Solid-state fermentation of *Pleurotus ostreatus* to improve the nutritional profile of mechanically-fractionated canola meal

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ABSTRACT

Canola meal is an abundant and economic source of energy and nutrients for production animals, but it contains more antinutrients, less protein, and has a lower nutrient digestibility compared to soybean meal. In this study, mechanical fractionation and solid-state fungal fermentation were conducted to improve nutrient profile and reduce antinutrient content of canola meal. Mechanical processing, including milling, sieving, and air classification, was used to fractionate canola meal and redistribute its components in different fractions. Then these fractions were inoculated for fungal fermentation. The results showed that 12-day solid-state fermentation with *Pleurotus ostreatus* increased the protein content in all fractions by 11-18%, decreased sinapine, glucosinolates, and phytate up to 99.8%, 98.8% and 75.8%, respectively, and increased the *in vitro* digestibility of selective fractions. Overall, *Pleurotus ostreatus*-based solid-state fermentation has the potential to be an effective treatment to improve the nutritional profile of canola meal.

Keywords: Canola meal, Fungal fermentation, Monogastric feed, *Pleurotus ostreatus* Solid-state fermentation

46 **1. INTRODUCTION**

47 Canola seed, with about 40% oil content, can yield 50-58% meal after oil extraction on a
48 dry weight basis [1]. Canola meal contains 38-43% of protein and with a balanced essential
49 amino acids profile, including methionine, cysteine, threonine, and tryptophan [1]. However,
50 canola meal is generally considered to be inferior to soybean meal due to differences in the
51 compositional profile [2, 3]. Compared with soybean meal, canola meal has lower metabolizable
52 energy, higher antinutrients, higher fiber, and slightly lower protein content. Among these
53 quality properties, the antinutrient content, including glucosinolates, sinapic acid, and phytate, is
54 the primary factor limiting the utilization of canola meal as animal feeds, especially for
55 monogastric animals [4]. Glucosinolates and sinapic acid are widely known to impede animal
56 growth by reducing feed palatability and causing cytotoxicity [5-9], while phytate decreases the
57 bioavailability of essential minerals [10-13].

58 To improve the nutritional values of canola meal as a feed and protein ingredient for
59 monogastric animals, different biological, chemical, thermal, and mechanical treatments have
60 been evaluated for their feasibility and efficacy to increase its protein content and decrease its
61 antinutrients without compromising its balanced amino acid profile. In general, thermal and
62 chemical methods can effectively reduce fiber and antinutrients content of canola meal, but this
63 can also decrease its protein content and digestibility [14-18]. Effects of mechanical treatments
64 for canola meal were rarely studied. Hansen et al. [15] examined the effects of different milling,
65 sieving, and air classification methods on the nutritional properties of rapeseed meal. They
66 applied ball milling and jet milling as common size reduction methods. In ball milling, trapped
67 particles repeatedly collide between colliding balls and the inner surface of the device, leading to

68 their deformation and milling [19, 20] . In comparison, jet milling process aids air jets or streams
69 in increasing the velocity of the particles from low to sonic range. This acceleration induces
70 collision between particles resulting in grinding and size reduction [21]. Both methods provide
71 fine fractions with a size distribution, a necessity for sieving and air classification [15].
72 Compared to ball milling, jet milling produces finer particles with narrower size distribution
73 while it has higher energy usage [15, 21]. The results showed that both ball milling and jet
74 milling followed by sieving and air classification effectively separated hulls and kernels,
75 producing fine and coarse fractions. Compared to unfractionated canola meal, the fine fraction
76 had higher protein level and digestibility and lower fiber and antinutrient contents, while the
77 coarse fraction had lower protein level and digestibility and higher fiber and antinutrient
78 contents. Mechanical fractionation separated a fine fraction of canola meal with better nutritional
79 profile and consequently higher potentials in animal industry [15].

80 Biological methods using fungi, bacteria, and enzymes have shown to achieve higher
81 efficiency in protein enrichment without compromising other nutrients and the digestibility of
82 seed meals [18, 22-25]. Bacterial fermentation, such as lactobacteria, has improved the suitability
83 of canola meal for monogastric animals [18, 26] . The capabilities of fungal fermentation to
84 detoxify antinutrients in agricultural residues and to degrade fiber and lignin in canola meal have
85 also been demonstrated [4, 27-32]. White-rot fungi such as *Pleurotus* spp., a group of food-
86 producing fungi, are known for the degradation of lignocellulosic structures. They also produce
87 laccase and peroxidases, which break down phenolic compounds such as sinapic acid and
88 tannins. All *Pleurotus* spp. known as oyster mushroom are edible white-rot fungi and generally
89 recognized as safe (GRAS) by the Food and Drug Administration (FDA) [33]. Unlike many

90 other fungi, such as *Aspergillus* spp., suffering from mycotoxins production, *Pleurotus* spp. have
91 even been used to degrade these secondary metabolites [34, 35]. *Pleurotus ostreatus* is the most
92 widely used GRAS-grade white-rot fungus in the feed industry to delignify lignocellulosic
93 materials. It also degrades sinapic acid in canola meal with a reduction rate up to 95% [36, 37].
94 Solid-state fermentation provides the optimal condition for growing this fungus. The lower
95 moisture content in solid-state fermentation compared with submerged liquid fermentation might
96 provide an easier path to commercialization [38].

97 In this study, we processed canola meal by combining mechanical fractionation with solid-
98 state fermentation of *P. ostreatus*. The effects of combined treatments on the nutrient
99 composition (i.e., protein, fiber and antinutrients content) and the digestibility of canola meal
100 were investigated. The focus of this study is on the quality improvement of the coarse fractions
101 from jet-milling and ball-milling separations. The biological treatment was also performed on the
102 fine fractions to examine the possibility of improving their nutritional quality.

103

104 **2. MATERIALS AND METHODS**

105 **2.1 Feedstocks Preparation**

106 Canola meal used in the first experiment to determine the fermentation duration
107 experiment was provided by CHS Inc. Inver Grove Heights, MN, USA. Mechanically treated
108 canola meal samples were obtained from the Norwegian University of Life Sciences. Hansen et
109 al. described the methods to generate these fractions in their paper published in 2017 [15].
110 Briefly, the samples from jet milling and ball milling went through air classification and sieving,

111 respectively. Parent samples from jet milling process (JP) were produced using a JMX-200 jet
112 mill at 650 rpm 103 (51 kg/h) to an average particle size of 35 µm. The meal was then air
113 classified using an ACX-200 classifier fitted with a CX-200 cyclone. Multiple air classification
114 at three different rotor speeds; 2200, 1900, and 1700 rpm separated the JP sample into two
115 categories of fine and coarse fractions. Parent samples from ball milling process (BP) were
116 produced using a 5.56-L ball mill equipped with 4.5 kg 20 mm and 2 kg 40 mm steel balls for 2
117 h at 30 rpm. The BP sample was then sieved through 150 and 300 µm sieves using a Haver &
118 Boecker sieving machine (RX-29-10, W.S.Tyler, OH, USA) which categorized the samples into
119 fine (0–150 µm), medium (150-300 µm) and coarse (>300 µm) fractions [15].

120 All samples were given an abbreviation which is identified as followings: meals not
121 milled any further than what received from the supplier (NM), parent samples obtained from jet
122 milling process (JP), fine samples obtained from jet milling process (JF), coarse samples
123 obtained from jet milling process (JC), parent samples obtained from ball milling process (BP),
124 ball milling samples with particle size ranging from 0-150 µm (B0), ball milling samples with
125 particle size ranging from 150-300 µm (B150), ball-milling samples with particle size ranging
126 from 150-300 µm (B300). Samples were not objected to any further treatment and were stored at
127 -21 °C prior to use. Moisture content of the meal was measured by drying the samples in a
128 105 °C oven overnight. The wet to dry weight ratio was used to calculate the initial moisture
129 content. The dry weight loss was calculated by subtracting the weight of the dried substrate after
130 incubation from the initial weight of the dried substrate.

131 **2.2 Cultures, Maintenance, and Inoculum Preparation**

132 *P. ostreatus* was obtained from the American Type Culture Collection (ATCC 32783). The
133 cultures were grown on potato dextrose agar (PDA) at 30 °C and were stored at 4 °C. New plates
134 were prepared every month. Inoculum for *P. ostreatus* was prepared by growing the fungus in
135 100 ml of sterile potato dextrose broth (PDB) in 250-ml Erlenmeyer flasks. Three mycelial discs
136 were cut and transferred to each flask and flasks were kept at 28 °C for 10 days. The grown
137 mycelia were harvested and inoculated to the new flasks with 100 ml of PDB. The flasks were
138 placed at 28 °C for 10 more days. The mycelia were then separated from the media by
139 centrifugation at 3724 ×g for 10 min and washed with sterile deionized (DI) water. The mass of
140 the fungi was estimated by drying 1 ml of the washed and homogenized fungi in the oven at 100
141 °C for 1 h. The inoculation ratio for solid-state fermentation was 0.5% w/w (g fungi / g of dry
142 substrate).

143 **2.3 Solid-State Fermentation**

144 Ten grams of canola meal (on a dry weight basis) were placed in each 250 ml Erlenmeyer
145 flask and covered with a plug and aluminum foil. Flasks with substrate were then autoclaved at
146 121 °C for 30 min and cooled down. *P. ostreatus* was inoculated with a volume of inoculation to
147 obtain the desired ratio of 0.5% w/w (fungi/dry substrate). The moisture content of the samples
148 was adjusted to 65% using sterile DI water. For controls (non-fermented samples), only sterile
149 DI water was added to the substrate. All solid-state fermentations were conducted at 28 °C. To
150 obtain the optimum fermentation duration, a preliminary study using canola meal provided by a
151 local plant was performed. In this experiment, the fungal incubation was carried out on untreated

152 canola meal for 4, 8, and 20 days. The incubation time resulting in the highest protein content
153 was selected to perform the solid culture using the mechanically fractionated materials.

154 2.4 Analytical Methods

155 After the fermentation, samples were freeze-dried and grounded. Moisture-free samples
156 were kept at 4 °C until they were analyzed. Protein content of the samples was determined by
157 measuring the organic nitrogen content of the samples based on Kjeldahl method using DK20
158 automatic Kjedahl Digestion Unit (VELP Scientifica, Inc., Bohemia, NY) followed by distillation in
159 UDK129 Distillation Unit (VELP Scientifica, Inc., Bohemia, NY) [39] The obtained value then
160 multiplied by 6.25 to calculate total crude protein [40]. Protein content was screened after days 4, 8
161 and 20 of incubation on fermented samples that had not been mechanically treated. On this basis,
162 protein content was then measured at day 8 and at day 12 of incubation on mechanically and
163 biologically treated samples. Day 12 of incubation was selected for further analysis.

164 Amino acid analysis was performed for the samples containing the highest protein level.
165 Sixty mg of samples were placed into 2 ml screw-cap tubes, followed by addition of 1 ml of 6 N
166 HCl containing 4% thioglycolic acid to each tube. Tubes were purged by nitrogen gas to remove
167 oxygen from them. They were incubated at 110 °C for 24 h and vortexed several times during the
168 incubation time. The samples were cooled down and centrifuged at 15,924 ×g for 10 min. The
169 supernatant was carefully removed and filtered using a 0.45 μm syringe filter. The filtered
170 supernatant was diluted 10 times and analyzed by HPLC using the method described in
171 Henderson and Brooks' study [41, 42].

172 Phenolic compounds including sinapic acid and tannic acid were extracted as described by
173 Khattab et al. [43] with slight modifications based on a method described by Doheny-Adams et

174 al. [44] to simultaneously extract glucosinolates. Briefly, 10 mg of the samples were mixed with
175 0.5 ml of 80% methanol using sonication (model 3510, Branson, Danbury, CT, USA) at room
176 temperature for 5 min. The solution was then mixed at 70 rpm for 30 min. The samples were
177 centrifuged at 15924 ×g for 10 min and the supernatant was collected. The process was repeated
178 once more before supernatants from each extraction were mixed and filtered using 0.45 syringe
179 filters. The extracts were analyzed using the modified HPLC method described by Khattab et al.
180 [43] and the UV absorbance for glucosinolates was set at 237 nm [45].

181 Phytate was measured following an enzymatic method derived from De Boland et al. [46]
182 and modified by McKie and McCleary [47]. For phytate extraction, 1 ml of 0.66 N hydrochloric
183 acid was added to 50 mg of sample in 2 ml microtubes. The tubes were covered and placed on a
184 shaker for 24 h at room temperature. The phytate was then measured using Megazyme (Ireland)
185 kit [48]. The hydrolysates were centrifuged at 15924 ×g for 10 min. The supernatants were
186 transferred to new microtubes and centrifuged again. Then, 0.5 ml of the supernatant was
187 transferred to new tubes and neutralized with 0.5 ml of 0.75 M NaOH. The following steps were
188 carried out as described in the Megazyme protocol [48].

189 The *in vitro* digestibility of the fermented fractions was investigated with a two-step
190 enzymatic hydrolysis followed by a filtration step that mimics pigs' gastric and small intestine
191 digestion processes [49]. For the first step, 2 g of dried samples were submerged in 100 ml of
192 phosphate buffer (0.1 M, pH 6) and 40 mL HCl solution (0.2 M) and the pH was adjusted to 2
193 using HCl (1 M). The hydrolysis was conducted for 2h with freshly prepared pepsin solution
194 (100 mg of pepsin / mL phosphate buffer 0.2 M). For the second step, 40 ml of phosphate buffer
195 (pH 6.8) and 20 ml of NaOH solution (0.6 M) was added, the pH of the solution was adjusted to
196 6.8 using NaOH, and samples were hydrolyzed using pancreatin for 4 h to mimic pigs' small

197 intestine. After enzymatic hydrolysis, samples were filtered using pre-weighed and labeled
198 Nylon bags (R510, 50 µm porosity, Ankom Technology, Macedon, NY), rinsed several times
199 with water, twice with ethanol, and twice with acetone. Each bag was then sealed and placed into
200 a 50 °C incubator for 72 h. Bags were cooled down in a desiccator and the weight was recorded
201 as residue dry matter (DM).

202 The *in vitro* digestibility coefficient of dry matter (DMdv) was calculated using the
203 following equation:

204 $$DMdv = (\text{feed DM} - \text{residue DM})/\text{feed DM}$$

205 where feed DM is the dry weight (dry matter) in the feed before the two-step enzymatic
206 hydrolysis and residue DM correspond to the dry weight of the residue after the two-step
207 hydrolysis [49-53].

208 **2.5 Statistical analysis**

209 A 2 by 8 two-way analysis of variance (ANOVA) was performed to study the main effect
210 of fraction and the main effect of fermentation. The independent variable fraction included eight
211 levels: NM, JP, JF, JC, BP, B0, B150, and B300. The independent variable fermentation
212 included two levels: fermented and control. The dependent variable is the amino acid
213 concentration protein, amino acid, antinutrients, phytate, and *in vitro* digestibility.

214 The 95% confidence intervals of each fraction before and after fermentation were
215 calculated to compare the significant difference between the control and fermented sample of
216 one fraction. The significance level of 0.05 was applied to all the analysis. All of the analyses
217 were completed using IBM SPSS Statistics 27.0 (IBM Corp. Armonk, NY).

218 **3. RESULTS AND DISCUSSION**

219 In this study, fractions of mechanically treated canola meal were used as substrate for
220 solid-state fermentation with the fungus *P. ostreatus*. It was visually observed that the fungus
221 successfully penetrated and colonized different canola meal fractions (**Figure S1**). The color of
222 cultures changed from dark brown (canola meal color) to white (*P. ostreatus* mycelia color) after
223 12 days of fermentation. No agitation was applied throughout the fermentation, though the
224 fungus successfully penetrated inside the substrates and even detached their particles from the
225 flasks' wall and colonized them (**Figure S1**). **Table 1** presents the initial moisture content of
226 fractions, and their final moisture content and the solid weight loss throughout the 12 days of
227 fermentation. Dry weight loss is a simple indirect indication of fungal growth and biomass
228 prediction in solid-state fermentation systems [54]. Analysis revealed that biological treatment of
229 these fractions impacts canola meal nutritional value presented in the following sections.

230 **Table 1.** Initial and final moisture contents of canola meal before and after fungal fermentation for different
231 mechanical fractions

Fraction[1]	Fermentation	Initial Moisture Content %	Final Moisture Content (Adjusted to 65% at the beginning of the fermentation)	Dry Weight Loss %
NM	Control	8.13	59.10	-
	Fermented		60.83	10.37
JP	Control	5.09	56.10	-
	Fermented		61.25	12.63
JF	Control	5.08	55.19	-
	Fermented		61.67	15.96
JC	Control	5.23	56.28	-
	Fermented		59.14	10.18

BP	Control	7.14	54.90	-
	Fermented		59.53	13.65
B0	Control	6.38	56.91	-
	Fermented		57.98	7.95
B150	Control	7.04	54.10	-
	Fermented		61.49	10.58
B300	Control	7.45	56.59	-
	Fermented		61.11	12.21

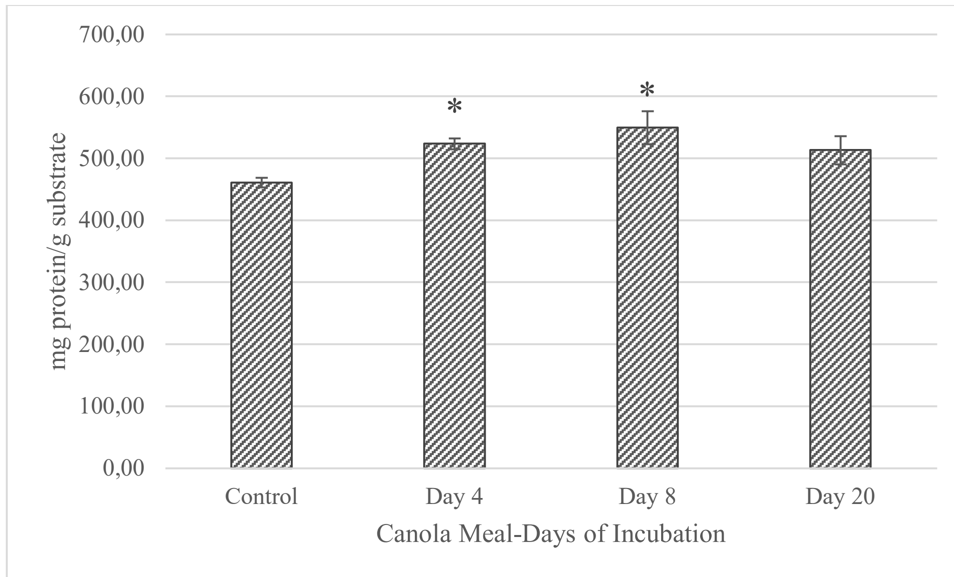
232 [1] Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
233 samples from jet milling process (JC), parent samples from ball milling process (BP), ball milling samples ranging
234 from 0-150 µm (B0), ball milling samples ranging from 150-300 µm (B150), ball milling samples ranging from 150-
235 300 µm (B300)
236

237 ### 3.1 Protein content of canola meal fermented with *P. ostreatus*

238 Canola meal was incubated for 4, 8 and 20 days. Twenty days was included in the initial
239 design as an extreme case to study the prolonged period of fermentation. The protein content
240 increased from day 4 to day 8 of incubation with respect to non-fermented samples (control).
241 However, it decreased after 20 days of the incubation (**Figure 1**), which indicates that prolonged
242 fermentation does not benefit this process. Therefore, the following experiments were designed
243 to last at most 12 days. The two-way ANOVA indicated that fermentation increased the protein
244 content of mechanically treated samples significantly ($p < 0.05$) after 8 and 12 days of incubation
245 compared to non-fermented samples (**Figure 2**). There was no significant interaction between
246 fermentation and fraction ($p > 0.05$). The increase in protein content was greater after 12 days of
247 incubation and it was significant for all samples compared to the controls ($p < 0.05$) (**Figure 2**),
248 this harvesting time was, therefore, selected for further analyses.
249 White-rot fungi are known for their ability to grow on lignocellulosic materials such as
250 canola meal. Croat et al. [45] performed a different set of experiments to examine the ability of

251 various white-rot fungi for canola meal protein enhancement. They used the meal produced
252 through different oil extraction methods and performed solid-state fermentation at 50% moisture
253 content. They observed 22.9, 16.9 and 15.4% protein enhancement by using *Trichoderma reesei*,
254 *Aureobasidium pullulans* (NRRL-58522), and *A. pullulans* (NRRL-Y-2311-1). They also
255 achieved the highest protein content of 47.5% in the samples subjected to solid-state
256 fermentation with *T. reesei*. In our study, growing *P. ostreatus* on mechanically fractionated
257 canola meal resulted in an increase in protein content, ranging from 11 to 18%. The highest
258 protein content of 51.9% was obtained from the microbial conversion of B0 samples, evidencing
259 an improvement from mechanical treatment.

260 Similarly, protein content of soybean meal increased from 47.1 to 57.6% after fungal
261 fermentation using *Aureobasidium pullulans* [55]. This increase was not solely due to microbial
262 fermentation since they had increased the protein content from 47.1 to more than 50% by adding
263 a high inoculation rate [55]. In contrast, in our study, the protein content of the inoculated
264 samples measured immediately after inoculation had not changed due to the low inoculation rate.



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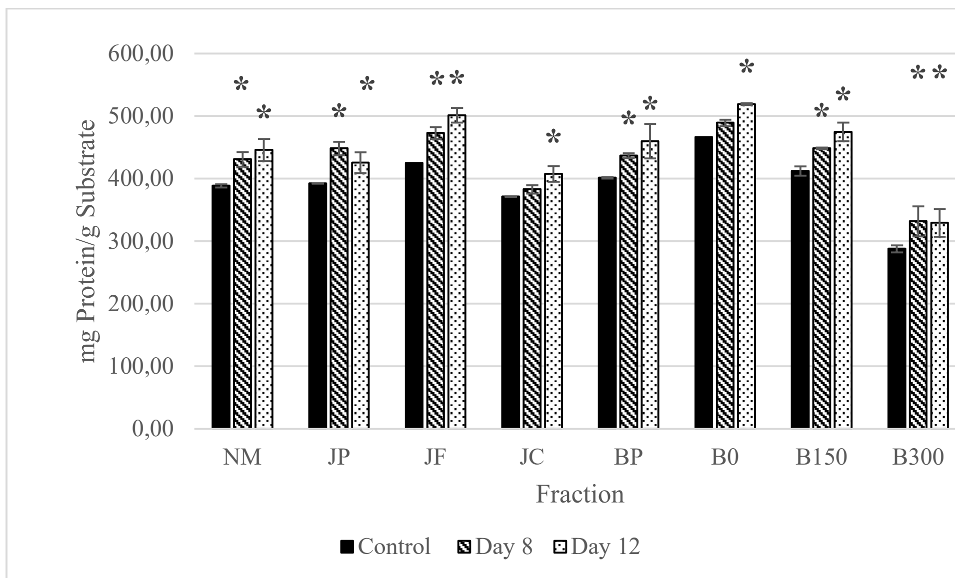
266 **Figure 1.** Protein content of canola meal treated with *P. ostreatus* for 4, 8 and 20 days compared to non-fermented
267 sample (control)
268 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
269 fraction, based on the 95% confidence intervals
270
271



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273 **Figure 2**. Protein content of canola meal treated with *P. ostreatus* for 8 and 12 days in different samples
274 Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
275 samples from jet milling process (JC), parent samples from ball milling process (BP), ball milling samples ranging
276 from 0-150 µm (B0), ball milling samples ranging from 150-300 µm (B150), ball milling samples ranging from 150-
277 300 µm (B300)

278 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
279 fraction, based on the 95% confidence intervals
280

281

282 **3.2 Amino acid profile of fermented canola meal**

283 Essential amino acids were analyzed for fermented and non-fermented (control) samples.
284 The two-way analysis showed that fermentation had a significant effect on all amino acids,
285 except valine and isoleucine. Arginine, leucine, lysine, and phenylalanine values increased
286 significantly ($P < 0.05$) in all fermented fractions as compared with controls, while histidine,
287 tyrosine and methionine decreased ($P < 0.05$). Despite adding 4% thioglycolic acid, tryptophan's
288 peak was not detected in our samples because it was hydrolyzed by hydrochloric acid through
289 the measurement [56, 57]. The interaction between fermentation and fraction was not significant,
290 except for tyrosine and lysine, proving that amino acid profile changes resulting from the fungal
291 growth is independent of canola meal particle sizes. Average value of these amino acids before
292 and after fermentation is presented in **Table 2**.

293 On average, arginine was subjected to the highest increase (28%) after the fermentation
294 with *P. ostreatus* compared to the non-fermented fractions. Arginine is considered a critical
295 amino acid in the swine industry. Its addition to gestating sows diet resulted in a higher number
296 of live-born piglets with greater birth weight, while its deficiency decreased the growth of the
297 piglets [58]. Supplementing higher levels of dietary arginine than what is recommended by the
298 National Research Council [59] is suggested due to the following benefits: maintaining gut
299 health and preventing intestinal disfunction in weanling piglets, enhancing the postnatal growth
300 of pigs, maximizing milk production by sows, and increasing fetal survival in pregnant pigs [60,

301 61]. Contrary to other studies, Bass et al. [62] found that adding 1% additional arginine to sows
302 diets during late pregnancy did not increase piglets body weight. However, their data
303 demonstrate that arginine supplementation increases the body weight gain in gilts and second
304 litter sows.

305 The two-way ANOVA also revealed that the main effect of fraction was significant for all
306 amino acids ($P < 0.001$). Finer fractions, in both fermented and non-fermented fractions, had
307 higher amino acid content compared to the coarser fractions and the parent meals. The B0
308 fraction had the highest average for all of the amino acids except for threonine and the B300
309 fraction had the lowest average for all of the amino acids except for threonine These data
310 confirm previous findings by Hansen et al. [15] on fractions of different sizes without biological
311 treatment, and highlight the effect of particle size in the nutritional value of defatted canola meal.

312 **Table 2.** Amino acid level of canola meal fermented with *P. ostreatus* for 12 days in different samples

Fraction[1]	Fermentation	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Tyr	Val	Sum[2]
		mg/g substrate										
NM	Fermented	18.10	6.76*	7.83*	21.84*	9.56	4.84*	10.63*	10.42*	5.50*	10.48*	238.28
	Control	18.96	8.38*	9.36*	20.11*	10.26	6.00*	11.66*	12.79*	7.13*	12.26*	262.98
JP	Fermented	25.99*	8.69	10.57	22.88	12.11	6.00	13.25	14.01	6.57*	13.60	287.20
	Control	19.95*	9.07	10.11	22.4	12.03	6.26	12.4	13.43	7.17*	13.02	278.61
JF	Fermented	27.72*	9.78	11.1	24.19*	13.12*	6.67	14.23*	14.99*	7.52	13.97	303.76
	Control	21.08*	9.19	10.17	22.73*	11.89*	6.56	12.74*	13.36*	7.24	12.98	280.85
JC	Fermented	24.58*	8.55	10.19	22.08*	11.59	5.91	12.7*	13.21	6.60	12.83	278.08
	Control	19.29*	8.50	9.40	20.76*	10.72	5.88	11.39*	12.27	6.50	12.13	260.10
BP	Fermented	24.83*	8.67	11.24	24.23*	12.11*	6.45	13.73*	14.14	7.24	14.35*	288.75
	Control	20.11*	9.13	10.25	22.17*	9.57*	6.40	12.53*	13.20	7.19	13.01*	273.52
B0	Fermented	27.20*	10.52	11.41	26.42	12.75	7.22*	15.13	14.62	7.89	14.32	316.62
	Control	23.70*	10.59	11.83	27.2	12.15	7.71*	15.01	14.37	8.28	14.86	319.41
B150	Fermented	25.36*	8.02*	10.58	22.43	11.77*	5.87	12.96	13.73	5.63*	13.39	286.67
	Control	19.13*	8.69*	10.05	22.01	10.02*	6.17	12.00	12.73	6.73*	12.91	264.33
B300	Fermented	17.81*	5.17	7.73	18.13*	8.69*	3.91	8.84	11.21	3.57*	10.41	210.24
	Control	12.89*	5.67	7.51	16.23*	7.58*	4.23	8.36	11.02	5.29*	10.43	200.12

Mean	Fermented	23.95	8.27	10.08	22.78	11.46	5.86	12.68	13.29	6.32	12.92	276.20
	Control	19.39	8.65	9.83	21.70	10.53	6.15	12.01	12.89	6.94	12.70	267.49
SEM[3]	Fermented	0.42	0.10	0.17	0.19	0.15	0.07	0.16	0.21	0.07	0.20	3.61
	Control	0.46	0.11	0.18	0.21	0.17	0.08	0.18	0.24	0.08	0.22	3.95
P value[4]	Fermented vs Control	<0.0001	0.01	0.33	0.001	0.0005	0.01	0.01	0.23	<0.0001	0.48	0.30

313 [1] Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
314 samples from jet milling process (JC), parent samples from ball milling process (BP), ball milling samples ranging
315 from 0-150 µm (B0), ball-milling samples ranging from 150-300 µm (B150), ball milling samples ranging from
316 150-300 µm (B300)
317 [2] Sum of essential and nonessential amino acids without tryptophan
318 [3] Standard error of means
319 [4] *P* value for the fermentation effect
320 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
321 fraction, based on the 95% confidence intervals
322
323

324 **3.3 Antinutrients**

325 Sinapic acid concentration of the treated samples decreased over 99% with 12 days of
326 incubation (**Table 3**). The sinapine concentration reduction ranged from 96.71 to 99.78 %.
327 Phenolic compounds such as sinapic acid add a bitter taste to the feed. It is also shown that hens
328 receiving sinapine, the choline ester of sinapic acid, lay eggs with a fishy odor or taste [7, 8].
329 This reduction is in agreement with prior observations where fermentation of rapeseed meal with
330 *P. ostreatus* and *Trametes versicolor* decreased the concentration of sinapic acid from the parent
331 meal by 93.2% and 93.0%, respectively [63].

332 The glucosinolates level decreased in a range of 61.3 to 98.8% in samples after fungal
333 fermentation (**Table 3**). The reported value corresponds to the sum of gluconapin and
334 glucotropaeolin. Sinigrin, another individual form of glucosinolates, could not be detected in the
335 samples. Glucosinolates are among the antinutrients in canola meal known to increase poultry
336 mortality, cause thyroid gland enlargement, perosis, and reduce growth and feed intake if
337 included in poultry diet [64]. Glucosinolates biodegradation by *P. ostreatus* was also measured

338 to assess the potential role of this fungus in reducing the risk associated with the presence of
339 these compounds in feedstocks. *P. ostreatus* exhibited a markedly high ability in decreasing
340 glucosinolates content. The presence of more than 2.0 and 2.5 µmol/g of glucosinolates can
341 cause issues for poultry and pigs, respectively. However, its effects on pigs are more severe than
342 poultry and can even be fatal [65]. The final glucosinolates levels for all samples fell
343 significantly below 2 µmol/g, representing a very safe usage of the final meal as an ingredient in
344 diets for animals.

345 Therefore, the results indicate that antinutrient concentration for different fractions of
346 mechanically treated canola meal had decreased significantly after 12 days of fermentation.
347 Besides biological treatment, heat treatment has also been used to decrease glucosinolates, with
348 reductions of up to 95% in glucosinolates content after toasting canola meal at 100 °C for 120
349 min [14]. However, heat treatment resulted in lower protein solubility, amino acid content, and
350 digestibility than the parent meal [14], which supports the use of mild alternative treatments such
351 as the fungal fermentation presented in this study.

352

353 **Table 3.** Antinutrient concentration in canola meal before and after fungal fermentation for different mechanical
354 fractions

Fraction[1]	Fermentation	Sinapic acid (nmol/g)	Sinapine (nmol/g)	Total glucosinolates (nmol/g)
NM	Control	171.18±25.84*	68948.28±913.79*	24.28±2.14*
	Fermented	0.38±0.49*	252.87±109.35*	0.72±0.7*
JP	Control	208.29±21.8*	132491.38±3801.72*	362.44±23.25*
	Fermented	0.13±0.07*	949.87±484.35*	4.33±0.18*
JF	Control	168.97±12.48*	133887.93±6750*	111.26±7.25*

	Fermented	0.18±0.18*	293.1±189.66*	4.77±1.24*
JC	Control	178.02±1.24*	69258.62±4224.14*	131.94±2.34*
	Fermented	0.12±0*	2275.86±1724.14*	13.76±7.34*
BP	Control	152.44±5.42*	91612.07±3353.45*	2.24±0.13
	Fermented	0.14±0.12*	1764.37±415.78*	0.86±0.39
B0	Control	243.96±33.3*	114560.34±1732.76*	0.85±0.01
	Fermented	0.33±0.10*	689.66±51.72*	0.11±0.03
B150	Control	139.68±2.33*	80508.62±1974.14*	4.33±0.33
	Fermented	0.05±0.04*	310.34±140.78*	0.26±0.18
B300	Control	81.89±0.4*	49405.17±3181.03*	4.54±0.1
	Fermented	0.04±0.03*	137.93±28.16*	0.10±0.05

355 [1] Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
356 samples from jet milling process (JC), parent samples from ball milling process (BP), ball milling samples ranging
357 from 0-150 µm (B0), ball milling samples ranging from 150-300 µm (B150), ball milling samples ranging from 150-
358 300 µm (B300)
359 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
360 fraction, based on the 95% confidence intervals

361

362 **3.4 Phytate**

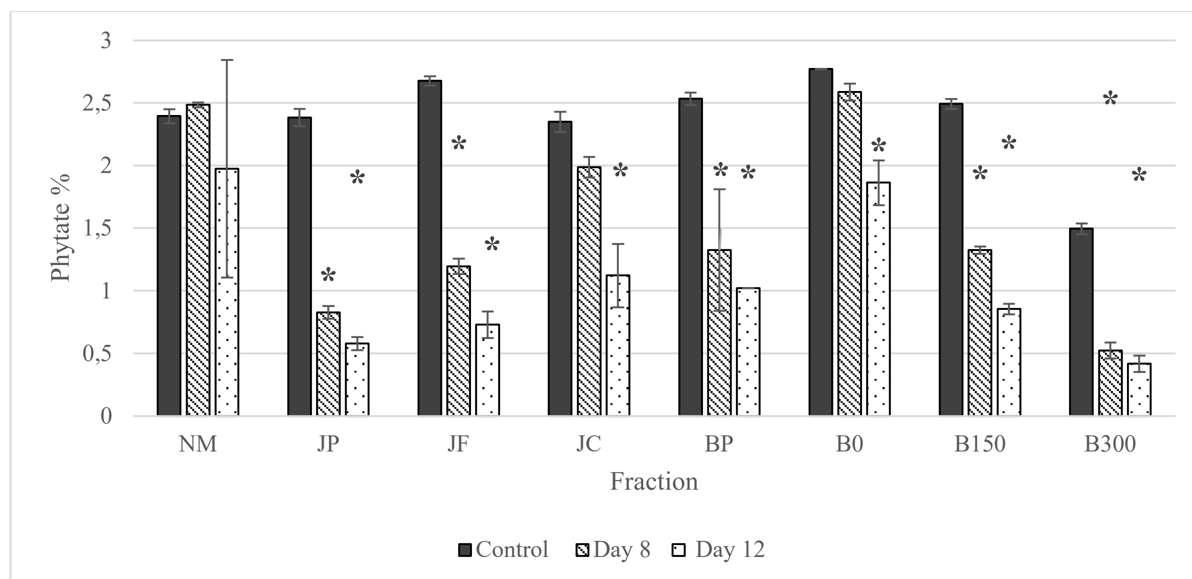
363 The analysis conducted in this study demonstrates that *P. ostreatus* has a high capability to
364 degrade phytate (**Figure 3**). Phytate content was measured on days 8 and 12 of incubation to
365 assess profile change throughout the fermentation progress. ANOVA analysis showed a
366 significant effect from fermentation ($P < 0.05$). An asterisk indicates significant reduction of
367 phytate content in a fermented fraction on days 8 and 12 of incubation compared to its control
368 (non-fermented). The analysis indicates that in all fractions, fermentation for 12 days
369 significantly ($P < 0.05$) reduce the phytate content of the samples except for NM. Not milled
370 (NM) samples were the coarsest samples compared to other fractions and we also observed a

371 noteworthy aggregation of canola meal particles, which was not removed to keep the samples a
372 good representative of what is received from the company. The larger size and aggregated
373 particles of NM samples could lead to limited accessibility of phytase to its substrate, which
374 could explain the lower phytate reduction in those samples. Similar decrease of fungal phytase
375 activity has been reported due to the presence of either large or very small particles, which result
376 in compaction and formation of agglomerates [66, 67]. Phytate plays a major role in the
377 determination of feed quality. Canola meal contains about 2-5% of phytic acid [13]. Phytic acid,
378 the primary form of phosphorus in plant seeds, binds with minerals such as K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+},
379 and Fe^{3+}, making both phosphorus and minerals biologically unavailable to monogastric animals
380 [10-13]. The presence of phytate not only results in the reduction of minerals and phosphorus
381 bioavailability and digestibility, the addition of the small trace of phytate, about 0.5%, to the feed
382 can also decrease feed conversion and protein availability [10, 68]. Consequently, phytate
383 reduction by combining mechanical treatment and fungal fermentation is a suitable and
384 potentially low-cost alternative to effectively improve feed quality.

385

386

387



388 **Figure 3.** Phytate content of samples from different mechanical fractions fermented with *P. ostreatus* for 8 and 12
389 days
390 Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
391 samples from jet milling process (JC), parent samples from ball milling process (BP), ball milling samples ranging
392 from 0-150 µm (B0), ball milling samples ranging from 150-300 µm (B150), ball milling samples ranging from 150-
393 300 µm (B300)
394 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
395 fraction, based on the 95% confidence intervals

396

397 3.5 *In vitro* digestibility

398 *In vitro* digestibility of mechanically treated samples was evaluated before and after
399 fermentation. The two-way ANOVA showed significant main effect from fraction and
400 fermentation. However, there was no significant interaction between fraction and fermentation
401 ($P > 0.05$). *In vitro* digestibility of non-fermented (control) and fermented samples are presented
402 in **Table 4** and an asterisk was applied if the improvement for a specific fraction was statistically
403 significant ($P < 0.05$). Finer fractions with higher nutritional quality resulted in higher
404 digestibility of dry matter, and JF showed the highest digestibility of all. In a previous *in vivo*
405 study performed by Hansen et al. [15], different fractions of mechanically separated canola

406 meals were included in mink diets, and they also determined that the fine fractions resulted in

407 higher digestibility.

408 **Table 4.** *In vitro* digestibility of different fractions of canola meal before (control) and after 12 days of fermentation.
409 Values represent means of *in vitro* digestibility coefficient of dry matter (DMdv)

Fraction[1]	DMdv	
	Control	Fermented
NM	52.9±0.6*	57.1±1.9*
JP	60.5±0.8	62.9±1.8
JF	72.7±0.9	74.0±1.1
JC	53.5±1.3	54.5±2.0
BP	52.8±0.8*	57.1±3.1*
B0	63.7±1.7*	66.9±1.4*
B150	47.9±0.6*	55.1±1.1*
B300	39.0±1.5	40.7±0.9

410 [1] Not milled (NM), parent samples from jet milling process (JP), fine samples from jet milling process (JF), coarse
411 samples from jet milling process (JC), parent samples from ball mill process (BP), ball milling samples ranging from
412 0-150 µm (B0), ball milling samples ranging from 150-300 µm (B150), ball milling samples ranging from 150-300
413 µm (B300)
414 * An asterisk shows significant difference between the fermented and control (non-fermented) samples, at a specific
415 fraction, based on the 95% confidence intervals
416

417 **Table 5** provides the nutritional values of treated and untreated samples and compares

418 them with soybean meal values as an ideal protein source for monogastric animals. The fungal

419 fermentation adds one step to the canola meal production flow chart. Taking the traditionally

420 made canola meal, NM, into a bioreactor and simply inoculate it with the edible *P. ostreatus* for

421 12 days improves its nutritional profile significantly. However, as shown in **Table 5** by

422 comparing the soybean meal values with fungal treated NM, the feed parameters still fall below

423 such a standard protein source. Mechanical treatment provides fine fractions with improved
424 nutritional value, including higher protein content, lower fiber content and higher digestibility.
425 However, the presence of antinutrients such as sinapine, glucosinolates and phytate remains
426 problematic even in fine fractions. Combining the two treatment methods, where mechanical
427 fractionation treatment is followed by a fungal fermentation, results in production of samples
428 such as fermented JF and fermented B0 with nutrient levels comparable soybean meal. Both
429 Fermented JF and B0 show the highest feed quality among the original fractions, to select the
430 superior sample for future studies and commercialization, their production cost should be
431 considered. JF is produced through a jet milling process followed by air classification, applying
432 both of these methods require high energy input [15]. Conversely, B0 is generated by applying
433 ball milling and sieving processes, both requires low energy usage [15]. Therefore, B0 could be
434 more suitable for large scale and industry purposes. Production of fermented B0 requires adding
435 three processes including milling, fractionation and fermentation to the existing canola
436 production plant (**Figure 4**). Hence, to have a realistic insight on the economic viability of the
437 proposed process, performing a techno-economic analysis is suggested.

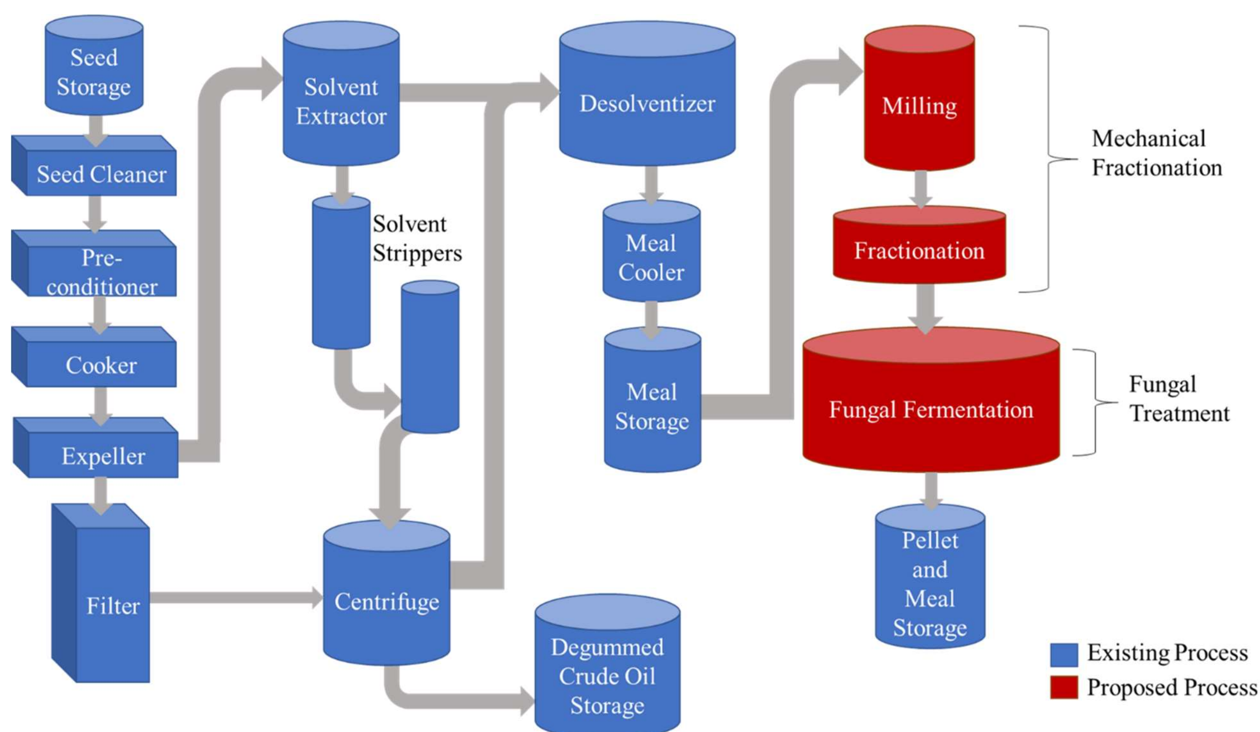
438

439 **Table 5.** Nutritional values of samples with best nutritional values before and after fungal fermentation compared to
440 soybean meal

Substrate[1]	Protein	Total Glucosinolates (nmol/g)	Sinapine (nmol/g)	Phytate (%)	DMdv
Soybean meal[2]	542.3 [69]	0	0 [70]	1.6 [71]	77.1 [72]
Control NM	388.55	24.28	68948.28	2.39	52.9
Fermented NM	445.95	0.72	252.87	1.97	57.1
Control JF	424.85	111.26	133887.93	2.68	72.7
Control B0	466.68	0.85	114560.34	2.77	63.7

Fermented JF	501.35	4.77	293.1	0.73	74
Fermented B0	519.33	0.11	689.66	1.86	66.9

441 [1] Not milled (NM), fine samples from jet milling process (JF), ball milling samples ranging from 0-150 µm (B0)
442 [2] Data gathered from other reports
443



444

445 **Figure 4**. Process flow diagram of canola meal production integrated with proposed processes [73]
446

447 Canola meal treatment has been researched in numerous studies with the primary goal of
448 converting the meal into a more nutritious feed, especially for monogastric animals [22, 24, 45,
449 74]. Mechanical treatments separate canola meal in size fractions that show different nutritional
450 values with potential for distinct feed applications. Coarse fractions with lower digestibility
451 could be included in ruminant diets. The finer fractions, with higher protein and nutritional
452 value, could be used as protein supplement in the diet of monogastric animals such as swine and

453 poultry diets. However, even the finest fractions contain a considerable amount of antinutrients
454 such as sinapine, glucosinolates, and phytate. Here, we demonstrated that *P. ostreatus* could
455 successfully grow on fractionated portions of canola meal through a solid-state fermentative
456 process with markedly positive outcomes. Our results also proved that fermentation of *P.*
457 *ostreatus* is an independent variable from the fractions obtained from mechanical treatment. *P.*
458 *ostreatus* fermentation resulted in higher protein content and lower phytate and antinutrients in
459 all sized-based separated fractions of canola meal compared with original non-fermented
460 fractions. Despite the significant effect of fermentation on most amino acid, compared to other
461 promising results obtained in this study, amino acid enhancement methods are worth future
462 research. Studies on other agro-industrial residues such as corn-based wet distiller's grains with
463 solubles (WDGS) showed that supplementing nitrogen sources such as urea to fungal solid-state
464 fermentation improve their protein and amino acid content [75, 76]. Sun et al. [76] observed that
465 supplementing urea to WDGS culture with *R. oryzae* and *M. indicus* improved the protein level
466 by 35 and 38%, and total amino acids concentration by 28 and 18%, respectively. This suggests
467 that if an additional source of nitrogen is provided, fungi could use it to synthesize critical amino
468 acids. Coarser fractions of canola meal with a lower protein content had lower amino acid levels;
469 therefore, adding nitrogen sources is proposed for future studies to assess the possibility of
470 further improvement of amino acid and protein content of these fractions.

471 **4. CONCLUSION**

472 This study demonstrated that combining fungal fermentation with mechanical treatment of
473 canola meal is a suitable strategy to improve its nutritional quality and significantly reduce its
474 antinutrient content. Even though the nutritional value of all canola meal fractions increased,

475 their quality did not increase equally. Growth of *P. ostreatus* was observed in all canola meal
476 fractions regardless of particle size, indicating that particle size did not limit the fermentation
477 process or the subsequent improvement in feed quality of the obtained fractions. Furthermore,
478 the fermentation process enhanced digestibility across different size fractions with no detrimental
479 effects in nutritional quality. We suggest in-vivo studies to further assess the potential benefits
480 and applicability of this alternative to enhance the feed quality of canola meal for monogastric
481 animals.

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487 **CONFLICT OF INTERESTS**

488 None

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