meals might require multiple enzymes. The lower reduction in the ANF levels in those studies compared to the present study could be explained by the lack of certain enzymes specific for degrading the particular ANF or the insufficient level of enzymes produced when using other strains. Cultivar differences in the starting materials (meals) should also be considered as cultivar and growing environment can greatly influence the composition and distribution of these compounds in the seed and therefore meals (Bueckert et al., 2011).

3.3 *In vitro* protein digestibility

The IVPD of control and fermented meals is presented in Table 4. Meal type, strain used, fermentation time and all their interactions were significant (p <0.05) factors affecting IVPD. At time 0 h, HE meals and the CP meal fermented by *A. oryzae* had similar IVPD of ˜72.7%, while that for CP by *A. niger*was much higher (80.2%). The *A. niger* fermented CP meal also followed a different trend than the other three meals after 24 h of fermentation as it had a decrease in IVPD (a ˜5.7% decrease) whereas the IVPD of all other meals increased (increases of ˜4.8, 10.6 and 11.9% for CP by *A. oryzae* , and HE by *A. oryzae* and *A. niger* , respectively). The three aforementioned meals reached their maximum IVPD value after 24 h, after which the values started decreasing as fermentation continued (towards 48 and 72 h). At the end of the fermentation period (72 h), all fermented samples had similar IVPD values of ˜69.7%, statistically lower compared to the respective controls for CP meals and the HE meal fermented by *A. oryzae* , whereas that for HE by*A. niger* remained relatively unchanged. The most substantial decrease was for *A. niger* fermented CP meal (by ˜12.8%), while those for CP meal fermented with*A. oryzae* (by ˜3.6%) and HE meal with *A. niger* (by ˜5.2%) were less consequential.

Despite the rather substantial drop in ANF contents (TPC and PAC with ˜65-85% reductions, Table 3), the IVPD decreased overall upon fermentation in the present study. Literature concerning changes in protein digestibility of fermented canola meals is scarce. Shi et al. (2015) reported a significantly enhanced amino acid *in vitro* digestibility of rapeseed meal (from China) after SSF with*A. niger* ; however, wheat bran was included with the meal as a carbon substrate for fungal growth. Fermentation, in general, has been reported to improve protein digestibility in various legume, cereal, and oilseed samples. Employing a similar fermentation approach (SSF by*A. oryzae* and *A. niger*), Kumitch et al. (2020) reported an increased IVPD of pea-protein enriched flour (from ˜74.8 to ˜80.2%) over the fermentation period (6 h). Natural fermentation (16-20 h) using a yogurt culture containing*Lactobacillus delbrueckii* subsp. *bulgaricus* and*Streptococcus thermophilus* improved the IVPD (a ˜9.5% increase) of desi chickpea flour; however, the fermentation process did not alter the values for kabuli chickpea or faba bean flours, according to Chandra-Hioe et al. (2016). An increased IVPD of sorghum flours coupled with reduced phytic acid, trypsin inhibitors, and tannins after traditional (natural) fermentation (24 h) was observed by Osman (2004).

The improved protein digestibility upon fermentation in the aforementioned studies is likely due to the breakdown of protein molecular structures that enhanced the accessibility of digestive enzymes, while the reduction/elimination of ANF also resulted in weakened crosslinking activities, allowing more intensive proteolytic attacks. However, depending on the nature of processing (e.g., types of fermentation and strains utilized) and the starting material (e.g., structural differences among species and cultivars), such microbial modification of matrix structures may also cause blockage in certain passages and limit the function of digestive enzymes (Chandra-Hioe et al., 2016; Emkani et al., 2022; Skalickova et al., 2022) which is hypothesized to have occurred in the present study. It is interesting to note that during the period where the IVPD of most samples initially increased (0-24 h), the DH was also increasing at its fastest rate (Table 1). We therefore hypothesize that within that time frame, effective hydrolysis of large protein molecules into smaller subunits might have overcome the influence of passage blockage of the digestive enzymes and as such an overall positive effect on the protein digestibility of the fermented meals was seen. The primary factor influencing IVPD in the canola meals was not the presence of ANF in our case but the specific actions of

the selected fungal strains and the sensitivity of sample materials to fungal fermentation using SSF.

4 CONCLUSIONS

SSF using *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 was applied on CP and HE canola meals to modify their nutritional value with ˜50% DH achieved after 72 h of fermentation. While HE meal naturally contains more protein than CP meal, 72 h SSF with *A. niger* increased the protein content of CP meal to levels comparable to unfermented HE meal. Similar fermentation conditions did not result in the same increase in protein in HE meals. The SSF lowered the lipid content of both meals. The levels of phytic acid and total phenolics decreased significantly after fermentation with % reduction (after 72 h) being 74-85% for PAC and 65-81% for TPC in both CP and HE meals. Enzymes from fungal cells, including lipase, proteinase and phytase, played critical roles in changing meal composition. In terms of fungal strain, *A. niger*performed better to reduce PAC in CP meals and TPC in HE meals. The effective reduction of PAC and TPC, as well as similar DH values between meals, indicates that the high residual oil content in CP meals did not interfere with the growth of the fungi. SSF for 72 h brought decreases in IVPD to all meals except for HE by *A. niger* (unchanged) whereas 24 h fermentation improved IVPD for both HE meals and the CP meal fermented with *A. oryzae* . The recommended fungal strain and fermentation time for canola meal will depend on the desired outcome as increased protein content, decreased ANFs, and increased IVPD could not all be achieved at the same time under the conditions tested.

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AUTHOR CONTRIBUTIONS

Chenghao Li carried out the experiments, analyzed data, and wrote the first draft of the manuscript. Dai Shi and Andrea K. Stone performed additional data analysis and interpretation, and critical revision of the manuscript. Janitha P. D. Wanasundara, Takuji Tanaka, and Michael T. Nickerson were responsible for the design of the study, supervision of the research, and revision of the manuscript drafts.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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LIST OF FIGURES

Fig. 1 Cold-pressed (CP) and hexane-extracted (HE) canola meals fermented with *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590

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