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ORIGINAL PAPER

Upcycling Canola Meal as Compared to Soybean Meal by Fungal Bioconversion for Potential Monogastric Animal Nutrition

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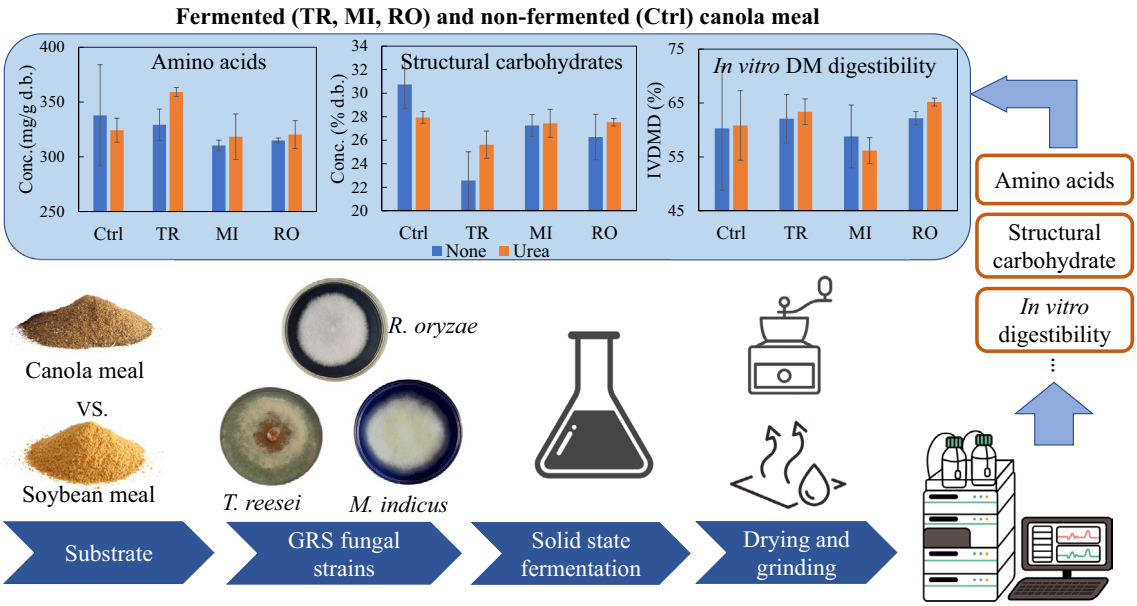
Abstract

Canola meal (CM) produced after oil extraction has potential to be used as a nutrient-rich feeding ingredient for monogastric animals if its fiber and anti-nutritional factors can be reduced while essential amino acids (AA) are increased. Fungal bioconversion provides a way of improving feeding value in CM for monogastric animals. This study explored the effects of three fungal strains namely, *Rhizopus oryzae* (*R. oryzae*), *Mucor indicus* (*M. indicus*), and *Trichoderma reesei* (*T. reesei*), via solid state fermentation, on profiles of AA, structure carbohydrates (SC), sinapic acid, and *in vitro* dry matter digestibility (IVDMD) of CM with and without supplement of urea as a nitrogen source. Soybean meal (SM) was investigated at the same condition as CM for comparison. Flask trials using each substrate with 70% moisture content (MC) were conducted for 192 h at 28 °C. *T. reesei* was determined as the most effective fungal strain due to its higher improvement of total AA by 10.7%, and threonine (Thr), methionine (Met), and lysine (Lys) by 19.2%, 20.4%, and 14.4%, respectively, when 1% N urea was supplied. *T. reesei* also degraded more SC (up to 26.6%) and produced more digestible sugars, compared to other strains. In addition, *T. reesei* treated SM and CM showed higher IVDMD than non-treated compared with other fungi. This study demonstrated the feasibility of fungal strain in improving feeding value of CM and SM for better monogastric animal diet.

Graphical Abstract



Keywords Canola meal · Soybean meal · Fungal bioconversion · Amino acids · Monogastric animals

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Statement of Novelty

As the world is facing more unpredictable supply of food, feed and fuel due to economic, political, and climate issues, exploring new resources or valorizing existing resources for these supplies are becoming urgent. As a recent mandate of enhancing biodiesel production in California, United States is effective, planting of more oil seeds especially soybean and canola seeds is required which will generate huge abundance of co-product mainly soybean meal and canola meal. Soybean meal has traditionally been used as main feeding materials due to its high protein and relatively balanced amino acids, while canola meal has lower feeding value due to its relatively imbalanced amino acids and antinutritional factors. Therefore, improving feeding value of canola meal to be more closed to soybean meal will have large benefits on both canola planters and animal feeding market. This study used several important GRAS fungal strains that have traditionally been used for human food production to process canola meal as compared to soybean meal with solid-state fermentation, to improve the feeding values and digestibility, reducing the impact of biofuel mandate and increasing potential revenues for the rural economy.

Introduction

Canola meal (CM) as a co-product from canola oil extraction has great potential as animal feed as it contains around 35–40% proteins, slightly lower than soybean meal (SM) [1], but a much higher B-vitamins and minerals. However, CM is a much lower valued feed due to its content of fiber and anti-nutrients compared to SM. Its usage is limited to ruminant animals and in some market, it is directly land-applied as fertilizer [2]. The anti-nutrients such as phenolic compounds and glucosinolates in CM can reduce growth performance in livestock animals [3]. The total dietary fiber in CM is on average 31.7% of dry biomass, higher than it in SM (21.8%) [4]. Fibers, especially hemicellulose, have low digestibility by monogastric animals [5]. CM has high content of several anti-nutritional factors such as glucosinolates, sinapic acid and phytate [6]. Glucosinolates and sinapic acid are toxic, with negative impact on animal growth. Phytate bounds minerals and phosphorus, preventing them from animal assimilation [7]. The amino acids (AA) profile of CM is also not well balanced especially for Lys which is usually limited in CM as compared to SM [8]. Indispensable AA such Lys, Met, and arginine (Arg) are critical for monogastric animal growth and health. All animals during the early stage of growth need as many indispensable AA as possible to produce sufficient proteins to support their body growth [9]. Lysine and Met are the two most important AA for pigs (non-ruminant) to support growth and maturity. In addition, Arginine is essential for young piglets which accounts for 40% of pigs, as most Arg supplied to their diet is used in the urea cycle of their livers during growth [9]. Arginine and Lys are also required in poultry diets and deficiency of these AA can lead to negative effect on feather growth [10]. Therefore, processes to degrade fiber and anti-nutrients, and increase protein content and indispensable AA are needed to transform CM into a high-quality feeding ingredient.

Bioprocessing of CM with bacteria, fungi, and enzymes for nutritional value improvement have been reported in many studies. The bacterial strains *Bacillus* and *Lactobacillus* are commonly utilized for bioprocessing of canola and rapeseed meal. Examples include using *Lactobacillus salivarius* in solid-state fermentation where 38% of glucosinolates and 16% of crude fiber in rapeseed meal were removed [11]. Fungal strains are also commonly used in CM bioprocessing. Croat and co-workers [1] investigated bioprocessing of different processed CM (hexane-extracted; and cold-pressed) using fungal strains *Trichoderma reesei* and *Aureobasidium pullulans*. All the fungal strains evaluated had increased protein by 15.4–22.9% in cold-pressed CM and reduced glucosinolates content by 89–99% in hexane-extracted CM and by 82–98% in cold-pressed CM, while the fiber content of the treated meal also increased. To address the issue of fiber enrichment, pretreatment of CM prior to fermentation had been explored. Pretreatment of the meal with extrusion method at 80 °C followed by treatment with fungal strain *T. reesei* resulted in protein increase by 42.8%, and reduction of neutral detergent fiber (NDF) by 19.5% in hexane-extracted CM. The same pretreatment followed by *A. pullulans* fermentation resulted in glucosinolates reduction by 84% in both hexane-treated and cold-pressed CM [12]. However, the effects of the thermal pretreatment on AA profile and digestibility were not evaluated. Therefore, developing bioprocessing that could reduce fiber and anti-nutritional factors while improve indispensable AAs and overall digestibility are required.

Fungal bioconversion is an eco-friendly way to improve AA profile, decrease fiber and ani-nutritional factors in animal feed. In addition, fermentation process has advantages of converting low-value carbon/nitrogen source into high-valued compounds. Nitrogen sources such as urea has long been considered as a high-quality nitrogen fertilizer, and it has been used in grape fertilization to improve the wine quality (Garde-Cerdán, et al., 2014). In addition, urea has been used as a replacement of vegetable and animal protein source in ruminant diet due to the presence of rumen uratolytic microorganisms in their gastrointestinal tracts that

convert urea into microbial protein and AA for ruminant nutrition. In comparison, monogastric animals such as swine [13] and poultry [14] have low quantity of uratolytic bacteria and are less likely to convert feeding urea into microbial protein. Since nitrogen source is key to AA synthesis, it is hypothesized that urea could help with amino acid synthesis in the animal feed fermentation process.

Urea had been proved as a low-cost nitrogen source to enrich AA during fungal fermentation of corn wet distiller's grains and soluble (WDGS) mixed with fiber-rich soybean hull [15]. However, urea supplement to protein-rich substrate such as CM and SM in fungal fermentation for potential monogastric animal feed production had not been evaluated. Three fungal strains *T. reesei*, *M. indicus*, and *R. oryzae* were selected due to their ability to produce hydrolytic enzymes to degrade indigestible fiber, reduce antinutritional factors such as phytate and phenolic compounds, and safe use in animal feed [16–18]. SM as a traditional high AA animal feeding ingredient with low phenolic compound was used as a control group to compare with CM. This study investigated effects of the three fungal strains, without and with urea supplement, on fiber degradation, AA enrichment, and sinapic acid reduction of CM and SM (Ctrl).

Materials and Methods

Selection of Substrates

CM and SM were purchased from local agricultural processing facility. The moisture content (MC), total solid (TS), ash, total structural carbohydrates (SC, containing glucan, xylan, arabinan, galactan and mannan) and total AA of CM and SM were shown in Table 1. Moisture content of CM and SM was determined based on the weight change before and after drying in 105 °C oven for 12 h. Total solid was calculated as percentage of the oven dry weight (ODW) of each sample over the sample before drying. Ash content was determined as the percentage of solids after burning the sample in 550 °C furnace for 4 h over the ODW of each sample. Total structure carbohydrates (SC) and total AA of each substrate was quantified based on methods described in the analytical methods section below.

Table 1 Major properties of the tested substrates

Meal	MC % w.b	TS % w.b	Ash % d.b	Total SC % d.b	Total AA % d.b
CM	7.4	92.6	7.9	30.7	33.8
SM	11.5	88.5	6.7	32.7	45.3

CM canola meal, *SM* soybean meal, *MC* moisture content, *TS* total solid, *SC* structural carbohydrates, *AA* amino acids, *w.b* wet basis, *d.b* dry basis

Fungal Strains Preparation

The original fungal strains of *Trichoderma reesei* (NRRL-3653) and *Mucor indicus* (ATCC-24905) (reclassified as *Mucor circinelloides* 24905), were purchased from American type culture collection (ATCC), and *Rhizopus oryzae* was isolated from evening primrose seeds [15]. All the fungal strains were maintained in potato dextrose agar (PDA) medium for 5 days at 28 °C for better development of spores and mycelia of each fungal strain. To prepare inoculation seed culture, five pieces of mycelia (0.5 × 0.5 square centimeter area for each) on the PDA medium for each fungal strain were cut and transferred aseptically to 50 mL of freshly prepared potato dextrose broth (PDB) medium (sterilized at 121 °C for 20 min) in 250 mL Erlenmeyer flask. The inoculated PDB for each fungal strain was then incubated shaken at 150 rpm for 2 to 4 days at 28 °C to achieve fungal growth at exponential phase so as to reduce lag phase after transferred to solid-state fermentation.

Solid-State Fermentation

For preparation of solid-state fermentation substrate, the amount of 11.3 g of SM (w.b.) or 10.8 g of CM (w.b.) (determined based on MC of each substrate) was added to 250 mL Erlenmeyer flask to ensure each flask having 10.0 g of dry solid. For treatment without urea supplement, deionized (DI) water was added to each flask so that 70% moisture of the substrate was reached after inoculation. For treatment with urea supplement, an aliquot of urea solution that can distribute 10 mg N per gram of dry substrate was prepared and added to substitute DI water in each flask. Each flask with substrate was inserted with foam stopper (JAECE Industries, Inc., NY, USA) and covered with alumina foil before autoclave at 121 °C for 30 min. The sterilized sample was cooled to room temperature and was then inoculated with 5 mL of the fully developed mycelia of the selected fungal strain. Treatments of Control (Ctrl, no fungal strain), *R. oryzae*(RO), *M. indicus*(MI), and *T. reesei* (TR) with and without urea were performed. Each treatment was conducted in triplicates.

The inoculated substrates with *R. oryzae* and *M. indicus* were incubated for 6 days and substrate with *T. reesei* was incubated for 9 days (due to slow growth of *T. reesei*) statically at 28 °C. After fermentation, each sample was dried at 60 °C for 48 h to reduce substrate MC to below 10%. To keep track on the substrate weight loss, the weight of each empty flask and the same flask with fermented and dried sample were measured. The weight loss was reflected in the product yield on wet basis: Yield

(%) = Substrate weight after fermentation/Substrate weight before fermentation × 100. The final dry weight of each sample was used to calculate yield of AA and SC based on initial dry substrate. The dried sample was grounded with a portable coffee grinder and stored frozen (− 20 °C) for further nutritional value testing.

Analytical Methods

Analysis of Amino Acids and Structural Carbohydrates

Each grounded sample was initially hydrolyzed with hydrochloric acid to breakdown the protein into AA based on reported method [19]. The hydrolyzed sample was then diluted and filtered with 0.22 µm PTFE filter. Derivatization of AA were performed by autosampler before analysis with high-performance liquid chromatography (HPLC) (1200 Infinity Series, Agilent Technologies Inc.) equipped with ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 3.5 µm) and diode array detector (DAD) [20]. A total of 17 types of AA for each sample were separated after 25 min running with injection volume of 40 µL, column temperature of 40 °C, and flow rate of 1.5 mL/min. Some of the most important AA such as Thr, Arg, Met, and Lys were selected to make further comparison between different treatments.

Structural carbohydrates of samples before and after fungal cultivation was determined based on NREL method [21]. Each grounded dried sample (or grounded raw material without treatment) was hydrolyzed with 72% wt. sulfuric acid at 30 °C for 1 h followed by further hydrolyzation using diluted sulfuric acid (4% wt.) at 121 °C for 1 h. The hydrolyzed sample was vacuum filtered. The solids were used for determination of acid insoluble residue (AIR) (including acid insoluble lignin and ash). The filtrate was firstly used to determine acid soluble lignin (ASL) with spectrophotometry method (320 nm wavelength). The remaining filtrate was neutralized with calcium carbonate and then filtered through 0.22 µm PTFE membrane. The hydrolyzed monomeric sugars (glucose, xylose, galactose, arabinose, and mannose) were separated and quantified with HPLC (1200 Infinity Series, Agilent Technologies Inc.) equipped with Biorad Aminex HPX-87P analytical column and refractive index detector (RID). The polymeric sugars (glucan, xylan, galactan, arabinan, and mannan) were converted from the monomeric sugars using an anhydro correction of 0.88 for C5 sugars (xylose, arabinose) and a correction of 0.9 for C6 sugars (glucose, galactose, mannose).

Sinapic Acid, Urea and Reducing Sugar Analysis

The modified method of phenolic compounds (mainly sinapic acid) extraction and determination was performed based on the reported study [22]. Briefly, a quantity of 40 mg of each ground and dried sample was mixed with 2.0 mL of 70% methanol for sinapic acid extraction. The supernatant after centrifugation at 13,000 rpm for 10 min was filtered (0.22 µm PTFE membrane) and quantified with HPLC (1200 Infinity Series, Agilent Technologies Inc.) equipped with ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 3.5 µm) and diode array detector (DAD).

Each ground dried sample was soaked with DI water at a ratio of 1:10 (w/v) at 4 °C overnight. The mixture was then centrifuged at 13,000 for 10 min to settle down the solids while the supernatant was collected for analysis of urea and reducing sugar. The concentration of urea was determined based on diacetyl monoxime method [23]. In this method, urea reacts directly with diacetyl monoxime under strong acidic conditions to give a yellow condensation product. The reaction was intensified to red color by the presence of ferric ions and thiosemicarbazide (to stabilize color) [24]. Reducing sugar was determined based on DNS method [25]. Briefly, a volume of 100 µL sample was mixed with 3 mL of regular DNS (3,5-dinitrosalicylic acid) solution (containing /L: 10 g NaOH, 2 g phenol, 10 g 3,5-dinitrosalicylic acid, 0.5 g sodium sulfite) followed by immediate heating in boiling (100 °C) water bath for 10 min for color development. The reacting mixture was diluted with deionized water before measuring absorbance in a spectrophotometer (DR 5000, Hach Company, Loveland, Colorado, USA) at 540 nm wavelength. Glucose was used as calibration standard for reducing sugar quantification.

In Vitro Dry Matter Digestibility

In vitro dry matter digestibility (IVDMD) of the fermented and non-fermented substrates was achieved via a sequential enzymatic hydrolysis using pepsin (P7000, 421 pepsin units per mg solids, Sigma-Aldrich) and pancreatin (P1750, four times the specifications of the United States Pharmacopeia, Sigma-Aldrich) to simulate gastric digestion and small intestine digestion of monogastric animals [26]. Briefly, each dry grounded sample was weighed into 500 mL Erlenmeyer flask with 100 mL of 0.1 M phosphate buffer solution (1/7 Na_2HPO_4/KH_2PO_4, pH 6.0) and 40 mL of 0.2 M HCl solution. The pH was adjusted to 2.0 by 1 M HCl or 1 M NaOH. An aliquot of 2 mL of 5 mg/mL chloramphenicol (C0378, Sigma-Aldrich Corp., St. Louis, MO, USA) solution (dissolved in 95% ethanol) was added to each flask to prevent microbial growth during hydrolysis. A volume of 4 mL of 100 mg/mL freshly prepared pepsin solution (dissolved in 0.2 M HCl) was added to each flask followed by incubation

in 39 °C incubator for 2 h with continuous shaking. Then, 40 mL of 0.2 M phosphate buffer (1/1 Na_2HPO_4/KH_2PO_4, pH 6.8) and 20 mL of 0.6 M NaOH were added to each flask with pH adjusted to 6.8, followed by adding 4 mL of 100 mg/mL fresh prepared pancreatin solution (dissolved in 0.2 M phosphate buffer). The flask was continuously hydrolyzed for 4 h under the same condition. The mixture in each flask after hydrolysis was filtrated through 50 µm pore size nylon bags (Ankom Technologies, Macedon, NY). The solids remained in the bag were dried in 50 °C for 72 h. The IVDMD was calculated based on the dry mass change of the sample before and after hydrolysis.

The calculation of IVDMD was shown below:

$$IVDMD\ \% = \frac{\text{Dry weight before hydrolysis} - \text{dry weight after hydrolysis}}{\textit{Dry weight before hydrolysis}} \times 100\%$$

Statistical Analysis

The statistical analysis for determination of pairwise statistical differences ($p < 0.05$) between treatments of AA, SC, sinapic acid, and reducing sugar were performed using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA). For analysis of key AA (Thr, Arg, Met, and Lys), given there were three categorical factors: treatment 1 (SM and CM), treatment 2 (Ctrl, TR, MI, and RO) and treatment 3 (No urea and urea), a three-way analysis of variance (ANOVA) was performed for each response variable: Thr, Arg, Met and Lys. The significance level was 0.05, and the analysis was done using SPSS.

Results and Discussion

Yield and Composition

The total solid recovery (TSR) of the meals varies between 90 and 100% based on different treatments with different fungal strains (Table 2). The TSR was calculated as the ratio of the weight of fermented meals to the weight of non-fermented meals (Ctrl) at dry basis. Generally, the fungal treated samples have shown a reduced TSR of meal due to the metabolisms of nutrients (protein, lipid or fiber) into volatile compounds such as CO_2 and NH_3 [27] which were emitted during the fermentation process. However, addition of urea as a nitrogen/carbon compounds showed a higher TSR after fermentation (averaged 95.6% in SM, 94.7% in CM) compared with treatments without urea (averaged 92.8% in SM, 91.2% in CM) (Table 2). This could be due to that urea partially supplied nitrogen/carbon source for fungal metabolisms, resulting in less utilization of nitrogen/carbon source from the substrate. Similar function of urea as both nitrogen and carbon source had also been reported for cyanobacteria metabolisms [28].

Table 2 The total and key amino acid concentration in SM and CM after fermentation with different fungal strains

Meal	Urea added	Strain	Dry matter g	Total solid recovery % d.b	Total AA mg/g d.b	*AA + %	Thr mg/g d.b	Arg	Met	Lys
SM	No	Ctrl	10.26 ± 0.01	100	452.77 ± 15.29	/	18.27 ± 0.65	30.82 ± 0.84	4.8 ± 0.22	24.16 ± 0.72
		TR	9.67 ± 0.02	94.25 ± 0.29	482.9 ± 27.98	6.65	22.03 ± 1.13	27.28 ± 1.03	4.75 ± 0.14	28.01 ± 0.51
		MI	9.63 ± 0.33	93.89 ± 3.24	454.24 ± 11.89	0.32	18.73 ± 0.42	31.13 ± 1.3	4.9 ± 0.47	26.18 ± 1.4
		RO	9.27 ± 0.03	90.38 ± 0.29	501.42 ± 13.27	10.74	20.86 ± 0.45	36.58 ± 0.54	4.51 ± 0.69	31.02 ± 0.73
	Urea	Ctrl	10.45 ± 0	100	455.44 ± 16.03	/	18.94 ± 0.62	34 ± 0.72	4.77 ± 0.36	20.05 ± 0.5
		TR	10.16 ± 0.03	97.19 ± 0.31	495.01 ± 3.18	9.33	22.7 ± 0.16	32.07 ± 0.9	5.6 ± 0.13	22.04 ± 0.32
		MI	10.11 ± 0.04	96.75 ± 0.42	446.96 ± 10.88	− 1.28	19.53 ± 0.79	34.06 ± 0.75	4.77 ± 0.16	20.3 ± 0.68
		RO	9.69 ± 0.04	92.76 ± 0.36	474.43 ± 8.14	4.78	20.83 ± 0.17	37.24 ± 0.35	4.49 ± 0.07	21.16 ± 0.38
CM	No	Ctrl	9.74 ± 0.01	100	337.87 ± 46.13	/	16.37 ± 2.52	22.38 ± 3.16	6.18 ± 1.67	18.11 ± 3.37
		TR	8.78 ± 0.08	90.11 ± 0.69	329.31 ± 14.34	− 2.53	18.01 ± 0.81	17.91 ± 0.73	4.84 ± 0.12	16.6 ± 0.7
		MI	9.03 ± 0.04	92.71 ± 0.47	310.42 ± 4.85	− 8.12	15.7 ± 0.32	20.76 ± 0.41	4.98 ± 0.13	16.92 ± 0.26
		RO	8.84 ± 0.05	90.73 ± 0.48	314.95 ± 2.24	− 6.78	15.14 ± 0.03	20.89 ± 0.15	4.79 ± 0.19	15.84 ± 0.1
	Urea	Ctrl	9.91 ± 0.03	100	324.29 ± 11	/	15.71 ± 0.29	22.59 ± 0.95	5.97 ± 1.79	13.22 ± 0.7
		TR	9.64 ± 0.1	97.31 ± 1.1	359.18 ± 4.09	6.31	18.73 ± 0.32	21.05 ± 0.3	7.19 ± 0.28	15.13 ± 0.09
		MI	9.38 ± 0.02	94.65 ± 0.39	318.35 ± 20.84	− 5.78	16.2 ± 1.05	22.75 ± 1.25	5.44 ± 0.34	13.72 ± 0.69
		RO	9.13 ± 0.03	92.16 ± 0.44	320.36 ± 12.59	− 5.18	15.7 ± 0.43	22.2 ± 0.76	5.14 ± 0.17	13.66 ± 0.52

*AA +: the improvement of amino acid content

Amino Acids Profile

Amino acids concentration after fermentation of SM and CM by three different fungal strain with and without urea addition was shown in Table 2. The total AA concentration in CM was 25% lower than in SM in non-fermented treatment, indicating the advantages of SM as protein-rich animal feed compared to CM. After treatment with fungi, the total AA concentration did not show much improvement ($p > 0.05$) by each strain in CM without urea supply. However, with supply of urea, *T. reesei* improved total AA concentration by 9.1% as compared with *M. indicus* and *R. oryzae*, although the improvement was not statistically significant ($p > 0.05$). In comparison, *R. oryzae* significantly ($p < 0.05$) improved total AA concentration by 10.8% without urea supply, while *T. reesei* enhanced total AA concentration significantly ($p < 0.05$) by 8.8% with supply of urea, when SM was used as substrate. Therefore, fungal treatment could be more effective in improving total AA concentration in SM than CM. When urea was supplied, *T. reesei* could be more preferable in improving total AA concentration of both CM and SM by converting urea into fungal protein, as compared to *R. oryzae* and *M. indicus* which, however, had been demonstrated to enhance total AA in corn distiller's grains and solubles (DDGS) with supply of urea [15, 29]. Different from *R. oryzae* and *M. indicus*, *T. reesei* produces significant quantity of carbohydrase and protease that could efficiently separate carbohydrates and protein in protein-rich substrate such as soybean flour [30].

The concentration changes of four essential AA: Thr, Arg, Met and Lys in non-fermented and fermented SM and CM by the three fungal strains were shown in Table 2. Fermentation of SM without urea supply showed improvement ($p < 0.05$) of Thr, Arg, and Lys by *R. oryzae*, and improvement ($p < 0.05$) of Thr and Lys by *T. reesei* as compared to Ctrl. When urea was supplied to SM, concentration increase ($p < 0.05$) was observed in Thr and Arg by *R. oryzae*, and Thr by *T. reesei* as compared to non-fermented SM. In comparison, fermentation of CM by three fungal strains without supplement of urea showed no difference ($p > 0.05$) in Thr, Met and Lys concentration as compared to Ctrl, indicating that supply of urea as additional nitrogen source could increase the net content of the four key AA via fungal fermentation. Threonine and Lys are considered metabolically indispensable AA which cannot be synthesized either by transamination of α-keto acid, or from their natural precursors, and therefore need to be supplied exogenously [31]. It was found that SM was more favorable for enrichment of Thr and Lys by fermentation with *R. oryzae* and *T. reesei* than CM, which could be due to refractory effects of phenolic compounds in CM on the fungal metabolisms [32]. It was noticed that CM treated with *T. reesei* with 1% N of urea supply was showing the best performance in improving the AA concentration. The supplement of urea provides nitrogen and carbon source for the synthesis of AA, and a significant advantage of AA improvement was shown in CM treatment with *T. reesei*. Thus, it could be concluded that urea is a valuable nitrogen source in the *T. reesei* fermentation of CM.

Structural Carbohydrate Profile

The degradation of the total SC (glucan, xylan, galactan, arabinan, and mannan) in each treatment is shown in Table 3. It was observed that total fraction of initial SC in SM (32%) and CM (30%) are similar. The SC in CM without urea supply were reduced significantly ($p < 0.05$) by 26.5% and 14.5% after fermentation by *T. reesei* and *R. oryzae*, respectively. When urea was supplied, only *T. reesei* showed significant reduction ($p < 0.05$) of the total SC by 8.3%. However, no significant ($p < 0.05$) reduction of total SC was found in SM after fungal fermentation in both urea supplied and non-supplied treatments as compared to non-fermented SM. The total SC contain cellulose and hemicellulose which have limited digestibility by monogastric animals due to their lack of gut bacteria that could secret endogenous cellulolytic and hemicellulolytic enzymes. However, some degradation products from fiber such as arabinoxylan and β-glucan can be used by gut bacteria and therefore serve as prebiotics for gut health of monogastric animals [33]. The higher fiber degradation in CM than SM by *T. reesei* was probably due to more carbohydrase production in CM than SM which induced more proteinase production due to high protein content in SM [30]. Supply of urea could simply promote synthesis of enzymes for urea utilization and AA accumulation by fungal strains [34], thus suppressing production of fiber degrading enzymes. It was noticed that the supply of urea showed some inhibitive effects on SC reduction by all fungal strains, especially in treatment with *T. reesei* (Table 3). This indicates that though urea is considered as effective N source for AA synthesis, it has potential adverse effect on SC degradation. Thus, the addition and amount of urea in the fermentation should be adjusted according to the type of fungal strains used in fermentation.

Without supply of urea, *T. reesei* showed significant reduction ($p < 0.05$) of glucan, galactan, and arabinan, while *R. oryzae* reduced galactan and mannan significantly ($p < 0.05$), when CM was used as substrate. Both *T. reesei* and *R. oryzae* were reported as producers of cellulase and xylanase [30, 35], which could effectively breakdown cellulose and hemicellulose into their corresponding subunits with one or more carbon backbones. These subunits consist of monosaccharides such as glucose, xylose, galactose, arabinose, and mannose, which are readily metabolizable by gastro-intestinal tracts and the gut microorganisms in swine. When urea was supplied, only galactan was reduced significantly ($p < 0.05$) by *T. reesei* and *R. oryzae* in CM, indicating

Table 3 Structural carbohydrate profile in SM and CM after fermentation of different fungi

Meal	Urea added	Strain	Glucan	Xylan	Galactan	Arabinan	Mannan	Total
			% d.b					
SM	No	Ctrl	11.99 ± 0.17	3.12 ± 0.28	10.05 ± 0.44	4.54 ± 0.28	3.09 ± 0.17	32.79 ± 0.17
		TR	10.25 ± 0.44	2.84 ± 0.49	7.64 ± 0.44	3.78 ± 0.16	3.77 ± 0.00	28.27 ± 0.76
		MI	11.50 ± 1.02	2.46 ± 0.43	9.67 ± 1.10	4.25 ± 0.28	2.71 ± 0.17	30.59 ± 1.54
		RO	8.80 ± 1.77	2.46 ± 0.16	9.47 ± 0.33	4.82 ± 0.28	3.19 ± 0.29	28.74 ± 2.69
	Urea	Ctrl	12.57 ± 2.47	3.02 ± 0.43	10.15 ± 0.58	4.06 ± 0.71	2.9 ± 0.29	32.71 ± 3.66
		TR	12.47 ± 2.77	2.84 ± 0.49	9.09 ± 0.89	3.69 ± 0.28	2.9 ± 0.29	30.98 ± 4.48
		MI	11.6 ± 0.00	2.08 ± 0.16	10.73 ± 0.58	4.73 ± 0.82	2.8 ± 0.17	31.94 ± 1.32
		RO	10.05 ± 1.17	2.74 ± 0.33	9.67 ± 0.60	4.63 ± 0.71	3.38 ± 0.17	30.48 ± 2.61
CM	No	Ctrl	13.24 ± 0.6	2.17 ± 0.33	4.64 ± 0.00	6.43 ± 0.16	4.25 ± 1.43	30.74 ± 2.03
		TR	9.86 ± 1.51	1.7 ± 0.28	3.48 ± 0.29	4.63 ± 0.59	2.9 ± 0.29	22.57 ± 2.45
		MI	11.6 ± 1.00	1.8 ± 0.33	4.74 ± 0.17	6.62 ± 0.16	2.51 ± 0.17	27.26 ± 0.93
		RO	10.34 ± 2.1	2.27 ± 0.28	4.35 ± 0.29	7.09 ± 0.28	2.22 ± 0.17	26.27 ± 1.93
	Urea	Ctrl	12.76 ± 0.77	2.08 ± 0.16	4.54 ± 0.17	6.33 ± 0.43	2.22 ± 0.33	27.94 ± 0.50
		TR	11.02 ± 1.05	1.7 ± 0.00	3.96 ± 0.17	6.52 ± 0.28	2.42 ± 0.17	25.62 ± 1.16
		MI	10.83 ± 0.44	2.36 ± 0.91	5.03 ± 0.17	6.9 ± 0.16	2.32 ± 0.00	27.44 ± 1.19
		RO	11.5 ± 0.44	2.65 ± 0.16	4.35 ± 0.00	6.9 ± 0.33	2.13 ± 0.17	27.53 ± 0.33

the inhibitive effects of urea on breakdown of SC. However, no significant reduction ($p > 0.05$) of each SC component, except for galactan, was observed in *T. reesei* fermented SM with either supply or non-supply of urea, suggesting that the degradation of SC also depends on the type of substrate used.

Sinapic Acid, Urea and Reducing Sugar Profile

Sinapic acid (4-hydroxy-3,5-dimethoxy-cinnamic acid) is predominant in the free phenolic acids contained in canola seed. The sinapic acid in CM was found causing bitter taste and astringency, which does negative effect to the fed cattle and swine. Though low level of sinapic acid existing in the feed would be less toxic to animals, it is hoped that phenolic compound content would be decreased after fermentation.

It was found that the sinapic acid content in CM is around 0.1 mg/g (Table 4) since the sample used for fermentation had already been treated to decrease sinapic acid level. The *T. reesei* and *M. indicus* both have shown good performance in degrading the phenolic compound in both urea-treated and non-urea treated cases. However, in the *R. oryzae* groups, the sinapic acid content did not show much difference after fermentation when 1% N of urea was added. The urea had probably prevented the strain *R. oryzae* from degrading the sinapic acid.

The only group with residual sinapic acid detected, treatment of *R. oryzae* with urea supply, was also showing a high standard deviation. Combined with many other measurements (AA, SC) showing same phenomena, it could also be expected that microorganisms in fermentation would probably show distinctive performance even in the same environment. *R. oryzae* would not be considered as a good choice in decreasing phenolic compound, as it did not show stable performance in the experiment.

In both CM and SM cases, *T. reesei* shows least urea residue after fermentation, which means it converted most urea in carbon metabolism into AA; the highest reducing sugar production indicates that *T. reesei* also degrades the cellulose and hemicellulose in the substrate in a highest amount as shown in the reduction of SC in Table 3. The treatment with *M. indicus* shows the lowest reducing sugar, which is potentially due to incapability of secreting hydrolytic enzymes that could degrade SC in the substrate. The treatment with *R. oryzae* shows highest urea residue and relatively high production of reducing sugar, which gives an interpretation that *R. oryzae* is efficiently degrading the polysaccharides into monomers, but urea is not well involved in its biosynthesis cycles. Instead, additional urea was produced in *R. oryzae* and *M. indicus* treated substrates, which could be due to degradation of proteaceous compounds in the substrates. Therefore, in fungal fermentation of CM, selection of fungal species and application of low-cost nitrogen source such as urea should be carefully considered to achieve the maximum nutritional value for production of feeding ingredients for monogastric animal.

In Vitro Dry Matter Digestibility

In vitro dry matter digestibility of the feeding material evaluated the digestibility of protein by pepsin in stomach and the remaining protein, carbohydrates, and lipid by pancreatin

Table 4 Urea residue, reducing sugar, and IVDMD in SM and CM after fermentation with different fungi

Meal	Urea addition	Strain	Urea residue mg/g d.b	Reducing sugar mg/g d.b	Sinapic acid mg/g d.b	IVDMD %
SM	No	Ctrl	0.000 ± 0.000	3.14 ± 0.498	–	83.53 ± 1.07
		TR	0.025 ± 0.008	10.89 ± 1.274	–	88.85 ± 0.64*
		MI	0.258 ± 0.008	4.07 ± 3.148	–	79.24 ± 0.75
		RO	0.323 ± 0.204	2.67 ± 2.222	–	78.96 ± 1.61
	Urea	Ctrl	0.961 ± 0.017	1.99 ± 0.549	–	82.59 ± 1.38
		TR	0.479 ± 0.032	10.61 ± 0.358	–	90.77 ± 1.06*
		MI	0.975 ± 0.472	1.28 ± 0.817	–	82.80 ± 0.78
		RO	1.558 ± 0.066	4.85 ± 0.622	c	82.91 ± 0.72
CM	No	Ctrl	0.013 ± 0.005	6.99 ± 0.493	0.12 ± 0.01	60.31 ± 11.49
		TR	0.024 ± 0.005	11.43 ± 0.751	0	62.08 ± 4.48
		MI	0.115 ± 0.05	2.34 ± 0.806	0	58.81 ± 5.83
		RO	0.353 ± 0.009	9.78 ± 0.875	0	62.17 ± 1.23
	Urea	Ctrl	1.066 ± 0.113	2.98 ± 0.254	0.06 ± 0.11	60.84 ± 6.44
		TR	0.377 ± 0.031	15.15 ± 1.68	0	63.39 ± 2.39
		MI	1.254 ± 0.1	2.75 ± 0.205	0	56.17 ± 2.40
		RO	1.583 ± 0.116	10.97 ± 1.773	0.06 ± 0.06	65.18 ± 0.73

*Represents significant improvement ($p < 0.05$) as compared to Ctrl

in small intestine of monogastric animals which include swine and poultry. The IVDMD provides basic information of whether the feeding material can be digested by animals without real animal feeding test. It was observed from Table 4 that non-treated SM had higher IVDMD (above 80%) than non-treated CM (around 60%). This is apparently due to higher proportion of protein in SM than in CM (Table 1). Fermentation by fungal strains showed either improvement or decrease in IVDMD. Among them, *T. reesei*-treated SM showed significant higher ($p < 0.05$) IVDMD than the SM treated by other strains in both urea-added and non-urea treatments. However, without urea, *R. oryzae* and *M. indicus* treated SM showed decreased IVDMD ($p < 0.05$) compared to non-fermented SM. Although the total AA of both *T. reesei* and *R. oryzae* treated SM were higher than in Ctrl, the IVDMD in *R. oryzae* treated SM didn't show improvement as *T. reesei* treated SM. This indicated that the fungal protein concentrated in SM and accumulated in *R. oryzae* was not as digestible as those with *T. reesei*. However, *M. indicus* treated SM in both urea-added and non-urea treatments did not show improvement of IVDMD. In CM, both *T. reesei* and *R. oryzae* showed some improvement of IVDMD ($p > 0.05$) after treatments. However, CM treated by *M. indicus* with both urea-added and non-urea conditions showed declined IVDMD as compared to Ctrl. *T. reesei*, as an ideal strain reducing non-digestible components such as fibers and converting urea into protein, performed better in the digestibility test as compared to *R. oryzae* and *M. indicus*. This indicates the potential of *T. reesei* in processing both protein-rich and low-protein substrates to increase their overall digestibility by monogastric animals.

The mechanism behind the solid-state fermentation with fungal strains is the interaction between components in the substrate and excreted chemicals and biocatalysts from fungi during colonization. As reported in a study, three fungal strains *Aureobasidium pullulans*, *Neurospora crassa*, and *Trichoderma reesei* and their combinations resulted in different nutritional profile of CM after fermentation. Fermentation with pure culture had higher protein improvement than co-culture. However, co-culture showed higher reduction of fiber and glucosinolates due to synergic catalytic effects of different enzymes produced by the fungi in co-culture [36]. Studies on feeding value improvement of CM are still in lab scale, while fermentation of SM has already been commercialized. SM has been successfully fermented by fungal and bacterial strains predominantly *Aspergillus oryzae* and *Lactobacillus subtilis*, respectively. Fungal fermented SM showed near complete removal of trypsin inhibitor, phytate, while increasing protein content, available phosphorous and zinc. Bacterial fermented SM usually showed higher essential and free AA, antioxidant activity, small sized peptides, and in vitro digestibility [37]. Including fermented SM to chicken and swine diets usually resulted in improved average daily gain and gain-to-feed ratio. The current study explored the feasibility of three fungal strains (*R. oryzae*, *M. indicus* and *T. reesei*) on nutritional profiles of CM as compared to SM, with and without supply of urea as low-cost N source, which will contribute towards commercialization of CM fermentation.

Conclusion

Fermentation with fungal strains *T. reesei*, *M. indicus* and *R. oryzae* could enhance the nutritional value of canola meal to different levels. The AA content was the key value tracked to determine the nutritional value of the meals. The sinapic acid, as one of the major phenolic compounds, was measured to evaluate the toxicity of the meals. By resulting in the highest AA improvement, conversion efficiency of urea, degradation of SC, and complete removal of sinapic acid, the treatment with *T. reesei* was found as the most effective fungal strain in improving feeding value of CM as compared with other strains used in this study.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing finantial or personal interests that could influence the work reported in this study.

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