

Sains Malaysiana 45(9)(2016): 1289–1297

## *Lactobacillus fermentum* LAB 9-Fermented Soymilk with Enriched Isoflavones and Antioxidants Improved Memory *In vivo*

(Susu Soya Difermentasi oleh *Lactobacillus fermentum* LAB 9 yang Kaya dengan Isoflavon dan Antioksidan Memperbaiki Daya Ingatan secara *In vivo*)

E.F. MOHAMAD FAKRI, S.M. LIM, N.H. MUSA, M. HAZIZUL HASAN, A. ADAM & K. RAMASAMY\*

### ABSTRACT

*This study examined lactic acid bacteria (LAB)-fermented soymilk for their ability in hydrolyzing glucosides to aglycones and corresponding antioxidant capacity and memory enhancing effect. Twelve LAB isolated from Malaysian fermented food and milk products were incubated in commercially available soymilk for 48 h. Generally, soymilk supported LAB growth and significantly increased ( $p < 0.05$ ) conversion to bioactive aglycone by 2.1-6.5 fold when compared to unfermented soymilk. Lactobacillus fermentum LAB 9-fermented soymilk, in particular, was presented with increased total phenolic content (+10%) as opposed to unfermented soymilk. Lactobacilli (LAB 10-12)- and pediococci (LAB 5)-fermented soymilk elicited maximal DPPH radical-scavenging activity. LAB 1, 7, 8, 9 and 12 exhibited significantly higher ( $p < 0.05$ ) ferrous ion chelating activity when compared to control. Interestingly, LAB 9 had significantly improved memory deficit ( $p < 0.05$ ) in LPS-challenged mice. LAB-enriched nutritional value of soymilk could be useful against oxidative stress and memory deficit.*

*Keywords: Antioxidant; bioactive isoflavones; lactic acid bacteria; memory enhancing; soymilk*

### ABSTRAK

*Kajian ini bertujuan menguji kebolehan susu soya yang difermentasi oleh bakteria laktik asid (LAB) dalam menghidrolisis glukosida kepada aglikon dan potensi daripada segi antioksidan serta peningkatan ingatan. Dua belas LAB yang dipencil daripada makanan Malaysia yang telah difermentasi telah dieram dalam susu soya komersial selama 48 jam. Secara amnya, susu soya menyokong pertumbuhan LAB dan meningkatkan secara signifikan ( $p < 0.05$ ) penukaran glukosida kepada aglikon bioaktif 2.1-6.5 kali ganda apabila dibandingkan dengan susu soya yang tidak difermentasi. Lactobacillus fermentum LAB 9, terutamanya, menunjukkan peningkatan jumlah kandungan fenol (+10%) berbanding dengan susu soya yang tidak difermentasi. Susu soya yang difermentasi oleh Lactobacilli (LAB 10-12)- dan pediococci (LAB 5) menunjukkan aktiviti DPPH radikal skaven yang maksimum. LAB 1, 7, 8, 9 dan 12 mempunyai aktiviti pengkelatan ferus ion yang tinggi secara signifikan ( $p < 0.05$ ) berbanding dengan kawalan. LAB 9 juga memperbaiki defisit daya ingatan mencit yang terdedah kepada LPS secara signifikan ( $p < 0.05$ ). Nilai nutrisi susu soya yang diperkayakan oleh LAB mungkin berguna terhadap tegasan pengoksidaan dan defisit ingatan.*

*Kata kunci: Antioksidan; bakteria asid laktik; isoflavon; peningkatan ingatan; susu soya*

### INTRODUCTION

Soy is a nutritious food type commonly consumed by Asians. Its health claims as low in fat, cholesterol free, high in protein, fiber, iron and essential fatty acids have been approved by the FDA (1999). Epidemiological evidence further suggests correlation of soy intake to improve health (Otieno et al. 2007). The beneficial effects of soy are often attributed to the action of isoflavonoids, the major phenolic phytochemicals found in soybeans which are effective in reducing oxidative degradation of DNA, prevention of premature aging and emergence of Alzheimer's disease (Wei et al. 2007).

Isoflavones possess chemical structures similar to oestrogen and exist in two basic forms, aglycones (primarily daidzein and genistein) and glucosides (primarily daidzin and genistin). Soybean-derived isoflavones are mainly

glucosides. Unfermented soymilk contains about 80-93% of total glucosides and about 7-15% aglycones (Ding & Shah 2010; King & Bignell 2000). Aglycones, however, are more bioavailable. They are being rapidly absorbed in great amounts in the intestine given their smaller molecular weight and greater lipophilicity when compared to glucosides (Ding & Shah 2010). Aglycones are also well documented for human health and disease prevention (Rekha & Vijayalakshmi 2011).

Probiotics are live microorganisms which confer health benefits on their host when consumed in adequate amounts (FAO 2002). Probiotic-fermented soymilk have been found to exhibit high antioxidant capacity through inhibition of ascorbate autoxidation, scavenging of DPPH radicals, superoxide anion radicals, hydroxyl radicals and reduction of hydrogen peroxide (Song et al. 2011; Wang et

al. 2006). There were also reports about probiotics (mainly lactic acid bacteria (LAB)) with  $\beta$ -glucosidase activity that were able to hydrolyse glucosides to biologically potent aglycones during soymilk fermentation (Rekha & Vijayalakshmi 2011; Zhao & Shah 2014). Most of these studies, however, involved mainly bifidobacteria and lactobacilli but not pediococci. The present study was undertaken to evaluate 12 LAB (lactobacilli and pediococci with probiotic characteristics derived from local fermented food or milk products (Ramasamy et al. 2012)) for their ability to ferment soymilk and hydrolyse glucosides to biologically potent aglycones. Antioxidant properties of probiotic-fermented soymilk were assessed. The memory enhancing effect of *Lactobacillus fermentum* LAB 9, which had yielded the highest total phenolic content in fermented soymilk, was also investigated using LPS-induced memory impaired mice.

## MATERIALS AND METHODS

### LAB STRAINS AND CULTURE CONDITION

The 12 LAB, namely *Lactobacillus plantarum* LAB 1 (Gene accession number: JN039357), *Pediococcus pentosaceus* LAB 2 (Gene accession number: JN039348) and LAB 3 (Gene accession number: JN039349), *Pediococcus acidilactici* LAB 4 (Gene accession number: JN039350) and LAB 5 (Gene accession number: JN039351), *Pediococcus pentosaceus* LAB 6 (Gene accession number: JN039352), LAB 7 (Gene accession number: JN039349) and LAB 8 (Gene accession number: JN039354), *Lactobacillus fermentum* LAB 9 (Gene accession number: JN039355), *Lactobacillus* sp. LAB 10 (Gene accession number: JN039356) and *Lactobacillus plantarum* LAB 11 (Gene accession number: JN039357) and LAB 12 (Gene accession number: JN039358) were maintained by routine propagation in de Man, Rogosa and Sharpe (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, England) using a 1% inoculum from an overnight culture and incubated at 37°C. Prior to experimental use, each strain was sub-cultured three times. The cultures were stored in 15% glycerol at -80°C between transfers.

### FERMENTATION OF SOYMILK

Each LAB strain (5%) was aseptically inoculated into sterile (121°C for 15 min) commercially available soymilk (Yeo's, Malaysia) and incubated at 37°C for 48 h. The composition of the soymilk included: energy 76 kcal/100 mL; fat 2.25 g/100 mL; carbohydrate 8.90 g/100 mL; total sugar 8.29 g/100 mL and protein 5.08 g/100 mL. During fermentation, aliquots from each batch of inoculated soymilk were taken at time intervals between 0 and 48 h for determination of cell growth, pH changes, isoflavones hydrolysis, total phenol and antioxidant content. LAB growth in soymilk was determined using the Pour Plate Method. Ten-fold serial dilutions of the samples were performed using sterile 0.1 g/L peptone water (Oxoid Ltd., Basingstoke, Hampshire,

England). The diluted samples were streaked onto MRS agar plates (Oxoid Ltd., Basingstoke, Hampshire, England) and LAB colonies (CFU/mL) were observed after incubation at 37°C for 48 h. Uninoculated soymilk was used as control throughout the study.

### HPLC ANALYSIS OF ISOFLAVONES

Isoflavone was extracted from soymilk fermented with or without LAB (control) as described by Rekha and Vijayalakshmi (2008). Briefly, 1 mL of each sample was mixed with 4 mL methanol (100%) and vortexed. The tubes were heated at 70°C for 30 min and inverted at intervals. The insoluble residue was separated by centrifugation (18000 g and 20°C for 30 min) and the supernatant was then filtered (0.45  $\mu$ m, Millipore Corp., Bedford, Massachusetts, USA) for HPLC analysis (UV-vis det., 265 nm, Water, Milford, USA) analysis. Standards used included glucosides (daidzin and genistin) and aglycones (daidzein and genistein) (Sigma-Aldrich Co., St. Louis, MO, USA). The mobile phase was 0.1% acetic acid in methanol (A) and 0.1% acetic acid in water (B). The samples or standards (20  $\mu$ L) were applied on a reverse-phase Phenomenex C18 column (4.6  $\times$  250 mm, Torrance, CA, USA). The gradient solvent system started with 80% solvent A and 20% solvent B at 0 min. Solvent A was then reduced to 75% within 30 min, held at 75% for 3 min and progressed to 80% within 5 min. The solvent flow rate was 1.0 mL/min. Retention time of standards (daidzin, genistin, daidzein and genistein) eluted as single peaks were integrated by Empower 2 Software (Waters, Milford, USA) and the peak areas were used for the quantification of the corresponding isoflavones in the sample. All samples were run in triplicates.

### DETERMINATION OF TOTAL PHENOLIC CONTENT IN FERMENTED SOYMILK

Total phenolic content was determined as described by Rekha and Vijayalakshmi (2008). Samples (0.1 mL) of soymilk fermented with or without LAB (control) were mixed with distilled water (0.9 mL) followed by 1 mL of Folin-Ciocalteu reagent (1:2 dilution in distilled water) and 2 mL of 10% Na<sub>2</sub>CO<sub>3</sub>. The mixture was centrifuged at 20000 g for 20 min and the supernatant filtered (Whatman No. 1 filter paper). Absorbance of the supernatant was measured at 765 nm (Ultraspec<sup>®</sup>2100pro, Amersham Biosciences, UK). Phenolic standard curve was generated using gallic acid and results were expressed as gallic acid equivalent (mg GAE/100 mL sample).

### DETERMINATION OF ANTIOXIDANT ACTIVITIES

**DPPH Radical-Scavenging Activity** The ability of each sample in scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was determined in accordance to McCue and Shetty (2005) but with some modifications. A 900  $\mu$ L ethanolic solution (96%) with or without DPPH (0.1 mM) was mixed with 100  $\mu$ L of sample, control or water (as blank), vortexed and then incubated at room

temperature ( $27 \pm 2^\circ\text{C}$ ) for 30 min. The samples were then centrifuged at 13500 g and  $28^\circ\text{C}$  for 5 min. Absorbance of each sample was measured at 520 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Infinite M200, Tecan, USA). Ascorbic acid was used as standard. The results were expressed as percentage (%) of DPPH scavenging activity calculated as follow:

$$\text{DPPH scavenging activity (\%)} = \frac{[(Aa-Ab)-(Ac-Ad)] \times 100}{(Aa-Ab)} \quad (1)$$

where Aa is the absorbance of blank solution mixed with DPPH solution; Ab is the absorbance of blank solution mixed with ethanolic solution without DPPH; Ac is the absorbance of the mixture solution containing both sample and DPPH; and Ad is the absorbance of the mixture solution containing both sample and ethanolic solution without DPPH.

**Ferrous Ion Chelating Activity** Ferrous ion ( $\text{Fe}^{2+}$ ) chelating activity of each sample was measured by inhibition of the formation of iron (II)-ferrozine complex after treating each sample with  $\text{Fe}^{2+}$  in accordance to Le et al. (2007). Briefly, the sample (100  $\mu\text{L}$ ) was diluted with 900  $\mu\text{L}$  of distilled water. The reaction mixture, which contained 500  $\mu\text{L}$  of the diluted sample or  $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$  (standard), 100  $\mu\text{L}$   $\text{FeCl}_2$  (0.6 mM in water) and 900  $\mu\text{L}$  methanol, was vortexed and incubated at room temperature ( $27 \pm 2^\circ\text{C}$ ) for 5 min. Ferrozine (5 mM in 100  $\mu\text{L}$  methanol solution) was then added, vortexed and incubated at room temperature for 10 min. The absorbance of  $\text{Fe}^{2+}$ -ferrozine complex was measured at  $\lambda = 562$  nm using a spectrophotometer (Ultraspec<sup>®</sup>2100pro, Amersham biosciences). The capability to chelate ferrous ions was calculated as follow:

$$\text{Percentage (\%)} = \left[ 1 - \frac{\text{absorbance of sample at } 562 \text{ nm}}{\text{absorbance of control at } 562 \text{ nm}} \right] \times 100.$$

#### ANIMALS

The *in vivo* experiment was approved by the Committee on Animal Research and Ethics (CARE), UiTM (reference number: 600-FF (PT.5/2)). A total of 24, two months-old male ICR mice, weighing 25-35 g were used. All mice, in group of six, were housed in polypropylene mouse cages ( $30 \times 20 \times 16$  cm) and maintained at ambient temperature ( $22^\circ\text{C}$ ). The rodents were allowed access to food pellet and water *ad libitum* and acclimatised for 7 days. The mice were randomly assigned to groups of untreated control (saline only, 0.2 mL), LPS-treated (LPS-saline), unfermented soymilk (0.2 mL) and soymilk + LAB 9 (0.2 mL of soy milk fermented with *L. fermentum*,  $10^9$  CFU/mL). Mice were administered with treatments via oral gavage daily for 32 days. LPS (*Escherichia coli*, serotype 055:B5, Sigma, St. Louis, MO, USA) was injected intraperitoneally (0.25 mg/kg, 0.2 mL) to induce memory impairment and neuroinflammation.

#### MORRIS WATER MAZE TEST

Habituation trials (once a day) were performed on days 26, 27 and 28 before the LPS challenge. The maximum trial length was 120 s. After the habituation trial, mice were injected with LPS for three consecutive days (day 29-31). Four hours after the LPS treatment, treated mice were allowed to swim until they found the escape platform. Escape latency and escape distance was monitored for one day. Escape latency and escape distance measures the time taken and distance travelled by the mice to find the platform. The shorter the time taken indicates improved memory. Data were analysed using a video tracking system (ANY-Maze, San Diego Instruments, San Diego, CA).

#### STATISTICAL ANALYSIS

Statistical analyses for growth study, isoflavones quantification, total phenolic content and antioxidant activities as well as escape distance and escape latency were performed using the one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiple comparison (SPSS 17.0, SPSS Inc, Chicago, IL, USA and GraphPad Prism version 6.07, GraphPad Software Incorporated, USA). Differences were considered as significant at  $p < 0.05$ .

#### RESULTS AND DISCUSSION

##### SOYMLK SUPPORTED PEDIOCOCCI AND LACTOBACILLI GROWTH

Table 1 indicates the growth profiles and pH changes of the 12 LAB incubated in soymilk at  $37^\circ\text{C}$  for 24 and 48 h. Both pediococci and lactobacilli yielded increased growth after incubation in soymilk (+2.10 to +3.14 log CFU/mL) for 48 h. The LAB growth rate in soymilk appeared to be strain specific. Whilst 7 LAB (LAB 1, 3, 5, 6, 7, 8 and 10) elicited increased growth percentage of +30.5 to +40.1%, the remaining LAB (LAB 2, 4, 9, 11 and 12) were relatively slow growing, with growth percentage ranging between +26.5 and +29.5%. The initial pH (0 h) of soymilk (pH 6.03 to 6.24) was found to decrease (i.e. increased acidity) after inoculation with LAB for 24 (pH 4.74 to 5.48) and 48 h (pH 4.58 to 4.89). The concomitant drop in pH values and increased LAB population indicated that lactobacilli and pediococci were able to adapt and survive in soymilk. This is consistent with previous studies that have described soymilk as a good medium for lactobacilli (Wei et al. 2007) and pediococci (Raghavendra et al. 2011).

##### PEDIOCOCCI AND LACTOBACILLI SIGNIFICANTLY INCREASED ACTIVE AGLYCONIC ISOFLAVONES IN SOYMLK

Figure 1 illustrates the elution profiles of glucosidic (genistin and daidzin) and aglyconic (genistein and daidzein) isoflavones in unfermented soymilk (control). Table 2, on the other hand, presents the quantitative analysis of isoflavones in soymilk fermented with the 12 LAB at  $37^\circ\text{C}$  for 48 h. Unfermented soymilk (control)

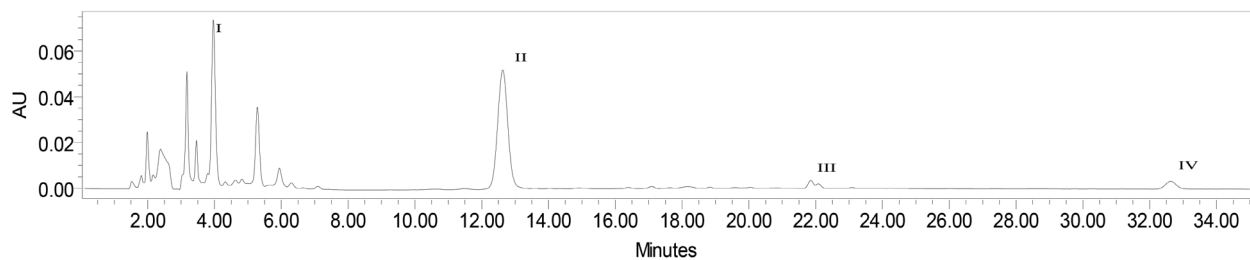
TABLE 1. Cell growth and changes of pH in soymilk fermented with or without LAB

Soymilk	Viable count (log CFU/ mL)			pH		
	Incubation period (h)			Incubation period (h)		
	0	24	48	0	24	48
Unfermented	-	-	-	6.68 ± 0.03	6.53 ± 0.06	6.39 ± 0.02
LAB 1	7.32 ± 0.07	9.59 ± 0.09	10.16 ± 0.13	6.10 ± 0.02	5.29 ± 0.02	4.88 ± 0.05
LAB 2	7.93 ± 0.10	9.70 ± 0.09	10.03 ± 0.08	6.03 ± 0.02	5.31 ± 0.01	4.71 ± 0.01
LAB 3	8.04 ± 0.10	9.37 ± 0.21	10.67 ± 0.06	6.03 ± 0.04	5.48 ± 0.02	4.73 ± 0.05
LAB 4	8.07 ± 0.12	9.92 ± 0.08	10.45 ± 0.16	6.12 ± 0.02	5.37 ± 0.02	4.78 ± 0.02
LAB 5	8.25 ± 0.12	10.26 ± 0.07	10.87 ± 0.20	6.15 ± 0.01	5.24 ± 0.01	4.70 ± 0.01
LAB 6	7.91 ± 0.09	8.95 ± 0.05	10.32 ± 0.06	6.06 ± 0.04	5.39 ± 0.02	4.89 ± 0.02
LAB 7	7.71 ± 0.14	9.71 ± 0.25	10.49 ± 0.05	6.06 ± 0.04	5.35 ± 0.02	4.84 ± 0.03
LAB 8	8.18 ± 0.11	10.05 ± 0.07	11.23 ± 0.10	6.10 ± 0.05	5.31 ± 0.03	4.67 ± 0.03
LAB 9	7.79 ± 0.04	9.92 ± 0.13	10.03 ± 0.07	6.09 ± 0.01	5.31 ± 0.02	4.72 ± 0.02
LAB 10	7.84 ± 0.05	9.84 ± 0.07	10.98 ± 0.15	6.16 ± 0.01	5.22 ± 0.02	4.63 ± 0.03
LAB 11	7.89 ± 0.09	9.47 ± 0.21	10.11 ± 0.03	6.15 ± 0.01	5.25 ± 0.01	4.83 ± 0.03
LAB 12	8.11 ± 0.05	9.15 ± 0.03	10.33 ± 0.02	6.24 ± 0.03	4.74 ± 0.02	4.60 ± 0.01

Each data represents mean ± standard error mean (SEM) of three measurements (in triplicates)

LAB 1, LAB 9; LAB 10, LAB 11 and LAB 12 are lactobacilli;

LAB 2, LAB 3, LAB 4, LAB 5, LAB 6, LAB 7 and LAB 8 are pediococci



I = daidzin; II = genistin; III = daidzein; IV = genistein

FIGURE 1. HPLC profile of isomeric isoflavones in unfermented soymilk

TABLE 2. Isoflavone isomers in soymilk fermented with or without LAB for 48 h

Soymilk	Glucosides (ppm)			Aglycones (ppm)		
	Daidzin	Genistin	Sub-total	Daidzein	Genistein	Sub-total
Unfermented	18.16 ± 0.35	12.44 ± 0.33	30.61 ± 0.56	1.09 ± 0.01	0.78 ± 0.00	1.78 ± 0.01
LAB 1	6.51 ± 0.33 <sup>#</sup>	4.96 ± 0.38 <sup>#</sup>	11.47 ± 0.03 <sup>#</sup>	2.96 ± 0.03 <sup>#</sup>	0.78 ± 0.00	3.74 ± 0.00 <sup>#</sup>
LAB 2	1.55 ± 0.09 <sup>#</sup>	0.77 ± 0.03 <sup>#</sup>	2.32 ± 0.04 <sup>#</sup>	8.68 ± 0.37 <sup>#</sup>	1.67 ± 0.10	10.35 ± 0.20 <sup>#</sup>
LAB 3	2.18 ± 0.03 <sup>#</sup>	0.49 ± 0.25 <sup>#</sup>	2.76 ± 0.25 <sup>#</sup>	8.15 ± 0.18 <sup>#</sup>	0.99 ± 0.02	9.14 ± 0.19 <sup>#</sup>
LAB 4	1.27 ± 0.03 <sup>#</sup>	0.00 ± 0.00 <sup>#</sup>	1.27 ± 0.03 <sup>#</sup>	4.63 ± 0.14 <sup>#</sup>	0.89 ± 0.01	5.52 ± 0.09 <sup>#</sup>
LAB 5	2.36 ± 0.10 <sup>#</sup>	1.24 ± 0.27 <sup>#</sup>	3.60 ± 0.34 <sup>#</sup>	8.02 ± 0.19 <sup>#</sup>	0.98 ± 0.02	9.00 ± 0.20 <sup>#</sup>
LAB 6	1.58 ± 0.01 <sup>#</sup>	0.52 ± 0.45 <sup>#</sup>	2.10 ± 0.26 <sup>#</sup>	9.70 ± 0.10 <sup>#</sup>	1.82 ± 0.02	11.52 ± 0.07 <sup>#</sup>
LAB 7	1.50 ± 0.06 <sup>#</sup>	0.79 ± 0.03 <sup>#</sup>	2.28 ± 0.09 <sup>#</sup>	5.18 ± 0.05 <sup>#</sup>	0.93 ± 0.00	6.11 ± 0.05 <sup>#</sup>
LAB 8	1.31 ± 0.03 <sup>#</sup>	0.00 ± 0.00 <sup>#</sup>	1.31 ± 0.03 <sup>#</sup>	4.98 ± 0.05 <sup>#</sup>	0.92 ± 0.00	5.90 ± 0.05 <sup>#</sup>
LAB 9	1.31 ± 0.04 <sup>#</sup>	0.00 ± 0.00 <sup>#</sup>	1.31 ± 0.04 <sup>#</sup>	4.74 ± 0.00 <sup>#</sup>	0.90 ± 0.00	5.64 ± 0.00 <sup>#</sup>
LAB 10	1.33 ± 0.02 <sup>#</sup>	0.00 ± 0.00 <sup>#</sup>	1.33 ± 0.02 <sup>#</sup>	4.66 ± 0.13 <sup>#</sup>	0.89 ± 0.02	5.55 ± 0.14 <sup>#</sup>
LAB 11	7.05 ± 0.09 <sup>#</sup>	3.91 ± 0.10 <sup>#</sup>	10.96 ± 0.17 <sup>#</sup>	7.16 ± 0.21 <sup>#</sup>	0.95 ± 0.02	8.11 ± 0.23 <sup>#</sup>
LAB 12	4.08 ± 0.06 <sup>#</sup>	2.32 ± 0.03 <sup>#</sup>	6.40 ± 0.08 <sup>#</sup>	6.64 ± 0.08 <sup>#</sup>	7.94 ± 0.22 <sup>#</sup>	14.58 ± 0.30 <sup>#</sup>

Each data represents mean ± SEM of three measurements (in triplicates)

<sup>#</sup> Means within a column differ significantly with unfermented soymilk ( $p < 0.05$ )

LAB 1, LAB 9; LAB 10, LAB 11 and LAB 12 are lactobacilli;

LAB 2, LAB 3, LAB 4, LAB 5, LAB 6, LAB 7 and LAB 8 are pediococci



contained higher levels of glucosides (31.0 ppm; 94% of total isoflavones) when compared to aglycones (1.8 ppm; 6% of total isoflavones). Interestingly, significant changes in the composition of isoflavone isomers took place after fermentation with LAB. Whilst glucosides were drastically reduced by 64.2-95.9%, aglycones in LAB-fermented soymilk were 2.1-6.5 fold higher as opposed to those in unfermented soymilk. The increased aglycones in LAB-fermented soymilk were attributed predominantly to the increased daidzein. Aglycones (i.e. daidzein and genistein) are important compounds with high bioavailability. When compared to glucosides, they were absorbed faster and in greater amounts (Izumi et al. 2000). Glucosides, on the other hand, were not absorbed intact across the enterocyte. Their hydrolysis to aglycones by  $\beta$ -glucosidase was therefore required to increase bioavailability (Sun 2011).

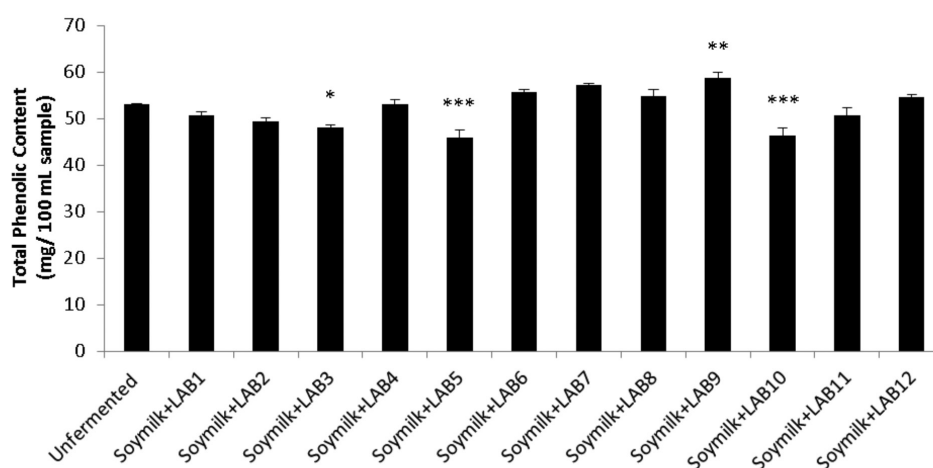
Probiotic LAB are known for their  $\beta$ -glucosidase activity that is essential for hydrolysis of glucosides into bioactive aglycones in fermented soymilk. Lactobacilli, in particular, are known for their ability in increasing bioactive aglycones in soymilk. The performances of the reported strains were, however, inferior when compared to the present results. Aglycones in soymilk was found to increase up to 90% after fermentation with *L. rhamnosus* (Marazza et al. 2009), by 71% with *L. casei* (Donkor & Shah 2008), by 75% with *L. delbrueckii* sp. *bulgaricus* ATCC 11842 (Prasad & Shah 2011) and by 98% with *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum* and *L. fermentum* (Rekha & Vijayalakshmi 2011). It is noteworthy to mention that the present study found that pediococci were also capable of hydrolysing glucosides into aglycones. Comparatively, the pediococci strains (increase by 3.7-9.7 ppm when compared to control) yielded higher amount of aglycones than the lactobacilli strains (increase by 2.0-6.33 ppm when compared to control).

#### LACTOBACILLUS FERMENTUM LAB 9 SIGNIFICANTLY YIELDED THE HIGHEST TOTAL PHENOLIC CONTENT IN SOYMILK

Phenolic compounds are plant-derived secondary metabolites known for their bioactivity as antioxidants, anti-oestrogens, anti-amyloidogenic aggregation effects and anti-proliferatives (Pandey & Rizvi 2009). Figure 2 depicts the total phenolic content of both unfermented soymilk and soymilk fermented with LAB for 48 h. It was found that unfermented soymilk contained considerably high amount of total phenolic content (53.04 mg GAE/100 mL), except for *L. fermentum* LAB 9 as none of the LAB confers significant advantage to the total phenolic content of soymilk. Fermentation of soymilk with the majority of LAB yielded either comparable or lesser total phenolic content when compared to that of unfermented soymilk. In spite of its positive outcome, the total phenolic content of soymilk fermented with LAB 9 has only significantly increased by 10%. Nevertheless, a higher total phenolic content suggests higher reducing capacity which may be translated into higher antioxidant potential (Stratil et al. 2007). Previously, Song et al. (2011) found the total phenolic content of soybean fermented with *L. plantarum* increased by 87% when compared to the unfermented form. In line with the present findings, previous studies indicated that the total phenolic content in LAB fermented soymilk may decrease depending on the strains used. Rekha and Vijayalakshmi (2011), for instance, reported decreased polyphenol contents in soymilk fermented with *L. acidophilus*, *L. bulgaricus*, *L. casei*, *L. plantarum* and *L. fermentum* with increased fermentation period.

#### ANTIOXIDANT CAPACITY

It is known that probiotic characteristics are strain dependent and each probiotic should be tested to know



Each bar represents mean  $\pm$  SEM of  $n = 6$   
 \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  when compared to unfermented soymilk  
 LAB 1, LAB 9; LAB 10, LAB 11 and LAB 12 are lactobacilli;  
 LAB 2, LAB 3, LAB 4, LAB 5, LAB 6, LAB 7 and LAB 8 are pediococci

FIGURE 2. Total phenolic content of soymilk fermented with or without LAB for 48 h

if it has specific beneficial effects and to describe the mechanism/s involved in their health-promoting properties (de Moreno de LeBlanc et al. 2015). In line with this notion, the antioxidant capacity of the 12 tested LAB appeared to be strain dependent. Also, not many mechanisms are usually associated with one individual strain (de Moreno de LeBlanc et al. 2015). The present study found no LAB to possess both excellent DPPH radical-scavenging and ferrous ion chelating activities. Whilst *P. acidilactici* LAB 5, *L. fermentum* LAB 10 and *L. plantarum* LAB 11 and LAB 12 exhibited excellent DPPH radical-scavenging activity, *L. plantarum* LAB 1, *P. pentosaceus* LAB 7 and LAB 8 as well as *L. fermentum* LAB 9 showed higher ferrous ion chelating activity. The mechanisms underlying the varying antioxidant capacity of the 12 tested LAB require further investigation in the future.

PEDIOCOCCI AND LACTOBACILLI FERMENTED SOYMILK SIGNIFICANTLY ELICITED EXCELLENT DPPH RADICAL SCAVENGING ACTIVITY

Figure 3 compares DPPH radical-scavenging activities of both unfermented and fermented soymilk. Soymilk fermented with LAB for 48 h resulted in significantly higher ( $p < 0.05$ ) DPPH radical-scavenging activity when compared to that of unfermented soymilk. Soymilk fermented with *P. acidilactici* LAB 5, *L. fermentum* LAB 10 and *L. plantarum* LAB 11 and LAB 12 demonstrated maximum DPPH radical-scavenging activity (100%), respectively. It was reported that soymilk fermented with lactobacilli possessed excellent DPPH radical-scavenging activity of over 50% as opposed to unfermented soymilk (Marazza et al. 2012). There is, however, no previous study on DPPH radical-scavenging activity of soymilk fermented with *pediococci*. The present study demonstrated for the first

time that fermentation of soymilk samples with *pediococci* elicited significantly higher DPPH radical-scavenging activity. The enhanced DPPH radical-scavenging activity of LAB fermented soymilk could be attributed, at least in part, to increased aglycone concentration. It was proposed that aglycones (mainly daidzein and genistein) generated through enzymatic action of *L. rhamnosus* CRL981 were able to act as good hydrogen donors that could effectively scavenge DPPH radicals in soymilk (Tachakittirungrod et al. 2007).

PEDIOCOCCI AND LACTOBACILLI (SELECTED STRAINS) FERMENTED SOYMILK EXHIBITED SIGNIFICANTLY HIGHER FERROUS ION CHELATING ACTIVITY

Ferrous ion ( $\text{Fe}^{2+}$ ) is the most abundant transition metal ion in our body. It may, however, contribute to pathological generation of highly reactive oxygen species such as hydroxyl radicals via the Fenton reaction. Catalysis by ferrous ions is correlated with incidents of cancer, arthritis and cardiovascular diseases (Yeh et al. 2011). Figure 4 highlights ferrous ion chelating activities of unfermented and LAB fermented soymilk. There were 4 out of 12 LAB (*L. plantarum* LAB 1, *P. pentosaceus* LAB 7 and LAB 8 as well as *L. fermentum* LAB 9) which had exhibited significantly higher ( $p < 0.05$ ) ferrous ion chelating activity when compared to control. These results were in agreement with ferrous ion chelating potential of various LAB (Ahire et al. 2013; Kullisaar et al. 2002; Lee et al. 2005; Liu & Pan 2010; Liu et al. 2011; Saide & Gilliland 2005). It is known that LAB genome contains genes that have been predicted to play vital roles in iron acquisition (Solioz et al. 2011). LAB could in fact produce high affinity chelators (siderophores) that can solubilise  $\text{Fe}^{3+}$  from the environment to meet cellular demands for iron.

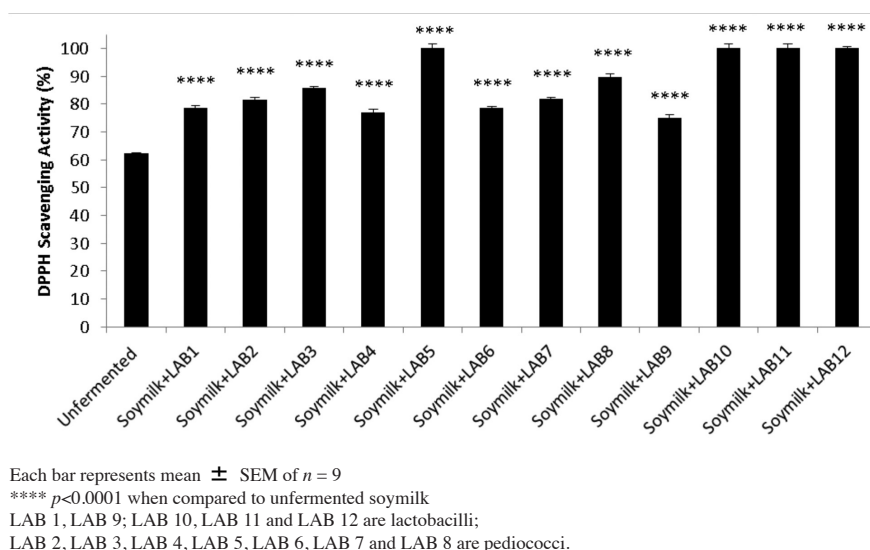
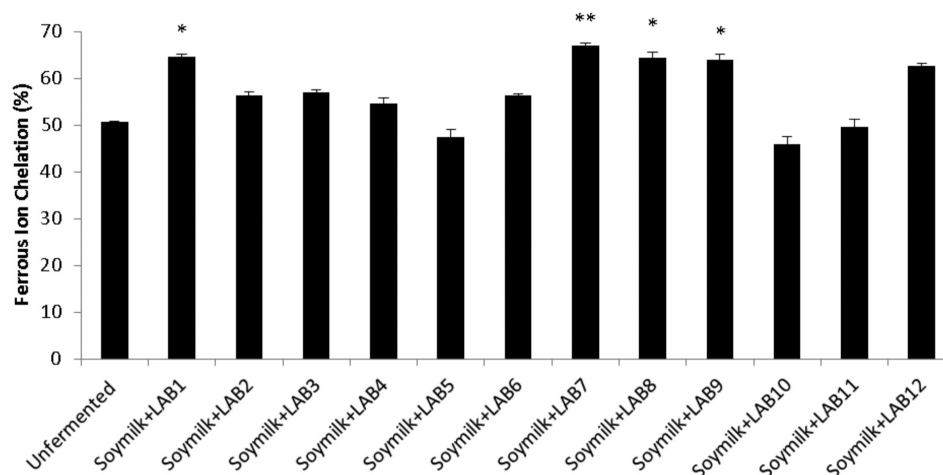


FIGURE 3. DPPH scavenging activity of soymilk fermented with or without LAB for 48 h



Each bar represents mean  $\pm$  SEM of  $n = 9$ ;  
 \*  $p < 0.05$ , and \*\*  $p < 0.01$  when compared to unfermented soy milk;  
 LAB 1, LAB 9; LAB 10, LAB 11 and LAB 12 are lactobacilli;  
 LAB 2, LAB 3, LAB 4, LAB 5, LAB 6, LAB 7 and LAB 8 are pediococci

FIGURE 4. Ferrous ion chelation by soy milk fermented with or without LAB for 48 h

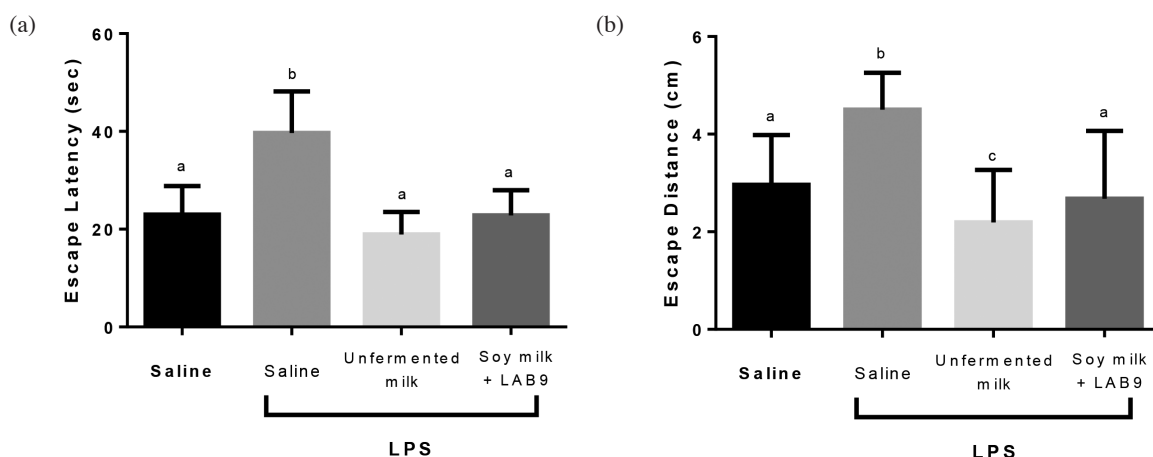
#### SOYMILK FERMENTED WITH *L. FERMENTUM* LAB 9 ENHANCE MEMORY IN LPS-INDUCED MEMORY IMPAIRED MICE

Polyphenols have been found to be associated with anti-amyloidogenic aggregation effects. As such, soy milk fermented with *L. fermentum* LAB 9 which had significantly yielded the highest phenolic content was selected for this study. Figure 5 illustrates the memory enhancing effect of soy milk fermented with *L. fermentum* LAB 9 against LPS-induced memory impaired mice. LPS-challenged mice were presented with increased escape distance and escape latency. LPS-challenged mice administered with either unfermented milk or LAB 9-fermented soy milk, however, exhibited significant ( $p < 0.05$ ) improvement of memory

deficit. This was evident by shorter time spent and shorter distance travelled to reach the hidden platform when compared to the LPS saline group. The results indicated that both unfermented and LAB-fermented soy milk has promising memory enhancing properties. The beneficial role of soy (unfermented and LAB-fermented) is consistent with that of previous studies (Ahmad et al. 2014; Kim et al. 2012).

#### CONCLUSION

Malaysian LAB has demonstrated promising potential in enriching the nutritional value of soy milk. The present study demonstrated increased hydrolysis of



Each bar represents mean  $\pm$  SEM ( $n = 6$ ); <sup>a,c</sup> Different superscript letters represent significant differences ( $p < 0.05$ ) amongst treatment groups

FIGURE 5. The effect of SM-LAB against LPS-induced memory impairment in ICR mice SM-LAB treated LPS-induced mice were subjected to behavioural study using the Morris Water Maze Test. The memory impaired rodents were assessed as follow: a) Escape latency and b) Escape distance

glucosidic isoflavones to their aglycone counterparts in LAB-fermented soymilk. This is also the first finding on biotransformation of glucosides to aglycones in pediococci fermented soymilk. This reaction was accompanied by significantly greater antioxidant capacity of fermented soymilk including maximal scavenging of DPPH radicals and enhanced chelation of ferrous ions. The LAB conferred beneficial effects appeared to be strain dependent. The use of multi-strain LAB may be useful in achieving greater efficacy. More interestingly, soymilk fermented with *L. fermentum* LAB 9 which had significantly yielded the highest phenolic content improved memory deficit in LPS-challenged mice.

#### ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Higher Education (Malaysia) and Ministry of Science and Technology (Malaysia) for the financial support under the Long Term Research Grant Scheme (600-RMI/LRGS 5/3 (2/2012) and e-Science (100-RMI/SF 16/6/2), respectively.

#### REFERENCES

- Ahire, J.J., Mokashe, N.U., Patil, H.J. & Chaudhari, B.L. 2013. Antioxidative potential of folate producing probiotic *Lactobacillus helveticus* CD6. *Journal of Food Science and Technology* 50(1): 26-34.
- Ahmad, A., Ramasamy, K., Jaafar, S.M., Majeed, A.B. & Mani, V. 2014. Total isoflavones from soybean and tempeh reversed scopolamine-induced amnesia, improved cholinergic activities and reduced neuroinflammation in brain. *Food and Chemical Toxicology* 65: 120-128.
- de Moreno de LeBlanc, A., Del Carmen, S., Chatel, J.M., Miyoshi, A., Azevedo, V., Langella, P., Bermúdez-Humarán, L.G. & LeBlanc, J.G. 2015. Current review of genetically modified lactic acid bacteria for the prevention and treatment of colitis using murine models. *Gastroenterology Research and Practice* 2015: Article ID. 146972.
- Ding, W.K. & Shah, N.P. 2010. Enhancing the biotransformation of isoflavones in soymilk supplemented with lactose using probiotic bacteria during extended fermentation. *Journal of Food Science* 75(3): M140-M149.
- Donkor, O.N. & Shah, N.P. 2008. Production of beta-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus casei* in soymilk. *Journal of Food Science* 73(1): M15-M20.
- FAO 2002. *Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria*. Joint Food and Agricultural Organization of the United Nations and World Health Organization Expert Consultation Report, Cordoba, Argentina, 2001.
- Food and Drug Administration. 1999. FDA survey of imported fresh produce. <http://vm.cfsan.fda.gov/~dms/prodsurv.html>. Accessed on 28 July 2015.
- Izumi, T., Piskula, M.K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y. & Kikuchi, M. 2000. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *The Journal of Nutrition* 130(7): 1695-1699.
- Kim, J.H., Choi, J.M., Lee, S. & Cho, E.J. 2012. Protective effects of purple sweet potato added to *Bacillus subtilis*-fermented soymilk against amyloid beta-induced memory impairment. *Journal of Agricultural Science* 4(4): 223-232.
- King, R.A. & Bignell, C.M. 2000. Concentrations of isoflavone phytoestrogens and their glucosides in Australian soya beans and soya foods. *Australian Journal of Nutrition and Dietetics* 57: 70-78.
- Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C. & Kilk, A. 2002. Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology* 72(3): 215-224.
- Le, K., Chiu, F. & Ng, K. 2007. Identification and quantification of antioxidants in *Fructus lycii*. *Food Chemistry* 105(1): 353-363.
- Lee, J., Hwang, K.T., Chung, M.Y., Cho, D.H. & Park, C.S. 2005. Resistance of *Lactobacillus casei* KCTC 3260 to reactive oxygen species (ROS): role for a metal ion chelating effect. *Journal of Food Science* 70(8): m388-m391.
- Liu, C.F., Tseng, K.C., Chiang, S.S., Lee, B.H., Hsu, W.H. & Pan, T.M. 2011. Immunomodulatory and antioxidant potential of *Lactobacillus exopolysaccharides*. *Journal of the Science of Food and Agriculture* 91(12): 2284-2291.
- Liu, C.F. & Pan, T.M. 2010. *In vitro* effects of lactic acid bacteria on cancer cell viability and antioxidant activity. *Journal of Food and Drug Analysis* 18(2): 77-86.
- Marazza, J.A., Nazareno, M.A., de Giori, G.S. & Garro, M.S. 2012. Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *Journal of Functional Foods* 4(3): 594-601.
- Marazza, J.A., Garro, M.S. & de Giori, G.S. 2009. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. *Food Microbiology* 26(3): 333-339.
- McCue, P.P. & Shetty, K. 2005. Phenolic antioxidant mobilization during yogurt production from soymilk using Kefir cultures. *Process Biochemistry* 40(5): 1791-1797.
- Otieno, D.O., Ashton, J.F. & Shah, N.P. 2007. Isoflavone phytoestrogen degradation in fermented soymilk with selected beta-glucosidase producing *L. acidophilus* strains during storage at different temperatures. *International Journal of Food Microbiology* 115(1): 79-88.
- Pandey, K.B. & Rizvi, S.I. 2009. Current understanding of dietary polyphenols and their role in health and disease. *Current Nutrition and Food Science* 5(4): 249-263.
- Prasad, L.N. & Shah, N.P. 2011. Conversion of isoflavone glycoside to aglycones in soy protein isolate (SPI) using crude enzyme extracted from *Bifidobacterium animalis* Bb12 and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842. *International Food Research Journal* 19(2): 433-439.
- Raghavendra, P., Ushakumari, S. & Halami, P. 2011. Phytate-degrading *Pediococcus pentosaceus* CFR R123 for application in functional foods. *Beneficial Microbes* 2(1): 57-61.
- Ramasamy, K., Abdul Rahman, N.Z., Sieo, C.C., Alitheen, N.J., Abdullah, N. & Ho, Y.W. 2012. Probiotic potential of lactic acid bacteria from fermented Malaysian food or milk products. *International Journal of Food Science and Technology* 47(10): 2175-2183.
- Rekha, C.R. & Vijayalakshmi, G. 2011. Isoflavone phytoestrogens in soymilk fermented with  $\beta$ -glucosidase producing probiotic lactic acid bacteria. *International Journal of Food Sciences and Nutrition* 62(2): 111-120.
- Rekha, C.R. & Vijayalakshmi, G. 2008. Biomolecules and nutritional quality of soymilk fermented with probiotic yeast and bacteria. *Applied Biochemistry and Biotechnology* 151(2-3): 452-463.



- Saide, J.A. & Gilliland, S.E. 2005. Antioxidative activity of lactobacilli measured by oxygen radical absorbance capacity. *Journal of Dairy Science* 88(4): 1352-1357.
- Solioz, M., Mermod, M., Abicht, H.K. & Mancini, S. 2011. Responses of lactic acid bacteria to heavy metal stress. In *Stress Responses of Lactic Acid Bacteria*, edited by Tsakalidou, E. & Papadimitriou, K. New York: Springer. pp. 163-195.
- Song, Y.R., Kim, Y.E., Kim, J.H., Song, N.E., Jeong, D.Y. & Baik, S.H. 2011. Preparation of fermented sugar-soaked black soybean snacks (FSBSS) and characterization of their quality changes. *Food Science and Biotechnology* 20(6): 1547-1553.
- Stratil, P., Klejdus, B. & Kubán, V. 2007. Determination of phenolic compounds and their antioxidant activity in fruits and cereals. *Talanta* 71(4): 1741-1751.
- Sun, X.D. 2011. Enzymatic hydrolysis of soy proteins and the hydrolysates utilisation. *International Journal of Food Science and Technology* 46(12): 2447-2459.
- Tachakittirungrod, S., Okonogi, S. & Chowwanapoonpohn, S. 2007. Study on antioxidant activity of certain plants in Thailand: mechanism of antioxidant action of guava leaf extract. *Food Chemistry* 103: 381-388.
- Wang, Y.C., Yu, R.C. & Chou, C.C. 2006. Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiology* 23(2): 128-135.
- Wei, Q.K., Chen, T.R. & Chen, J.T. 2007. Using of Lactobacillus and Bifidobacterium to product the isoflavone aglycones in fermented soymilk. *International Journal of Food Microbiology* 117(1): 120-124.
- Yeh, J.Y., Hsieh, L.H., Wu, K.T. & Tsai, C.F. 2011. Antioxidant properties and antioxidant compounds of various extracts from the edible basidiomycete *Grifola frondosa* (Maitake). *Molecules* 16(4): 3197-3211.
- Zhao, D. & Shah, N.P. 2014. Changes in antioxidant capacity, isoflavone profile, phenolic and vitamin contents in soymilk during extended fermentation. *LWT - Food Science and Technology* 58(2): 454-462.
- E.F. Mohamad Fakri, S.M. Lim, N.H. Musa, M. Hazizul Hasan, A. Adam & K. Ramasamy\*  
Faculty of Pharmacy  
Universiti Teknologi MARA  
42300 Bandar Puncak Alam, Selangor Darul Ehsan  
Malaysia
- E.F. Mohamad Fakri, S.M. Lim, N.H. Musa & K. Ramasamy\*  
Collaborative Drug Discovery Research (CDDR) Group  
Pharmaceutical and Life Sciences Community of Research  
Universiti Teknologi MARA  
40450 Shah Alam, Selangor Darul Ehsan  
Malaysia
- M. Hazizul Hasan & A. Adam  
Research on Affinity, Safety and Efficacy Studies (OASES)  
Pharmaceutical and Life Sciences Community of Research  
Universiti Teknologi MARA  
40450 Shah Alam, Selangor Darul Ehsan  
Malaysia
- N.H. Musa  
Faculty of Applied Sciences, Universiti Teknologi MARA  
Tapah Campus, Tapah Road  
35400 Tapah, Perak Darul Redzuan  
Malaysia

\*Corresponding author; email: kalav922@salam.uitm.edu.my

Received: 3 January 2016

Accepted: 3 May 2016