

## Antioxidative activity and polyphenol content in fermented soy milk supplemented with WPC-70 by probiotic Lactobacilli

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### Abstract

Soymilk is fermented with probiotic lactobacilli (*Lactobacillus rhamnosus* NCDC 19, 24 and cheese isolates: *L. rhamnosus* C2, C6 and *L. casei* NCDC 17, 297) @1.0% for 24 h at 37°C. We find that in fermented soymilk both the inhibition of ascorbate autoxidation, and the reducing activity of polyphenol content and increasing of proteolysis varied with the starters used, but nevertheless are significantly higher than those found in unfermented soymilk. Thus, soymilk was supplemented with 2.0% whey protein concentrate 70 (WPC70). WPC70 enhances the bacterial growth in soy milk medium and also increases the antioxidative activity and proteolysis activity of these six probiotic lactobacilli cultures. Antioxidative activity was measured following three methods (ABTS, DPPH and FRAP). *L. rhamnosus* C6 strain showed maximum antioxidative activity and proteolytic activity as well as reduced polyphenol content among these six lactobacilli cultures. However, soy based probiotic foods can be nutritionally beneficial with these health benefits.

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### Keywords

Antioxidant

Polyphenol

Soy milk

Fermentation

Lactobacilli

Probiotic

### Introduction

Soymilk has a high amount of protein, iron, unsaturated fatty acids and niacin, but it is low in fat, carbohydrates, calcium. It has little saturated fat and no cholesterol; hence it is known to reduce the risk of heart disease (Kim *et al.*, 2006; Jinapong *et al.*, 2008). It is safe for people with lactose intolerance or milk allergy and for children with galactosemia. However, the beany flavor due to the presence of aldehydes and alcohols and flatulence caused by indigestible oligosaccharides limit the widespread consumption of soymilk (Blagden and Gilliland, 2005; Yang and Zhang, 2009). Soy-based foods are promising supplements to overcome existing protein-calorie-malnutrition problems (Khare *et al.*, 1994). However, its consumption may lead to digestive problems associated with the presence of raffinose and stachyose. One method of overcoming such problem and also to improve the acceptability is by fermentation (Hati *et al.*, 2013). Fermentation improves the bioavailability of isoflavones, assists in digestion of protein, provides more soluble calcium, enhances intestinal health, and supports immune system. Soybeans contain the highest concentration of isoflavones among all foods (Ismail and Hayes, 2005; Kuo *et al.*, 2006). The range of health benefits such as cancer prevention, reduced risk of osteoporosis, valuable role in chronic renal disease, and protection against cardiovascular disorders are

claimed to be due to the presence of these isoflavones (Orhan *et al.*, 2007). However, soymilk is the aqueous extract of whole soybeans (*Glycine max*). Soymilk is considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for lactose-intolerant population (Dhananjay *et al.*, 2006). Soybean is a rich source of isoflavone, which are reported to have beneficial estrogenic effects (Adlercreutz, 2002; Brouns, 2002; Corn *et al.*, 2004) with potential bioactive antioxidant properties. Probiotic lactic acid bacteria when grown in soy milk with whey protein concentrate have the ability to utilize phenolic components producing phytase enzyme and reduce the polyphenol content after fermentation. Free radicals and other reactive oxygen species are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body. The radicals may cause oxidative damage by oxidizing biomolecules and results in cell death and tissue damage (Kehrer, 1993). Atherosclerosis, cancer, emphysema, cirrhosis, and arthritis have been correlated with oxidative damage (Kehrer, 1993; Jacob, 1994). Therefore, oxidative damage plays a significant pathological role in human disease. However, ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human body (Lin and Yen, 1999). Soybean, the most important legume in the traditional Chinese diet, is rich in high-quality protein. Despite its ubiquity in the Asian diet,

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soybean has some limitations such as a disagreeable bean flavor for some food uses, and contains raffinose and stachyose that are not digested by human beings and may cause flatulence (Thananunkul *et al.*, 1976). Beans contain phenolic compounds that exhibit antioxidative activity (Murakami *et al.*, 1984; Drumm *et al.*, 1990). Previous research has demonstrated that the antioxidative activity of fermented soyfoods, such as miso, natto, and tempeh, was remarkably stronger than unfermented steamed soybeans (Murakami *et al.*, 1984; Esaki *et al.*, 1994; Berghofer *et al.*, 1998; Sheih *et al.*, 2000). Results obtained from these studies suggest the possibility and potential of developing soy based fermented food products with higher antioxidative activity and reduced polyphenol content after fermentation by probiotic lactobacilli. In this study, antioxidative activity was shown due to inhibition of ascorbate autoxidation, the scavenging effect of superoxide anion radicals and hydrogen peroxide, as well as the reducing activity exerted by various fermented soymilk were compared with unfermented soymilk. In addition, the effect of whey protein concentrate on the changes of antioxidative activity and polyphenol content of the fermented soymilk was also examined.

## Material and Methods

### Soybean sample

Soybeans were obtained from Local Market, Karnal, India.

### Whey protein concentrate 70 (WPC 70)

WPC 70 has been purchased from Mahan Proteins Ltd., New Delhi, India.

### Source of cultures

Bacterial strains (*L. rhamnosus* NCDC19, *L. rhamnosus* NCDC24 and *L. rhamnosus* C2, *L. rhamnosus* C6 and *L. casei* NCDC17, *L. casei* NCDC297) used were taken from National Collection of Dairy Cultures (NCDC), Karnal, India and C2 and C6 strains were cheese isolates in our Laboratory (Bioactive peptides Laboratory, NDRI, Karnal, India-132001). The cultures were maintained by biweekly transfers into sterile litmus milk or soymilk and held at 5°C between transfers.

### Soymilk preparation

To prepare soymilk, 100 g of soybeans were soaked for 14 to 16 h in 1.0 L of distilled water at room temperature (28°C) in a 2.0 L beaker. The soak water was drained from the soybeans and the beans thereafter were blanched at 98°C in boiling

distilled water for 30 min. The drained beans were hand washed thoroughly to remove their testa. They were then placed in a warring blender and 600 ml of boiled distilled water at 87 to 90°C was added and then blended for 3 min. The boiled water inactivated the enzyme, lipoxygenase during blending (Wilkins *et al.*, 1967). The resulting slurry was filtered through two layers of moslin cloth and approximately 600 ml of soymilk was obtained per 100 g of soybeans in 600 ml of water.

### Inoculum development

The following lactobacilli cultures were graciously supplied by National Collection of Dairy Cultures (NCDC), Karnal, India. Stock cultures were prepared by mixing MRS-grown cultures with sterile (sterilization at 120°C for 15 min at 15 psi) rehydrated skim milk 20% (w/w) and glycerol 20% (w/v) in a 2:5:5 ratio, placing 1 mL in Cryovials and storing at -20°C. After two successive transfers of the test organisms in MRS broth (HiMedia, India) and incubation at 37°C for 24 h, each activated culture was inoculated into MRS broth which was then incubated at 37°C for 16 h. These working cultures of *L. rhamnosus* NCDC19, *L. rhamnosus* NCDC24 and *L. rhamnosus* C2, *L. rhamnosus* C6 and *L. casei* NCDC17, *L. casei* NCDC297 which were then transferred in soymilk medium to check their activity in this medium. Lactobacilli cultures were selected because of their potential use for making traditional indigenous fermented curd to improve health by providing various health benefits like antimicrobial, anti-hypertension, antioxidative, anti-diarrhea etc. For the preparation of the inocula, 100 ml of sterile soy milk medium was inoculated with 1 mL of active working culture and incubated at 37°C until a pH of 4.5 was reached.

### Conditions

When fermentation was performed, 100 ml of sterile soymilk was placed in a 150 ml screw cap, Erlenmeyer flask and was inoculated with 1.0 ml of an inoculum of the Lactobacilli cultures. In these experiments, the initial population of each organism in the soymilk was between 3 and 4 log cfu/ml. Inoculated soymilk was incubated without shaking at 37°C for 24 h. During that period, samples were taken at pre-determined intervals to determine the antioxidative activity and polyphenol content of probiotic lactobacilli in the soymilk with supplemented with whey protein concentrate (WPC70) @ 2.0%.

### Fermentation of soymilk

In these studies, soymilk fermentations were

conducted in 500 ml capacity flasks using all six probiotic lactobacilli cultures separately. Inoculum at the rate of 1.0% active culture was added to the each of 200 ml soy milk in 500 ml flasks and placed in an incubator at 37°C for 24 hrs.

#### **Antioxidative activity**

##### *ABTS [2, 2'-Azinobis (3-ethylene benzothiazoline) 6-Sulphonicacid] Assay*

Total radical scavenging capacity was based on the ability of a compound to scavenge the stable ABTS radical in 10 min (Re *et al.*, 1999) with some modifications. The ABTS working solution was prepared by mixing 88 µL of 140 mM potassium persulphate with 5 mL of 7 mM ABTS stock solution and incubating overnight in dark bottles for generation of radicals. Then it was diluted with phosphate buffer saline (PBS) to adjust the absorbance at 734 nm to 0.7 ± 0.02. An aliquot of 10 µL of product supernatant, collected after centrifuging at 14,000 x g for 30 min, was coated in 96 wells microplate and to this 100 µL ABTS in PBS solution was added and were mixed for 10 sec. The decrease in absorbance at 734 nm was recorded over period of 10 min at 10 sec interval using Multiplate reader (Tecan-Infinite® 200 PRO, Switzerland). The results were expressed as trolox equivalent antioxidant capacity (TEAC) values.

##### *DPPH (2, 2-diphenyl - 1-picryl hydrazyl) Assay*

Antioxidant capacity based on DPPH radical for extracts of product was analyzed following the method given by (Brand-Williams *et al.*, 1995) with some modifications. Stock solution was prepared by taking 27.7 mg of DPPH (Sigma-Aldrich, molecular weight -394.32) in 50 mL amber colored reagent bottle and dissolving in 25 mL methanol by stirring over magnetic stirrer overnight at 4°C. The final volume was adjusted with methanol to 25 mL using volumetric flask and it was stored at -20°C. Working solution was prepared by dissolving 142 µL of stock in 9.858 mL of methanol. Working solution was prepared freshly prior to analysis and kept in amber glass bottle. Hundred microliter of appropriate dilution of product supernatant was loaded in 96 wells microplate and was mixed with 100 µL of freshly prepared DPPH working; the contents were mixed for 10 sec and incubated in dark for 120 min at 37°C after covering the microplate with aluminium foil. The absorbance of the solution was measured at 517 nm against methanol using Multiplate reader (Tecan-Infinite® 200 PRO, Switzerland). For blank determination 100 µL methanol was taken instead of sample and absorbance was measured immediately against methanol. The experiment was performed in

triplicate. The results were expressed as:

$$\% \text{DPPH scavenging activity} = \frac{(A_{515 \text{ nm blank}} - A_{515 \text{ nm sample}}) \times 100}{A_{515 \text{ nm blank}}}$$

##### *FRAP (Ferric Reducing Antioxidant Power) Assay*

FRAP values of the product was analyzed by method of Benzie and Strain (1996) with some modifications. Stock solution of acetate buffer (300 mM, pH 3.6) was made by mixing 3.1 g of sodium acetate trihydrate (molecular weight-136.08) in 16 mL of glacial acetic acid and final volume was made up to 1000 mL with distilled water. FRAP reagent was prepared by mixing 10 mL of 300 mM acetate buffer (pH 3.6), 1 mL of 10 mM TPTZ (molecular weight-312.34) solution (10 mM TPTZ in 40 mM HCl) and 1 mL of 20 mM FeCl<sub>3</sub> (molecular weight-270.30) solution (i.e., in the ratio 10:1:1 v/v). Fresh reagent was prepared immediate before analysis. Ten microliter of product supernatant was loaded to 96 wells microplate and mixed with 100 µL of FRAP reagent. The samples were mixed and then incubated at 37°C for 30 min. The increase in absorbance was measured at 593 nm against acetate buffer using Multiplate reader (Tecan-Infinite® 200 PRO, Switzerland). For reagent blank preparation 10 µL of distilled water was taken instead of sample and subtracted from the sample reading to calculate increase in absorbance for each sample. The results were expressed as ferrous sulphate equivalent values.

##### *Polyphenol content*

Determination of polyphenols was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). The sample (0.1 ml) was mixed with 0.9 ml of distilled water and was extracted for 2 h at room temperature on a mechanical shaker. To this, 1 ml of Folin-Ciocalteu reagent (1:2 dilution) and 2 ml of 10% Na<sub>2</sub>CO<sub>3</sub> was added. The mixture was centrifuged at 20,000×g for 20 min, and the supernatant was decanted and filtered through Whatman No. 1 filter paper. The absorbance of the clear supernatant solution was measured at 765 nm (Shimadzu 160 uv A). Gallic acid was used as a standard. Each sample was analyzed twice with duplicates. Results were expressed as milligram gallic acid equivalent per 100 g dry weight.

##### *Determination of proteolytic activity by OPA method*

Proteolytic activities of probiotic lactobacilli cultures grown in soy milk medium were assessed by measuring liberated amino acids and peptides using the o-phthaldialdehyde (OPA) method of Church *et al.*

(1983) with some modifications. Cultures (2.50 mL) were added to 5 mL of 0.75% trichloroacetic acid and the mixture was filtered using Whatman filter paper 42 (MFS. Inc., CA, USA). Permeate (150  $\mu$ L) was added to 3 mL of OPA reagent and the absorbance of the solution was measured spectrophotometrically at 340 nm after 2 min at room temperature (20°C). The proteolytic activity of these bacterial cultures was expressed as the free amino groups measured at 340 nm in samples containing culture.

### Preparation of serine standard

Serine was dissolved in double distilled water (100  $\mu$ g/ml). 25  $\mu$ l, 50  $\mu$ l, 75  $\mu$ l, 100  $\mu$ l, 125  $\mu$ l, 150  $\mu$ l, 175  $\mu$ l, 200  $\mu$ l, 225  $\mu$ l and 250  $\mu$ l of serine was added in clean test tubes and sterilized distilled water was added to make final volume 1 ml. 150  $\mu$ l of sample was taken from each dilution in separate test tubes. 3 ml of OPA reagent was added to each test tube and kept for two minutes. Absorbance was taken thereafter of each test tube at 340 nm with OPA as blank.

### Statistical analysis

The mean values and the standard deviations were calculated from the data obtained with triplicate trials. Analyses of variance using ANOVA were conducted. Differences between the sample means were analyzed by Duncan's Multiple Range tests at  $\alpha = 0.05$  by SPSS software.

## Results and Discussion

### Antioxidative capacity of fermented soymilk

Total antioxidative potential of six lactobacilli cultures were determined by three methods namely, ABTS, DPPH and FRAP method. In ABTS method of antioxidative determination (Table 1), inhibition had shown by unfermented soymilk and soymilk fortified with WPC70 @2.0 %. After 24 h incubation, the extent of inhibition had increased comparatively among these lactobacilli cultures within these three methods. The TEAC (Trolox Equivalent Antioxidative Capacity) of unfermented soymilk and fermented soymilk with WPC70 was found to be 71.65% inhibition and for NCDC19, NCDC24, C2, C6, NCDC17, NCDC297: 71.65, 91.97, 88.62, 89.09, 97.05, 88.05% inhibition respectively. Among them, *L. rhamnosus* C6 strain had provided highest antioxidant activity in ABTS method, DPPH method and FRAP method (89.09, 50.09 and 801.25% inhibition. However, NCDC19 and NCDC17 showed good amount of inhibition in ABTS method (91.97, 90.16% inhibition and 971.69 and 968.69 TEAC ( $\mu$ M) respectively). However, in this study *L.*

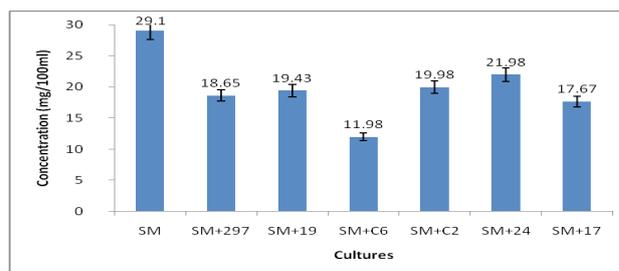


Figure 1. Polyphenol content in soy milk supplemented with WPC70 @2.0% during fermentation by probiotic lactobacilli

*rhamnosus* C6 strain was selected for its maximum antioxidant activity as well as polyphenol content reduction in soy milk medium supplemented with WPC70 @1.5% under the similar growth conditions (at 37°C for 24 h). The inhibition of ascorbate autoxidation observed with soymilk may be attributed to the action of isoflavones and tocopherols, the main phenols found in soybean (Persky and van Horn, 1995). On the other hand, liberation of aglycone genistein and diadzein through the catalytic action of beta-glucosidase during fermentation (Chien, 2004) and the intracellular antioxidants of starter organism (Lin and Yen, 1999) may account for the increased ascorbate-autoxidation inhibition found with the fermented soymilk. To prepare the probiotic fermented soy curd, we previously found that the bifidobacteria and lactic acid bacteria were beneficial to each other when grown simultaneously (Wang *et al.*, 2002). Soymilk fermented with of lactobacilli (*L. casei* and *L. rhamnosus*) resulted in a higher viable population of starter organisms and higher reduction of the oligosaccharide content in soymilk. It was also noted in the present study that soymilk supplemented with whey protein concentrate always enhanced higher antioxidant value, in general, exhibited a significantly higher inhibition rate of ascorbate autoxidation than soy milk without addition of whey protein concentrate. Whey protein concentrate supplementation in soy milk added extra nutrition to the microorganisms and also increased antioxidative activity shown by the lactobacilli cultures as well as reduced less whey separation in the soy curd after fermentation.

### Changes in polyphenol content

The changes in polyphenol content were shown in Figure 1. Polyphenols are present in considerable amount in soymilk. This content decreased from 29.10 mg/100 ml to 11.98 mg/ 100 ml during fermentation by six probiotic lactobacilli cultures, which were incubated for 24 h at 37°C. During SM+24 culture fermentation, not much reduction was observed (21.98 mg/100 ml). SM+C6 culture showed

Table 1. Antioxidative capacity of fermented soymilk

Samples	Total Antioxidative Capacity			
	ABTS		DPPH	FRAP
	% Inhibition <sup>1</sup>	TEAC ( $\mu\text{M}$ ) <sup>1</sup>	% reduction <sup>2</sup>	FRAP ( $\mu\text{M}$ ) <sup>3</sup>
Soymilk	71.65 $\pm$ 0.41 <sup>b</sup>	937.99 $\pm$ 1.30 <sup>a</sup>	No reduction <sup>c</sup>	0722.57 $\pm$ 0.067 <sup>d</sup>
Soymilk +NCDC19	91.97 $\pm$ 0.14 <sup>a</sup>	971.69 $\pm$ 0.46 <sup>a</sup>	45.89 $\pm$ 3.65 <sup>b</sup>	0782.64 $\pm$ 0.006 <sup>c</sup>
Soymilk +NCDC24	88.62 $\pm$ 0.54 <sup>b</sup>	897.98 $\pm$ 1.70 <sup>b</sup>	48.04 $\pm$ 0.44 <sup>ab</sup>	0719.79 $\pm$ 0.006 <sup>c</sup>
Soymilk+C2	89.09 $\pm$ 0.29 <sup>b</sup>	967.48 $\pm$ 0.95 <sup>a</sup>	49.96 $\pm$ 0.15 <sup>ab</sup>	0780.07 $\pm$ 0.003 <sup>b</sup>
Soymilk+C6	97.05 $\pm$ 1.04 <sup>a</sup>	975.47 $\pm$ 3.40 <sup>a</sup>	50.09 $\pm$ 0.18 <sup>ab</sup>	0801.25 $\pm$ 0.021 <sup>d</sup>
Soymilk +NCDC17	90.16 $\pm$ 0.14 <sup>a</sup>	968.69 $\pm$ 0.45 <sup>a</sup>	46.40 $\pm$ 0.33 <sup>a</sup>	0789.81 $\pm$ 0.001 <sup>c</sup>
Soymilk + NCDC297	88.05 $\pm$ 0.12 <sup>b</sup>	941.53 $\pm$ 0.40 <sup>a</sup>	42.72 $\pm$ 0.36 <sup>a</sup>	0759.33 $\pm$ 0.041 <sup>c</sup>

<sup>a-d</sup> same column bearing different superscripts differ significantly, the level of significance was preset at  $\alpha = 0.05$  and significant  $***P < 0.001$ ,  $\pm$  standard error of three replicates <sup>1</sup>Trolox Equivalent Antioxidative Capacity, studied at 750 nm, <sup>2</sup>Free radical-scavenging activity (FRSA) studied at 517 nm, <sup>3</sup>Ferric-reducing anti-oxidative power (FRAP) studied at 593 nm

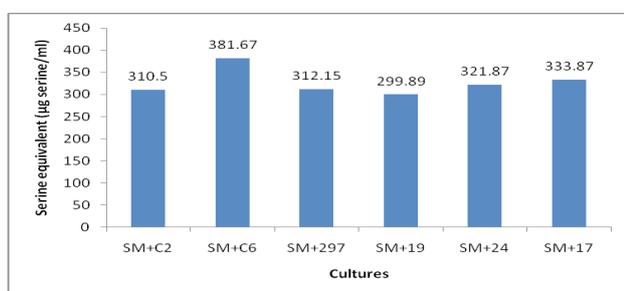


Figure 2. Proteolytic activity of probiotic lactobacilli in soy milk medium supplemented with WPC70 @2.0%

maximum reduction in polyphenol content (11.98 mg/100 ml), followed by SM+17 (17.67 mg/100 ml) and SM+297 (18.65 mg/100 ml). Similar observation was reported by Rekha and Vijayalakshmi (2008) in fermented soymilk with different Lactobacilli. These antinutrients interfere with mineral bioavailability and digestibility of proteins (Serraino *et al.*, 1985) and carbohydrates. Reduction in polyphenol content through fermentation may imply improved digestibility of proteins and carbohydrates and also enhance bioavailability of minerals in the fermented product, thereby improving the nutritive value. Besides nutritional benefits, probiotic lactobacilli have a role in improving metabolism, reducing constipation, lowering cholesterol levels in the blood, and increasing phenol tolerance (Sindhu and Khetarpaul, 2003). Fermentation with different probiotic lactobacilli resulted in reduction of polyphenols. The diminishing effect of fermentation on polyphenols may be due to the activity of polyphenol oxidase present in the food grain or microflora. This reduction in polyphenolic content by fermentation results in less astringency.

### Protein hydrolysis

The proteolytic activity of soymilk fermentation is shown in Figure 2. Probiotic lactobacilli organisms are rich in proteolytic activity. The rate of protein hydrolysis ranged from 381.67 to 299.89  $\mu\text{g serine/ml}$  with different strains of probiotic lactobacilli grown in soy milk supplemented with WPC70 @1.5%. The highest was seen in SM+C6 (381.67  $\mu\text{g serine/ml}$ ) followed by SM+17 (333.87  $\mu\text{g serine/ml}$ ), SM+24 (321.87), and SM+297 (312.15  $\mu\text{g serine/ml}$ ); the least was in SM+19 (299.89  $\mu\text{g serine/ml}$ ). The addition of probiotic organisms to soymilk results in increased free amino acid content. The degree of protein hydrolysis is expressed as content of  $\mu\text{g serine equivalent}$  in soymilk after 24 h of fermentation at 37°C in soy milk medium. Similar observation was reported by Rekha and Vijayalakshmi (2008) in soy fermentation with different lactobacilli and yeast culture increased free amino acid content after 24 h incubation.

### Conclusions

Soybean is a rich source of isoflavone, which are reported to have beneficial estrogenic effects with potential bioactive antioxidant properties. Probiotic lactic acid bacteria when grown in soy milk with or without added whey protein concentrate have the ability to utilize phenolic components producing phytase enzyme and reduce the polyphenol content after fermentation. Due to production of  $\beta$ -glucosidase activity by Probiotic lactobacilli, isoflavone aglycones increase the antioxidative activity in soy milk during fermentation. Practically, Lactobacilli cultures can be used for the development

of functional soy based food products with different health benefits. Based on the findings obtained from the present study, we concluded that an enhanced inhibition effect on ascorbate autoxidation and a reducing effect on polyphenol content in soy milk can be obtained through fermentation with probiotic lactobacilli cultures. However, the extent of increased antioxidative activities varied with the starter organism employed. In addition, six lactobacilli also showed highest proteolytic activity and reduced polyphenol content by producing phytase enzyme. Finally, the increased antioxidative activity and reduced polyphenol content of soymilk after fermentation with probiotic lactobacilli that was observed further increases the potential of developing a probiotic diet adjunct with fermented soymilk having these health beneficial attributes.

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