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Functional and technological application of probiotic *L. casei* PLA5 in fermented soymilk

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Abstract

This study was designed with an objective to develop a probiotic dietary adjunct using soymilk (Harit soya variety). Soymilk is fermented with probiotic strain *Lactobacillus casei* PLA5 (Accession no KJ726650) which have been isolated from Churpe -a traditional fermented milk product. During various fermentation time, changes in pH, titratable acidity and viable count were evaluated and were compared with the soymilk fermented with commercial probiotic *L. casei* strain Shirota. Changes in β-glucosidase, β-galactosidase and proteolytic activity during the fermentation of probiotic soymilk were examined. In addition, antioxidative activities including DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity, total flavonoids and polyphenols determination were also investigated. Growth of probiotic *L. casei* PLA5 in soymilk via supplementation with prebiotics was also studied and shelf life of probiotic soymilk was periodically checked for 14 days. Log CFU/ml of soymilk fermented with *L. casei* PLA5 was found to be significantly higher than soymilk fermented with *L. casei* strain Shirota. β-glucosidase (3.40 U/mg) activity was found to be higher than β-galactosidase (0.14U/mg) activity in soymilk during fermentation. Antioxidative activity increased during fermentation; however, H_2O_2-scavenging effect decreased. Polyphenols content declined from 14.01 to 6.01 mg/100 ml whereas, flavonoid content varied between 1.46-3.52 mg/ml during fermentation. Increase in bioavailability of minerals was also observed in fermented soymilk. A significant enhanced growth ($p<0.05$) of *L. casei* PLA5 was observed with maltodextrin and fructooligosaccharide by 1.24 and 1.09 log CFU/ml respectively. Bacterial counts remained constant and above 10^7 CFU/ml during the storage period of 14 days at refrigeration temperature. These results suggest that soymilk prepared from probiotic *L. casei* PLA5 strain had high antioxidant capacity and promising enzymatic potential and could be commercialized as probiotic beverage.

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Introduction

Soymilk, a traditional inexpensive oriental food drink provides a plentiful supply of protein and energy (Bressani and Elias, 1968). It is considered as a suitable economical substitute for cow's milk and an ideal nutritional supplement for lactose-intolerant population (Dhananjay *et al*., 2006). Soy-based foods are usually consumed by the Asian population and are promising supplements to overcome protein calorie malnutrition problems. Due to its undesirable beany flavor and some digestive problems associated with the presence of non-digestible oligosaccharides it is not a popular drink (Thananunkul *et al.,* 1976). Fermentation by lactic acid bacteria (LAB) overcomes the problem of beany flavor and increases the acceptability of soymilk by reduction of oligosaccharides (Otieno and Shah, 2006). The use of Lactic acid bacteria (LAB) in preparing fermented soy products has received much attention due to its positive health effects like improving bioavailability of isoflavones, digestion of proteins, provides more soluble calcium, enhances intestinal health, improves antioxidant activity and supports immune system (Wang *et al*., 2003). A health benefit can also arise from the ability of an ingested microorganism to contribute an enzyme to the small intestine e.g. β-galactosidase (lactase) that many adults lack (Kumari, Angmo and Bhalla, 2016).

Prebiotics are defined as 'non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and activity of colonic microbiota (Manning and Gibson, 2004). Therefore, incorporation of probiotic Lactic acid bacteria along with the prebiotics into food products to increase the therapeutic and nutritional value has become a popular trend. Fructooligosaccharides (FOS), Maltodextrin, Mannitol and Inulin have

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been considered as a commonly used prebiotics. Soybean is an inexpensive vegetarian food which is cholesterol and lactose free source of protein for human consumption (Scalabrini *et al.*, 1998). It is traditionally grown as a food crop which is oilseed crop of great promise. Besides its use as a source of oil, it is used to prepare high protein milk, curd, cheese and many other useful foodstuffs. Soymilk has a high nutritional value and after addition of probiotic bacteria to the soymilk it exert beneficial and therapeutic health effects including the activation of the immune system, reduction of serum cholesterol, and inhibition of the growth of enteric pathogens in the host (Holzapfel *et al.,* 2001; Ishibashi and Yamazaki, 2001). Various research has showed that consumption of soy-derived food has potential health benefits related to cardiovascular diseases, menopausal symptoms, osteoporosis, breast and prostate cancers because they are rich sources of bioactive phenolic compounds (Devi *et al.,* 2009). It has been demonstrated that the fermented soyfoods/ soymilk has remarkably higher antioxidative activity and lower polyphenol content than its unfermented counterpart (Otieno *et al.*, 2007; Subrota *et al.*, 2013).

Therefore, keeping in view of nutritional benefits of soymilk present study was focused on the preparation of probiotic soymilk from variety Harit soya (P-4-2). Microbiological analysis, mineral bioavailability, enzymatic characterization (β-galactosidase, β-glucosidase and proteolytic activity) and anti-oxidative activities (hydrogen peroxide and DPPH scavenging activity, polyphenol and flavonoid content) during the fermentation of probiotic soymilk were assessed and being reported in this research communication. Additionally, the effect of various prebiotics such as maltodextrin, FOS, inulin, mannitol on the viability of probiotic bacteria in fermented soymilk was evaluated.

Materials and Methods

Strain and culture conditions

Pure culture of probiotic *Lactobacillus casei* PLA5 strain was isolated from the Churpe (dried cottage cheese) of north western Himalayas and its probiotic characteristics have been extensively studied (Kumari *et al.*, 2016). Reference Probiotic Strain *Lactobacillus casei* Shirota was obtained from the Department of Food and Environmental Sciences, Viikki Biocentre, University of Helsinki, Finland. The strains were maintained in de Man, Rogosa, and Sharpe (MRS) Elliker broth (Hi-Media) and stored at -80°C in sterile containing 40% glycerol. Working cultures were cultivated in MRS slant agar at 30°C for 24 hr.

Preparation of fermented probiotic soymilk

Soybean *(Glycine max)* variety Harit soya P-4-2 developed by Y.S Parmar University Nauni, Solan, India was purchased from the local market. Preparation and fermentation of soymilk was performed according to the procedure described by Wang *et al.* (2002). 100 ml of autoclaved soymilk was placed in a 250-ml screw-cap Erlenmeyer flask and inoculated with probiotic *L. casei* strain PLA5. The initial population of lactic acid bacteria was 4log CFU/ml in soymilk. Soymilk containing LAB was incubated at 30°C till the maximum population of lactic acid bacteria could be obtained. The fermented soymilk was stored at 4°C for 10 days. During the storage period, the pH and viable counts of lactic acid bacteria in the fermented soymilk were determined.

Microbiological and chemical analysis of probiotic product

Viability of L. casei

The growth of lactobacilli cultures was determined by the pour plate method using MRS agar (Hi Media) and incubated at 30°C for 48hr. One millilitre of appropriate serial dilution of each sample was pour-plated onto the appropriate MRS medium. After 48 hr of incubation at 30°C, the colonies that appeared on the plates were counted and the CFU/ml was calculated.

pH and Titratable acidity

Changes in pH were monitored during fermentation of soymilk at 0, 12, 24, 36, and 48hr using a pH meter. Titratable acidity was determined using the method of AOAC (1984) by titration with 0.01N NaOH solution and expressed as percent lactic acid.

$$\text{Titrable acidity (\% Lactic Acid)} = \frac{\text{Volume of NaOH x N of NaOH x Mol wt of acid}}{\text{Volume of sample x 100}}$$

Milli-equivalent weight of acid: lactic acid = 0.0090

Moisture (or total solids) content

Fermented soymilk (5 ml) was weighed in moisture dishes and these were transferred to the oven maintained at 100±1°C and dried for 2-3 hr. Dishes with dried samples were cooled, weighed and expressed as % moisture in the sample.

$$\% \text{ Moisture} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

W_1 (g) =Weight of empty moisture dish; W_2 (g) = Weight of dish + sample; W_3 (g) =Weight of dish + dried sample

Determination of ash content

Well mixed fermented soymilk (5 ml) was added into crucibles and weighed. Samples were first incinerated on slow flame of burner followed by their ignition in a muffle furnace (550±20°C) until light grey ash is obtained (about 4 to 6hr). Dishes were cooled and weighed and the results were expressed as % ash in sample.

$$\% \text{ ash} = (W_{ash}/W_{wet} \text{ sample}) \times 100$$

W- Weight in grams (g)

Total carbohydrate (Dubios et al., 1956)

Soymilk (100 µl) was blended with 5 ml of distilled water. To 1ml of properly diluted sample, 1 ml of phenol (5%) and 5 ml of H_2SO_4 (96%) was added. After 10 mins, the content of tube was vortexed and placed in water bath at 30°C for 20 mins. Absorbance was read at 490 nm.

Mineral content

Mineral content in food sample was analyzed using wet ashing method (Dennis *et al.*, 1981). Weighed dried food sample in a flask containing strong acids and oxidizing agents (e.g., nitric, perchloric and/or sulfuric acids) and then heated for 10-20 minutes at 150°C till organic matter is completely digested and leaving the mineral oxides in solution. The resulting solution is then analyzed by atomic absorption spectroscopy (AAS) for corresponding elements i.e. Zn, Ca, Fe and Mg.

Enzyme activities of L. casei *in fermented soymilk*

Proteolytic activity

Proteolysis during fermentation of soymilk was determined by measuring free NH_3 following the o-phthaldialdehyde (OPA) method (Donkor *et al.*, 2005). The proteolytic activity of *L. casei* PLA5 was expressed as the absorbance of free amino group, measured using the untreated soymilk as a blank.

β-Galactosidase activity

β-Galactosidase activity was determined according to the method of Bhowmik and Marth (1989). Cells were harvested by centrifugation at 4000 X g for 10 mins at 4°C at different fermentation time. Specific activity was then expressed as the amount of o-nitrophenyl released per mg of protein.

β-Glucosidase activity

Fifty milliliters of aliquot was withdrawn aseptically from each sample at 0, 12, 24, 36 and 48 hr of incubation and β-glucosidase activity was determined using modified method of Matsuda *et al.* (1994) by measuring the rate of hydrolysis of 1mM of p nitrophenyl- β-D glucopyranoside (p-NPG). One unit of enzyme activity was defined as the amount of enzyme that released 1µmol of p-nitrophenol from the substrate per min.

Determination of antioxidative activity

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured by the method of Pick and Keisari (1980) with minor modification. One ml of sample or distilled water (control) was first mixed with 1ml of 5mM H_2O_2 solution and incubated at room temperature for 20 mins. Reaction mixture was then supplemented with 2 ml of horseradish peroxidase phenol red solution (100 mM phosphate buffer containing 300 mg/ml HRPase and 4.5mM phenol red). After incubation at 30°C for 10 mins, the absorbance was recorded at 610nm. The scavenging effect was then calculated using the following equation:

$$\text{Scavenging effect \%} = 1 - \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

DPPH free radical scavenging activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity was assessed according to Moon and Terao (1998). To 1.0ml DPPH (500µM in ethanol), 200 µl of sample was added, and the reaction mixture was made to 2.0ml with Tris-HCl buffer (100 mM, pH 7.4). The mixture was shaken vigorously and incubated at room temperature for 30 mins. The absorbance of the resulting solution was measured at 517 nm. Reaction mixture without DPPH was used as a control.

$$\text{Scavenging effect \%} = 1 - \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Determination of total flavonoids

The total flavonoids of the soybean extracts were assayed according to Yang *et al.* (2012). Soybean extract (0.25 ml) was mixed with 1.25 ml of distilled water and 75 µl of 5% sodium nitrite. After 6 mins, 150 µl of 10% aluminum chloride was added and kept for 5 mins at room temperature prior to mixing of 0.5 ml of 1M sodium hydroxide and 775 µl of distilled water. The absorbance of the reaction mixture was

determined at 510 nm. Quercetin standard in range of 5-50 µg/ml was made.

Determination of polyphenols

Polyphenols were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). The sample (0.1 ml) was mixed with 0.9 ml of distilled water and extracted for 2 hr at room temperature on a mechanical shaker. To this, 1 ml of Folin-Ciocalteu reagent and 2 ml of 10% Na_2CO_3 was added. The mixture was centrifuged at 20,000rpm for 20 mins, and the supernatant was filtered. The absorbance of the clear supernatant solution was measured at 765 nm. Results were expressed as milligram tannic acid equivalent per 100 g dry weight.

Effect of prebiotics on viability of L. casei

Autoclaved soymilk was supplemented with 10g/L of individual prebiotics including Mannitol, FOS, Inulin and Maltodextrin. Bacterial culture was washed twice with phosphate-buffered saline (pH 7.4) prior to adjustment of the cell density (optical density of 1.0 at 600 nm) in the same buffer. After centrifugation at 12,000 rpm for 10 mins at 4°C, the cell pellet was inoculated into 50ml of soymilk with the initial population of *L. casei* to 4 CFU/ml and fermented at 30°C for 24 hr.

Viability of LAB during storage at 4°C

The final product was stored for 14 days at 4°C. During storage of the fermented products the changes in pH and viable cells count of *L. casei* PLA5 were periodically observed and viability was compared with soymilk prepared from commercial probiotic strain *L. casei* strain Shirota.

Results

Changes in Viability, pH and titratable acidity (TA) during fermentation

Changes in viable counts, pH and TA during the fermentation of soymilk inoculated with *L. casei* PLA5 and *L. casei* Shirota are summarized in Table 1. Viability of fermented soymilk increased significantly ($p< 0.05$) with a range of viable counts from 4.21 to 8.01log CFU/ml in *L. casei* PLA5 and 4.13 to 7.83log CFU/ml in reference probiotic strain *L. casei* strain Shirota at 24hr of incubation at 30°C as shown in Table 1. Furthermore, a significant increase in viability ($p< 0.05$) were seen in both the isolates i.e. 9.42 and 9.19log CFU/ml in *L. casei* PLA5 and *L. casei* Shirota, respectively at the end of 48hr of incubation. An appreciable decrease in pH and increase in TA were noted in soymilk inoculated with single culture of *L. casei* PLA5 and *L. casei* Shirota at 48 hr of fermentation. At the end of 48hr of fermentation, the pH declined from 6.79 to 4.03, while TA increased from 0.01% to 0.09% in sample fermented with *L. casei* PLA5. In case of soymilk fermented with *L. casei* Shirota, a decrease in pH (6.80 to 4.64) and an increase in TA (0.01% to 0.07%) was recorded.

Moisture and ash content

The ash content of fermented soymilk ranged between 0.31 and 0.42% which is a reflection of the mineral compositions of the soymilk samples. Although it was an indication that all the samples analyzed had micronutrients. However, it was observed that the ash content value of 0.42% at 48hr of fermentation was highest.

No significant difference in moisture content was observed among the sample at different incubation time which ranged between 86.12% - 88.5% (Table 2). The moisture content of soymilk was the highest (88.5%) at 0hr of fermentation it declined during fermentation to 86.12% at 48 hr. The total carbohydrates in fermented soymilk during fermentation were assayed at an interval of 12 hr till 48hr of fermentation and it decreased from 5.5 g/100 ml to 2.57g/100ml (w/v) at 48 hr of fermentation (Table 2).

Enzyme activities of L. casei *PLA5 in fermented soymilk*

Although there was increase in amount of liberated amino groups and peptides during the fermentation from 0-12 hr; yet the significant ($p<0.05$) increase was observed from 12-48 hr. Highest proteolytic activity was observed at 48 hr and after that it started decreasing (Table 2). The highest activity of β-galactosidase (0.14U/mg) was observed at 24 hr of fermentation. An abrupt decrease in β-galactosidase activity was observed after 24 hr of fermentation. The profile of β-glucosidase activity in fermented soymilk at 30°C for 48hr is depicted in Table 2. Highest β-glucosidase activity (3.40U/mg) was recorded after 24 hr of fermentation. An increase in β-glucosidase activity up to 24 hr was observed but it declined with the progress of fermentation.

Determination of antioxidative activity

Hydrogen peroxide (H_2O_2) and DPPH free radical scavenging effect

Soymilk (control) exhibited a significantly high H_2O_2-scavenging effect (%) of 13.89 in comparison to fermented soymilk (Table 4). The H_2O_2 scavenging

Table 1. Viable counts, pH and titratable acidity of soymilk during fermentation (0-48 h) with *L. casei* PLA5 and *L. casei* strain Shirota

	L. casei PLA5			*L. casei* Shirota		
Fermentation time (h)	Log CFU/ml	pH	Titrable acidity	Log CFU/ml	pH	Titrable acidity
0	4.21±0.2	6.79±0.5	0.01±0.001	4.13±0.2	6.8±0.4	0.01±0.001
12	6.13±0.5	5.33±0.2	0.06±0.002	5.45±0.25	5.91±0.45	0.03±0.002
24	8.01±0.5	4.36±0.2	0.06±0.002	7.83±0.45	5.05±0.5	0.04±0.001
36	9.13±0.45	4.17±0.3	0.08±0.005	8.92±0.5	4.97±0.25	0.06±0.001
48	9.42±0.5	4.13±0.3	0.09±0.002	9.19±0.5	4.64±0.25	0.07±0.002

The results are expressed as the mean of triplicate samples from three independent experiments ±SD

Table 2. Moisture, ash, carbohydrate content and enzymatic activities of *L. casei* PLA5 in soymilk at different fermentation time

Fermentation time (h)	0 h	12 h	24 h	36 h	48 h
Moisture content (%)	88.5±0.5	87.5±0.45	86.33±0.35	86.2±0.25	86.12±0.4
Ash content (%)	0.31±0.02	0.34±0.02	0.36±0.01	0.4±0.02	0.42±0.12
Carbohydrate content (g/100 ml)	5.5±0.05	5.05±0.04	3.29±0.02	3.15±0.01	2.57±0.12
Proteolytic activity (U/mg protein)	0.02±0.01	0.15±0.01	0.24±0.02	0.26±0.02	0.33±0.03
β-galactosidase (U/mg protein)	ND	0.10±0.001	0.14±0.001	0.05±0.002	0.02±0.001
β-glucosidase (U/mg protein)	ND	1.18±0.01	3.39±0.02	2.13±0.05	1.23±0.01

The results are expressed as the mean of triplicate samples from three independent experiments ±SD

effect at different fermentation time (12-48 hr) was found to be in the range of 12.78 ± 0.41 to 1.96 ± 0.35 (%), and their chelating capacity on ferrous ions decreased with the increase in fermentation time. The highest scavenging effect of 12.78% was recorded at 12 hr of fermentation. The DPPH values of fermented soymilk at different time interval increased from 13.91 to 30.61% (v/v) at 24 to 36 hr of fermentation. DPPH activity was significantly ($p \leq 0.05$) affected by the fermentation time (Table 4). At 24 to 36hr of fermentation, the product had shown reduced DPPH activity in comparison to soymilk (control).

Polyphenol and flavonoid content

The changes in polyphenol content are shown in Table 4. Polyphenols are present in considerable amount in soymilk. This content decreased from 14.01 mg/100ml to 6.01 mg/100ml at 48hr of fermentation. A reduction of 57% in polyphenol content was observed during the 48 hr of fermentation.

The content of flavonoids in fermented soymilk varied between 1.46 and 3.52 mg/ml during first 24 hr of fermentation (Table 4). Higher flavonoid content (60%) was achieved in fermentation time of 24 hr than that of soymilk (control). However, change in flavonoids content was not significantly different ($p>0.05$) in soymilk fermented for 24 hr to 36 hr and after that it decreased to 2.05 mg/ml at 48 hr of fermentation.

Minerals content

Mineral bioavailability of soymilk (unfermented) and soymilk fermented with *L. casei* PLA5 are shown in Table 3. Increase in level of calcium content (4.89 to 20.32 mg/100g) and magnesium (220.03 to 245.48 mg/100g) was found in soymilk fermented with *L. casei* PLA5 as compared to soymilk (control). Not much significant difference in the zinc levels was observed in soymilk fermented with *L. casei* PLA5. In contrast, iron content decreased from 5.95 to 4.30 mg/100g in fermented soymilk ranging.

Effect of prebiotics on viability of L. casei

L. casei PLA5 grew well in soymilk supplemented with prebiotics, with viable counts ranging from 7.89 to 9.25 log CFU/ml. Prebiotic supplementation affected growth significantly ($p<0.05$); the effect was most prevalent in fermented soymilk with maltodextrin and FOS supplementation which enhanced the growth of *L. casei* PLA5 by 1.24 and 1.09 log CFU, respectively as compared to control ($p<0.05$) after 24 hr as shown in Table 5. Supplementation with mannitol also increased the growth of *L. casei* PLA5 after 24 hr of fermentation (1.04 log CFU). It was found that inulin has least effect in the growth of *L. casei* PLA5

Table 3. Mineral content (mg/100g) of soymilk and fermented soymilk at 24 h of fermentation

Minerals	Soymilk (control) (mg/100 g)	Fermented soymilk (mg/100 g)
Calcium (Ca)	4.89±0.5	20.32±0.45
Iron (Fe)	5.95±0.2	4.30±0.15
Magnesium(Mg)	220.03±15.8	245.48±15.2
Zinc (Zn)	33.42±1.5	40.06±1.3

The results are expressed as the mean of triplicate samples from three independent experiments ±SD

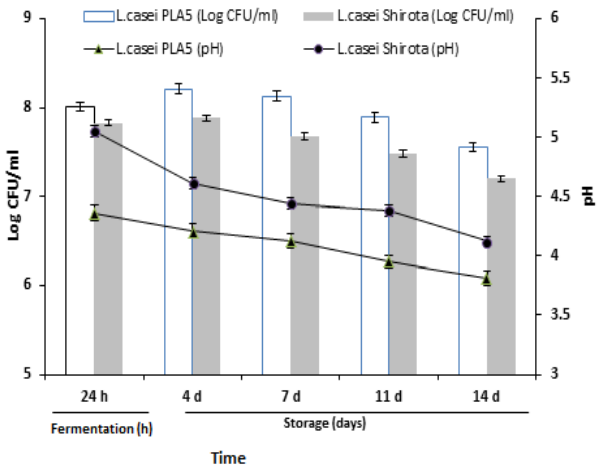

Figure 1. pH and viable count of *L. casei* PLA5 and reference strain *L. casei* Shirota during soymilk fermentation (24 h at 30 °C) and cold storage (14 days at 4 °C). Error bars are standard deviations with respect to the mean values of triplicate analyses.

(0.22 log CFU/ml) as compared to control. The pH of fermented soymilk supplemented with maltodextrin was decreased from 6.75 to 4.23, while pH dropped to 4.32, 4.38 and 4.89 in soymilk supplemented with FOS, mannitol and inulin respectively at the end of 24hr of fermentation (Table 5). The highest increase in TA of soymilk from 0.01% to 0.12% was observed after 24hr of fermentation with maltodextrin. However, marginal increase was observed with other prebiotics i.e. FOS (0.09%), mannitol (0.08%) and inulin (0.08%).

Viability of LAB during storage

L. casei PLA5 and *L. casei* strain Shirota revealed 0.51log CFU/ml and 0.63log CFU/ml reduction in viability during the 14d of storage (Figure 1). pH of fermented soymilk continued to decrease and reached 3.81 (*L. casei* PLA5) and 4.12 (*L. casei* strain Shirota) on 14d of storage.

Discussion

Lactic acid bacterial count should be viable and abundant (>10^6 CFU/ml) in the probiotic product at the time of consumption to exert a beneficial health benefits (Angmo *et al*., 2016). Soymilk was considered to be a better substrate for the growth of the probiotic LAB strains as probiotic strains grow more quickly in it than in cow's milk (Li *et al*., 2012). It has been demonstrated that probiotic organisms are capable of utilizing sucrose, a major disaccharide found in soymilk (Wang *et al*., 2003).

Coagulation of sterilized soymilk occurs at pH 5.7 (Chou and Hou, 2000) and optimum pH of fermented soymilk was reported at 4.2 to 4.3 (Oberman, 1985). In general, a decrease in pH was followed by increase in TA which corresponds to acidity of a sample. Significant increases in TA (0.01-0.09%) and decreases in pH (6.79-4.13) in soymilk during fermentation was observed which were similar to the work reported by Rekha and Vijayalakshmi (2008) and Wang *et al.* (2000). This is due to the production of acids (mainly lactic acids) during the fermentation.

The ash content increased during fermentation may be due to the bioavailability of micronutrients (Rekha and Vijayalakshmi, 2008). Fermentation resulted in higher amount of mineral content in the Ca, Mg and Zn. An increase in mineral bioavailability after fermentation might be due to the presence of phytase enzyme produced by *L. casei* PLA5 which breaks mineral-phytate complex during fermentation. However, the probiotic fermented soymilk could therefore be referred to as good sources of calcium, magnesium and zinc. This observed increase in mineral composition and ash content may be due to the contribution from fermentation microorganisms (Oyeleke *et al.*, 2012). High moisture could affect the stability and safety of food with respect to microbial growth and proliferation hence the products will require cold storage (Ladokun and Oni, 2014).

An increase in proteolytic activity was observed during fermentation which was found to be similar to the pattern observed by Donkor *et al*. (2007) and Rekha and Vijayalakshmi (2008). Some amino acids and peptides were used by the organisms during fermentation for cell growth and survival (Nielsen *et al*., 2001). Therefore high proteolytic activity of these organisms in soymilk may have contributed to appreciable cell growth, in addition to their ability to metabolize disaccharides present in soymilk as energy sources.

The two endogenous enzymes (β-glucosidase and β-galactosidase) were responsible for the hydrolysis of β-1-6-glycosidic bonds in the conjugated isoflavone glycoside thus releasing the bioactive isoflavone aglycone in soymilk (Otieno *et al*., 2007). β-galactosidase activity (0.03-0.14 U/mg dcw) was

Table 4. Antioxidative activities of *L. casei* PLA5 in probiotic soymilk at different fermentation time

Fermentation time (h)	0 h	12 h	24 h	36 h	48 h
Polyphenol (mg/100ml)	14.01±0.2	12.34±0.25	8.03±0.3	6.13±0.25	6.01±0.15
Flavonoid (mg/ml)	1.46±0.05	1.53±0.04	3.52±0.07	3.24±0.04	2.05±0.06
DPPH activity (%)	3.65±0.5	13.91±0.41	43.65±0.51	30.61±0.45	2.43±0.35
H_2O_2 scavenging activity (%)	13.89±0.45	12.78±0.35	5.00±0.25	2.00±0.15	1.96±0.05

The results are expressed as the mean of triplicate samples from three independent experiments ±SD

Table 5. Effect in viable count, pH and titrable acidity of *L. casei* PLA5 in fermented soymilk supplemented with prebiotics

	Log CFU/ml			pH			Titrable Acidity (%)		
	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h
FOS	4.91±0.1	7.89±0.2	10.1±0.1	6.71±0.2	4.51±0.2	4.32±0.2	0.01±0.12	0.08±0.15	0.09±0.25
Mannitol	4.19±0.3	7.72±0.5	9.05±0.1	6.74±0.3	4.57±0.5	4.38±0.3	0.01±0.21	0.07±0.12	0.08±0.15
Maltodextrin	5.15±0.2	8.2±0.4	10.25±0.2	6.75±0.5	4.27±0.5	4.23±0.5	0.01±0.25	0.07±0.25	0.01±0.15
Inulin	4.12±0.2	7.41±0.2	8.23±0.2	6.8±0.2	5.25±0.2	4.89±0.3	ND	0.01±0.15	0.01±0.12

The results are expressed as the mean of triplicate samples from three independent experiments ±SD

found to be much lower than β-glucosidase activity (1.19 to 3.40 U/mg dcw) in the soymilk during fermentation. Possible reason behind the lower β-galactosidase activity could be related to the low concentration of β-D-galactopyranosides (such as lactose) found in the soymilk as compared to β-D-glycopyranoside substrate which is present in higher amount (Otieno *et al.,* 2006). Decrease in H_2O_2 scavenging effect of soymilk during fermentation may be attributed to the formation of H_2O_2 by the LAB (Teraguchi and Ono, 1987). Since LAB is devoid of catalase, a key enzyme for the breakdown of H_2O_2, thus it has to depend on NADH oxidase and NADH peroxidase to scavenge environmental oxygen (Amanatidou *et al*., 2001). Increase in DPPH radical scavenging activity with fermentation suggested that each fermentation extract might react as free radical scavengers by contributing hydrogen from their phenolic hydroxyl groups thereby forming stable free radicals that do not initiate or propagate further oxidation of lipids (Caillet *et al.*, 2006).

Probiotic LAB when grown in soymilk has the ability to utilize phenolic components by phytase enzyme and polyphenol oxidase present in the food grain or microflora and reduce the polyphenol content after fermentation. Polyphenol content decreased from 14.01 mg/100ml to 6.01 mg/100ml during fermentation period of 48hr. Similar observation was reported by Rekha and Vijayalakshmi (2008); Subrota *et al.* (2013). Soybean has been found to contain high contents of isoflavones and flavonoids which possess biological and antioxidative activity (Correa *et al*., 2010). Increase in flavonoid content (1.46-3.52 mg/ml) was observed in 24 hr of fermentation which was in accordance with the observations of Juan and Chou (2010). This may be due to the action of β-glucosidase produced by LAB during fermentation which catalyses the release of total phenolics and flavonoids from the soybean substrate leading to an increase in the content of flavonoid compounds (Dajanta *et al.,* 2013).

Maximum growth of *L. casei* PLA5 was observed in FOS and maltodextrin which was similar to the work done by Yeo and Liong (2010). Supplementation with maltodextrin increased the growth of *L. casei* PLA5. Enhanced growth of *L. casei* PLA5 in the presence of maltodextrin was probably due to its ability to produce various glycosyl hydrolases that hydrolyses maltodextrin to glucose for growth (Liong and Shah, 2006). To maintain beneficiary effect of probiotic products, it is important to demonstrate viability of bacteria in the products throughout the shelf-life of the products. Final population of the probiotic organisms in fermented product at the end of the shelf life, should be anywhere between 5-8 log CFU/ml to maintain its beneficiary effect (Svensson, 1999). Stable LAB counts and pH was observed during the 14 days storage at refrigeration temperature.

Conclusion

Soymilk has been widely accepted as a probiotic carrier. Based on the findings obtained from the present study, we conclude that the fermented soymilk has improved chemical composition and mineral profile than its unfermented counterpart. It was demonstrated that fermented soymilk had a higher β-glucosidase, β-galactosidase and proteolytic activity. Therefore, a probiotic soymilk drink with increased antioxidative, calcium bioavailability and probiotic properties can be prepared by fermentation with *L. casei* PLA5 at 24 hr. Future studies should be focused on preparation and characterization of synbiotic soymilk product and optimizing the sensory characteristics of product. Specific beany flavor of soymilk can be masked by the addition of sugars, aromas, and fresh fruit paste and flavors leading to more acceptable probiotic and nutritionally improved product.

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Declaration of interest

The authors declare that there is no conflict of interest.

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