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Lactobacillus salivarius Fermentation Reduced Glucosinolate and Fibre in Canola Meal

Aljuobori, A.[1], N. Abdullah[1,2], I. Zulkifli[1], A. F. Soleimani[1], J. B. Liang[1] & E. Oskoueian[1]

[1] Laboratory of Animal Production, Institute of Tropical Agriculture, Universiti Putra Malaysia, Selangor, Malaysia

[2] Department of Biochemistry, Faculty of Biotechnology & Biomolecular Sciences, Universiti Putra Malaysia, Selangor, Malaysia

Correspondence: N. Abdullah, Department of Biochemistry/Institute of Tropical Agriculture, Universiti Putra Malaysia, Selangor, Malaysia. Tel: 60-389-471-147. E-mail: norhani@upm.edu.my

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Abstract

Local traditional fermented foods consisting of seven samples of *tempoyak* (made from fresh durian fruit), six samples of *budu* (made from boiled fish), four samples of *tempeh* (made from boiled soybean), two samples of vegetable pickle (made from raw cabbage) and one sample of *tapai* (made from cooked glutinous rice), were used as samples for the isolation of lactic acid bacteria (LAB). A number of samples for different fermented foods like *tempoyak*, *budu* and vegetable pickle were screened as they were fermented by microorganisms naturally present in the food. One hundred isolates were obtained and by sequential screening for catalase activity and Gram-staining, 20 isolates were determined to be *Lactobacillus*. Ten isolates with the highest enzymatic activities were selected based on the biochemical reactions of the API ZYM kit. Canola meal (CM) was treated with the ten *Lactobacillus* isolates in solid state fermentation for 30 days. The most efficient LAB isolate was identified as *lactobacillus salivarius* and significantly reduced the total glucosinolate and crude fibre content of CM by 38% and 16%, respectively.

Keywords: canola meal, glucosinolate, solid state fermentation, LAB

1. Introduction

Fermentation by lactic acid bacteria offers promise for detoxification and breakdown of undesirable factors present in vegetable protein sources. Verbiscar et al. (1981) reported that lactic acid bacteria (LAB) reduced cyano toxicants in jojoba seed meal and increased its palatability. Fermentation by LAB also reduced trypsin inhibitor in black bean and soybean meal (Granito et al., 2002; Gao et al., 2013) and non-starch carbohydrates in wheat and barley whole meals (Skrede et al., 2001). Yang et al. (2006) observed that fermentation of food waste using *L. salivarius* enhanced breakdown of fiber. The advantage of using LAB as the fermenting agent over yeast or fungi is that these microorganisms are notably nontoxic and as "potential probiotics" (Bao et al., 2010) aid in immune response stimulation and prevention of infection by enteropathogenic bacteria (Reid, 1999).

The presence of glucosinolates and high fiber content reduced the utilization of canola meal (CM) as a protein source in animal feed. Previous methods such as inactivation of glucohydolase and steam stripping had some disadvantages like loss of protein, high cost and non-feasibility of the process. Therefore, solid state fermentation by LAB could be an alternative method in improving CM nutritive values. Hence, the present study was conducted to evaluate the ability of LAB isolated from various local fermented foods as potential agents in reducing glucosinolates in CM.

2. Materials and Methods

2.1 Samples and LAB Isolation

Fermented foods consisting of seven samples of *tempoyak* (made from fresh durian fruit), six samples of *budu* (made from boiled fish), four samples of *tempeh* (made from boiled soybean), two samples of vegetable pickle (made from raw cabbage) and one sample of *tapai* (made from cooked glutinous rice), purchased from the local market in Serdang, Malaysia, were used as samples for the isolation of lactic acid bacteria (LAB). A number of samples for different fermented foods like *tempoyak, budu* and vegetable pickle were screened as they were

fermented by microorganisms naturally present in the food. One gram of each sample was added to 9 ml of peptone water (0.01%) and vortexed for 30 s and serially diluted (10^3 to 10^7). One hundred µl of each dilution was spread on the MRS (De-Man, Ragosa and Sharp) agar plate (Merck, Darmstadt, Germany). The plates were incubated for 48 h at 37 °C in an anaerobic jar (Oxoid, UK). Several colonies were selected from each plate and sub-cultured for three times. Bacterial colonies were then individually picked and streak on fresh MRS agar plates by dilution-streaking to obtain single colonies. This procedure was repeated three times in order to purify the isolates. One hundred isolates were selected and maintained in MRS broth (Merck, Darmstadt, Germany) for immediate use and in 20% glycerol for storage at -80 °C. The isolates were subjected to two transfers in MRS broth for activation before use.

All isolates were subjected to catalase activity test by adding a drop of 3% hydrogen peroxide solution on the cultures. Immediate formation of bubbles was considered as the presence of catalase. Accordingly, the catalase-negative and Gram-stained positive isolates were selected for further screening.

2.2 Enzymatic Activities of Isolates

Enzyme activities of the selected isolates were determined using the API ZYM system (BioMerieux, Marcy-L'Etoile, France) for 19 enzyme reactions (Herreros et al., 2003; Levett, 1985). Briefly, the bacterial suspensions (2 mL) from an overnight culture were prepared with the turbidity of a McFarland standard 5-6. Five mL of distilled water were distributed into each cupule of the honeycomb wells of the tray, followed by the bacterial suspension (65 µL). The strips were incubated for 4 h at 37 ºC. The reaction was terminated by the addition of the API ZYM reagents. After 5 min of color development, enzymatic activities were graded from 0 to 5 with the API ZYM color reaction chart. The API ZYM system consisted of cupules to detect the following enzymes activity: alkaline phosphatases, acid phosphatase, butyrate esterase (C4), esterase lipase (C8), lipase (C14), leucine, valine aminopeptidases, cystine aminopeptidases, trypsin, chymo- trypsin, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The API ZYM test was conducted twice to confirm the results. Ten isolates with the highest enzymatic activities were selected, propagated and freeze dried for future use.

2.3 Fermentation of Canola Meal by LAB Isolates

Canola meal was obtained from a commercial feed mill (Gulf Feed Mill, Al Ghurair Foods - A Part of Al Ghurair Investment, Dubai, United Arab Emirates). The meal was separately inoculated which each of the ten freeze dried LAB at 0.1% based on dry matter of canola meal. The inoculation was first prepared by suspending the appropriate weight of LAB powder in water to increase the moisture content of CM to 70% (Rodriguez-Leon et al., 2008). The inoculant suspension was sprayed over 2-kg batches of CM and was mixed thoroughly manually. The colony forming units (c.f.u) of LAB used were 10^7/g CM. The treated CM was placed in bottle (0.5-L Scott) in triplicates and incubated for 30 days at room temperature (28 to 32 ºC). Control CM was prepared at the same time without inoculant addition. At the end of the fermentation period, samples of the untreated and inoculated CM were dried at 50 ºC for 3 days and ground into 3-mm particles for chemical analyses.

2.4 Identification of Isolate FS10

The isolate FS10 which showed the highest percentage of glucosinolate reduction was identified by PCR-based method. Bacterial cells were harvested from overnight culture in MRS broth. The total genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Identification was carried out by amplification of 16S rRNA using forward 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R reverse (5'-GGCTACCTTGTTACGACTT-3') primers. The PCR amplification was performed with i-StarTaq DNA polymerase kit (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea) in a thermal cycler (BioRad MyCycler™) with the following program: 1 cycle at 94 ºC for 4 min, 30 cycles of 94 ºC for 1 min, 55 ºC for 30s, 72 ºC for 2 min and a final extension at 72 ºC for 5 min. The PCR product was extracted from the gel using MEGA quick-spin PCR and Agarose Gel Extraction kit (iNtRON Biotechnology) and then sequenced (1st Base Co., Malaysia). The contigs were visually checked for the forward and reverse sequences of isolate by contig assembly program of Bioedit software. The assembled sequence was checked to detect chimeras using Bellerophon and Mallard software. Approximately 1400 bp segment of the 16S rRNA gene were analyzed using BLAST (NCBI, Washington, USA.

2.5 Fermentation of Canola Meal by Isolate FS10

Fermentation of larger amount of CM was conducted by using isolate FS10 which showed the highest activity in removing glycosinolate. Ten kg of CM were fermented according the method described above. In this CM treatment, the pH values and c.f.u. were determined at the initial and the end stage of fermentation. The sample

was homogenized with distilled water (1/10 w/v) and the pH was measured with a portable pH meter (Hanna Instruments, Woonsocket, RI). For c.f.u. determination, 1 g of the sample was added to 9 ml of peptone water and vortexed for 30s and serially diluted. One hundred µl of 10^3 to 10^7 dilutions were streaked on MRS agar plate. The plates were incubated for 48 h at 37 ºC in an anaerobic jar (Oxoid, UK). Colonies on the plates were counted by using a colony counter (Funke Gerber). The fermented canola meal was oven dried at 50 ºC for 3 days, and stored for further use.

2.6 Chemical Analyses

Dried samples were finely ground using a grinder (Panasonic, Malaysia). Dry matter (DM), crude protein (CP) and ether extract (EE) of samples were determined according to the procedures of AOAC (1990). The DM was determined by drying at 105 ºC overnight, CP (N × 6.25) was determined by the Kjeldahl method and ether extract (EE) was determined by using the 2025 Soxtec Auto Analyser system. Crude fiber(CF) was determined according to Van Soest et al. (1991). Gross energy (GE) was measured with an adiabatic oxygen bomb calorimeter (C 2000 basic IKA, Germany). Total glucosinolate was determined based on alkaline degradation and subsequent reaction of released 1-thioglucose with ferricyanide (Jezek et al., 1999; Gallaher et al., 2012). Amino acid concentrations were determined by high performance liquid chromatography according to the procedures described by Strydom and Cohen (1994). Pre-column derivatisation was done with AQC reagent (6-aminoquinolyl-N-hydroxysuc-cinimidyl carbamate, Waters, Milford, MA, USA). Cys and Met were analysed as cysteic acid and methionine sulfone by oxidation with performic acid for 16 h at 0 °C and neutralisation with hydrobromic acid before hydrolysis. Trp content was determined following alkaline hydrolysis of sample with 4.3 M $LiOH.H_2O$ for 16 h at 120 ºC and neutralization with 6 M HCl. Quantification of the other amino acids was done by hydrolysing the sample in 5 ml 6 M HCl for 22 h at 110 ºC.

2.7 Statistical Analysis

Data were analyzed by one way analysis of variance (ANOVA) by using the SAS software package (V 9.1, SAS Institute Inc., Cary, NC). Means were tested by Duncan's multiple range test and level of significant difference was $P < 0.05$. The results are presented as means ± SEM. The observation relating to enzyme activities, as monitored using the API-ZYM assay, were not statistically analyzed.

3. Results and Discussion

The enzymatic activities results showed that ten LAB isolates had β-galactosidase, α-glactosidase, β-glucuronidase, α-glucosidase and β-glucosidase activities (Table 1). According to Lu et al. (2008), isolates with α-glucosidase and β-glucosidase activities indicate the ability to digest polysaccharides. These isolates include *Pediococcus pentosaceus*, *L. lactics*, *L. fermentum*, *L. delbruckii. delb* and *L. curvatus.* Herreros et al. (2003) also observed that most of the strains of *L. plantarum* showed a high β-galactosidase activity. Skrede et al. (2001, 2003) also showed that fermentation by *Lactobacillus* spp. successfully reduced the levels of total and soluble dietary fibre and non-starch carbohydrates in wheat and barley whole meals. In the present study, most of the of the LAB isolates evaluated showed the ability to breakdown polysaccharides.

Table 1. Enzyme activity of bacterial isolates detected by API ZYM kit

	α - glactosidase	β - glactosidase	β- glucuronidase	α– glucosidase	β - glucosidase
D1	+	+	+		+
B12	–	+	+	+	+
D6	–	+	+	+	+
T14	+	+	–	+	–
FS36	+	+	–	+	+
D10	+	–	–	+	+
D5	+	–	–	–	+
FS10	+	+	+	+	+
B40	+	+	–	+	+
D3	–	+	–	–	+

+Positive reaction; – negative reaction; D: LAB isolates from *tempoyak*; B: LAB isolates from *budu*; T: LAB isolate from *tapai* ; FS: LAB isolates from *tempeh*.

Table 2 shows the results of DM, CP and CF content of CM after fermentation for 30 days with the ten LAB isolates. The DM content decreased significantly ($P < 0.05$) after fermentation by isolates B12, D1, D10, D3, D5, D6, FS10 and T14. In the case of CP content, no significant difference was observed between fermented and control CM. Significant ($P < 0.05$) reduction in CF was observed in CM fermented with isolates B12, D6, FS10, FS36 and T14. Canola meal fermented with isolate FS10 showed the lowest CF content. The reduction in CF content was due to the polysaccharidases produced by the bacteria.

Table 2. Proximate analyses of fermented canola meal on dry matter basis by different LAB isolates after 30 days incubation

Isolates	DM%	CP%	CF%
Control	51.3 ± 0.49^{a}	40.5 ± 0.69^{abc}	13.0 ± 0.22^{a}
B12	47.9 ± 0.03^{de}	41.3 ± 0.74^{ab}	11.1 ± 0.62^{bcd}
B 40	50.7 ± 0.49^{ab}	41.0 ± 0.17^{abc}	12.0 ± 0.27^{abc}
D1	50.0 ± 0.01^{bc}	40.1 ± 0.21^{bc}	11.7 ± 0.31^{abcd}
D10	48.7 ± 0.13^{d}	40.2 ± 0.08^{bc}	12.8 ± 1.18^{a}
D3	50.4 ± 0.12^{bc}	39.7 ± 0.21^{c}	12.6 ± 0.37^{ab}
D5	48.5 ± 0.15^{d}	41.1 ± 0.21^{ab}	11.8 ± 0.41^{abcd}
D6	50.1 ± 0.20^{bc}	39.7 ± 0.62^{c}	11.0 ± 0.11^{bcd}
FS10	49.8 ± 0.12^{c}	41.7 ± 0.28^{a}	10.4 ± 0.46^{d}
FS36	50.8 ± 0.11^{ab}	41.3 ± 0.42^{ab}	10.7 ± 0.22^{cd}
T14	47.4 ± 0.39^{e}	40.1 ± 0.16^{bc}	11.0 ± 0.17^{bcd}

Control: CM without LAB; D: LAB isolates from *tempoyak*; B: LAB isolates from *budu*;

T: LAB isolate from *tapai* ; FS: LAB isolates from *tempeh*.

CP, crude protein; CF crude fibre.

Means ± SEM (n=3) with different superscripts within a column are significantly different ($P < 0.05$).

The glucosinolate content was reduced significantly ($P < 0.05$) in CM treated by all the bacterial isolates, except for isolates D3 and D5 (Figure 1). The highest reduction was observed in CM fermented with isolate FS10. The reduction was due to the degradation of glucosinolates into glucose and sulphur moieties by microbial enzymes during fermentation. The nucleotide sequence of isolate FS10 showed 99% similarity to *Lactobacillus salivarius* and this isolate was deposited as a new entry in GenBank database (accession number: KF303794).

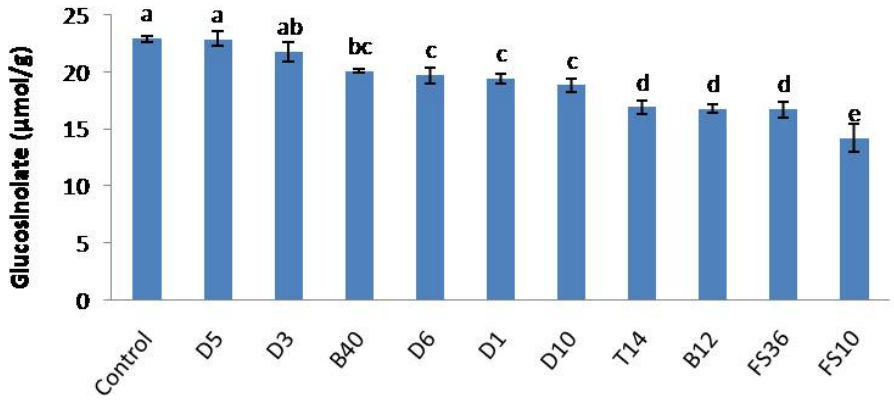

Figure 1. Effect of LAB fermentation on glucosinolate content of CM on dry matter basis

Control: CM without LAB; D: LAB isolates from *tempoyak*; B: LAB isolates from *budu;* T: LAB isolate from *tapai* ; FS: LAB isolates from *tempeh*. a-e indicates significant difference ($P < 0.05$) compared to control.

Table 3 shows the pH values and c.f.u. at the initial and end stage of fermentation of CM by isolate FS10 after 30

days of fermentation. The pH value decreased significantly from 5.6 to 4.0 at the end of the fermentation period. This is due to the presence of organic acids produced from sugar fermentation (Correia et al., 2005). The results of the present study are in accordance with the findings of Gao et al. (2008) that after 30 day fermentation, the fermented straw by LAB had an acid-fragrant smell, and the pH was 3.8. A pH of 4–5 is desired for fermented feed ingredients because below pH 4, feed intake is decreased and over pH 5 microbial spoilage is likely to occur (Lee et al., 2004). The c.f.u were 2×10^9 per g at the initial stage and decreased to 1×10^5 per g DM of CM. The final pH was within the range of LAB fermentation in silage making (Filya et al., 2007) and the reduction in c.f.u. was as expected due to the diminishing nutrients for bacterial growth as well as the low pH. Yang et al. (2006) also reported reduction in LAB numbers at 30 days fermentation in waste food inoculated with 0.2% *L. salivarius* when compared to 10 days fermentation. Hence, reduction in LAB numbers is a general occurrence in fermentation.

Table 3. Fermentation of canola meal by isolate FS10 after 30 days

FCM	pH	c.f.u/ g CM
0 day	5.6 ± 0.057^a	2×10^9
30 day	4.0 ± 0.133^b	1×10^5

FCM: treated CM with isolate FS10.

Means ± SEM (n=3) with different superscripts within a column indicate significant difference ($P < 0.05$).

The results of CM fermentation by isolate FS10 showed that DM, CF and glucosinolate contents were significantly ($P < 0.05$) reduced in treated CM when compared to that of the untreated CM (Table 4). The CP content was significantly ($P < 0.05$) increased in the treated CM, but differences in EE and gross energy were not significant. The increase in CP in the present study is in agreement with the finding reported earlier by Rozan et al. (1996), that the loss of dry matter at the expense of fermentable sugars during fermentation with bacteria, would result in higher percentage of CP. Later, Chiang et al. (2010) also reported that CP increased slightly after fermentation, and this increase was most likely a reflection of the decline in dry matter content rather than an actual increase in protein content. In the case of amino acid contents, elevated levels were observed for some amino acids like Asp, Glu, Pro and Leu in treated CM, but the differences were not significant when compared to the control. However, the amino acid percentage values of CM in the present study were within the range of values reported by Woyengo et al. (2010).

Solid state fermentation has been used widely for treatment of plant materials. Vig and Walia (2001) conducted their study by using *Rhizopus oligosporus* for rapeseed meal treatment and observed a reduction in CF as well as other anti-nutritional factors including glucosinolate, hiooxazolidones and phytic acid, which declined by 25.5%, 43.1%, 34%, and 42.4%, respectively. The reduction in the level of glucosinolate in their study was higher than the reduction by isolate FS10 (38%) observed in the present study. However, fermentation by LAB is advantageous when compared to the *R. oligosporus* as the later may increase accumulation of undesirable end products such as aflatoxins (Mienda et al., 2011). The ability of LAB to alleviate anti-nutritional factors and toxic compounds in plant materials by fermentation have been observed in various studies (Verbiscar et al., 1981; Fuchs et al., 2008).

Table 4. Composition of canola meal (CM) and fermented canola meal (FCM) by isolate FS10 (dry matter basis)

Items	CM	FCM
DM (%)	91.9 ± 0.2[a]	88.8 ± 0.2[b]
CP (%)	41.2 ± 0.7[b]	42.2 ± 0.03[a]
CF (%)	12.0 ± 0.2[a]	10.1 ± 0.4[b]
EE (%)	3.3 ± 0.2	3.5 ± 0.3
Ash (%)	6.2 ± 0.2	6.3 ± 0.5
Glucosinolate (μmol/g)	22.0 ± 1.5[a]	13.6 ± 0.8[b]
GE (kcal/kg)	4421 ± 15	4437 ± 14
Amino acids (%)		
Asp	2.57 ± 0.10	2.70 ± 0.16
Ser	1.81 ± 0.05	1.83 ± 0.05
Glu	6.59 ± 0.24	6.89 ± 0.34
Gly	2.13 ± 0.06	2.15 ± 0.06
His	1.22 ± 0.02	1.20 ± 0.02
Arg	2.54 ± 0.07	2.50 ± 0.05
Thr	1.81 ± 0.05	1.83 ± 0.04
Ala	1.59 ± 0.05	1.66 ± 0.09
Pro	2.37 ± 0.07	2.45 ± 0.11
Cys	1.07 ± 0.01	1.03 ± 0.06
Tyr	1.03 ± 0.02	1.04 ± 0.02
Val	1.90 ± 0.07	1.94 ± 0.08
Met	0.77 ± 0.01	0.76 ± 0.05
Lys	1.65 ± 0.07	1.72 ± 0.12
Ile	1.35 ± 0.04	1.39 ± 0.05
Leu	2.63 ± 0.08	2.71 ± 0.10
Phe	1.79 ± 0.04	1.76 ± 0.02
Trp	0.39 ± 0.01	0.39 ± 0.01

DM: dry matter; CP: crude protein; CF: crude fiber; EE: ether extract; GE: gross energy.

Means ± SEM (n=3) with different superscripts within a row are significantly different ($P < 0.05$). Means ± SEM without superscripts within a row are not significantly different ($P > 0.05$).

4. Conclusion

Solid state fermentation of CM with *L. salivarius* reduced CF by 16% and glucosinolate content by 38%. This treatment improves the overall nutritive value of CM. Therefore, fermented CM is a promising alternative protein source and could partially replaced soybean meal in poultry diets.

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