**Evaluation of Total Antioxidant Activity of Soy Yoghurt**

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Soymilk is a well known protein enriched biofunctional food, but its acceptability was reduced due to the presence of complex sugars which gives soymilk beany flavor. However, fermentation had proven earlier reduction of such off-flavor in soymilk. Thus, soymilk was supplemented with 4% skim milk powder, 1.5% inulin and 10% strawberry pulp and fermented with combination of yoghurt culture NCDC-262 and L. acidophilus NCDC-195 (1:2) at 42°C for 6h. The yoghurt thus formed was evaluated for its antioxidative potential by ABTS, DPPH and FRAP method and was found to show 92% ABTS inhibition, 983.15 µM of TEAC, 54% DPPH reduction and 1364.25 µM FRAP reduction. Thus, soy based probiotic yoghurt can be nutritionally beneficial nutraceutical with persuasive antioxidative potential.

Keywords: Soymilk, Strawberry, Probiotic, Antioxidant, Isoflavones

INTRODUCTION

Free radicals generated by exogenous chemicals or endogenous metabolic processes in food systems may cause oxidative damage by oxidizing biomolecules and result in cell death and tissue damage (Kehrre 1993). Oxidative damage plays a significant pathological role in human diseases like cancer, emphysema, cirrhosis, atherosclerosis, and arthritis (Halliwell and Gutteridge 1984). Almost all organisms are well protected against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol and glutathione (Niki et al. 1994). When the mechanism of antioxidant protection becomes unbalanced by factors such as aging, the deterioration of physiological functions may occur, which result in diseases and accelerating aging (Yang et al., 2000). Therefore, some traditional foods have aroused a great deal of attention mainly due to their various functionalities such as scavenging free radicals, anti-diabetic, antihypertensive and antithrombotic properties. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have limited use in foods, as they are suspected to be carcinogenic (Namiki 1990). Soymilk is a potent antioxidative food due to the presence of phenolic components and isoflavones but its consumption has been limited due to the presence of "beany flavor" which gives off-flavor to it. Fermentation tends to enhance antioxidant activity of the food by releasing by-products (Yang et al., 2000). The beneficial effect of soymilk by-products can be attributed to various phenolic compounds present in them (Amin and Mukhrizah 2006). Phenolic compounds are plant-derived antioxidants that possess metal-chelating capabilities and radical-scavenging properties (Lopes et al., 1999). Soybean and soybean products, containing various amounts of phenolic compounds have been shown to possess antioxidative ability. Generally, the traditional soybean foods are usually fermented by Bacillus, Aspergillus, Rhizopus and Mucor strains respectively or mixed culture fermentation. Isoflavones have been found to increase the activities of some antioxidative enzymes in the liver (Wei et al. 1993). It was reported that the liberation of lipophilic aglycones of isoflavone glucosides such as daidzein and genistein by the catalytic action of β-glucosidase during fermentation increased the antioxidative activity of miso and tempeh, a significant increase in the formation of a water-soluble antioxidative fraction, not the free aglycone, lead to the enhanced antioxidative activity of natto. Furthermore, the use of daidzein and genistein

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2012-033 Received:April 2012; Accepted:May 2012
during the fermentation of Japanese soybean has yielded o-hydroxyisoflavones, potent antioxidants. Tocopherols (vitamin E) are lipid soluble molecules that are also present in significant amounts in soybean seeds. Soy intake aids against oxidative stress, as indicated on the basis of measurements of conjugated dienes in the LDL fraction (Jenkins et al., 2002) and (Jenkins et al., 2000) lag time for copper-induced LDL oxidation (Ashton et al., 2000) and plasma concentrations of F2-isoprostanes. In the past few years, a great interest has been developed to study the health functional properties of traditionally fermented soybean foods such as miso, natto, tempeh, sufu, and douche as they possess many advantageous properties including free radical scavenging ability, reducing ability (Chung et al., 2002) but reports regarding the fermented soymilk are scarce. Keeping this into consideration, strawberry fortified soy based probiotic yoghurt was prepared and evaluated for its total antioxidative potential in vitro.

MATERIALS AND METHODS

Microorganisms

Yoghurt culture NCDC-262 (Yg 262) and L. acidophilus NCDC-195 (La 195) were procured from National Collection of Dairy Cultures (NCDC) at N.D.R.I., Karnal, India and used for the fermentation of soymilk. Yoghurt culture (Yg 262) was maintained in M17 broth at 42°C and probiotic culture (La 195) was maintained in MRS broth at 37°C.

Preparation of Soymilk

Soymilk was prepared as per the protocol of Nelson et al., 1976. For preparation of soyculture I, soymilk was fermented with combination of Yg 262 and La 195 (1:2) at 42°C for 6h and soyculture II was prepared by supplementation of soymilk with 4% skim milk powder (SMP), 1.5% inulin and 10% strawberry pulp and fermented with combination of Yg 262 and La 195 (1:2) at 42°C for 6h.

Total Antioxidative Capacity of Soy Yoghurt by Microplate Method

ABTS [2, 29-Azinobis (3-ethylenbenzothiazoline) 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:

%DPPH scavenging activity = (A515 nm blank - A515nm sample) x 100

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 FeCl3 (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 ?L 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 ?L 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.

Statistical Analysis
All results presented in this paper are the average of three independent assays. Results are expressed as mean ± standard error, and their significance was analyzed by one way anova using SAS System ('Local', W32_VSPRO) version 9.2.

RESULTS AND DISCUSSION
Total antioxidative potential of both the soy yoghurts i.e. soyghurt I and soyghurt II were determined by three methods namely, ABTS, DPPH and FRAP method. In ABTS method of antioxidative determination (Table 1), inhibition shown by unfermented soymilk and soymilk fortified with skim milk powder, inulin and strawberry was found to be 80.65% and 87.05%, respectively. After fermentation, the extent of inhibition had increased, with soyghurt II having comparatively higher inhibition of 92.34% than soyghurt I, with ABTS inhibition of 91.09%. The TEAC (Trolox Equivalent Antioxidative Capacity) of unfermented soymilk and strawberry soymilk was found to be 859.99 µM and 927.47 µM, which was further enhanced after fermentation, with soyghurt II having TEAC value of 983.15 µM and soyghurt I with TEAC value of 980.48 µM. In DPPH method, the antioxidative capacity was expressed in terms of percent reduction of free radicals. On studying antioxidative capacity of the yoghurts by DPPH method (Table 1), again fermented product had shown higher reduction evaluated against unfermented one (where no reduction was observed). On the other hand, reduction of DPPH radicals by soyghurt II and soyghurt I was found to be 54.12% and 52.96%, respectively. Obviously, there were more antioxidant components present in fermented soymilk and strawberry soymilk than unfermented one, which could react rapidly with DPPH radicals, and reduce almost all DPPH radical molecules corresponding to available hydroxyl groups (Brand-Williams et al., 1995). In FRAP method (Table 1), antioxidative capacity of the product was expressed in µM of FRAP reagent utilized. Here again fermented product had shown higher FRAP reducing potential compared to unfermented one. FRAP reduction by soyghurt II was significantly higher (P<0.0001) than soyghurt I, 805.07 µM was significantly higher by former and 1364.25 µM by later. In unfermented product, strawberry soymilk had 730.25 µM FRAP reduction potential and soymilk 705.57 µM. similar findings of the enhanced reducing power of fermented soybean and soybean products have been reported previously (Wang et al., 2006). After studying total antioxidative potential of unfermented and fermented product of soymilk and strawberry soymilk with probiotic cultures and yoghurt cultures individually and in combinations, we concluded that highest antioxidation was shown by combination of probiotic cultures, followed by probiotic culture alone, yoghurt culture alone and unfermented product. This difference could be due to different proteolytic activity of individual cultures, which results in release of antioxidative peptides, with combination showing symbiosis, hence more proteolysis and resulted in high antioxidative activity. Since unfermented product lack such peptides, lower antioxidation was observed than fermented one. Among unfermented soymilk also, strawberry soymilk had more antioxidation than soymilk because of the presence of SMP, inulin and most importantly strawberry. Soymilk possesses innumerable bio-molecules like isoflavones, tocopherols, vitamin C, soy peptides, lecithin, saponins, and sterols, which quench free radicals species by donating hydrogen atom or an electron. It was also noted
that fermentation increases antioxidative capacity of the product. Pyo et al. (2005), reported that the extract obtained from soybean fermented with B. thermophilum KFRI 00748 had shown the highest antioxidant activity (94.1%±1.5% scavenging, 19.8 mM 0.4 TEAC) and also had the highest concentrations of isoflavone aglycones (159.2 mg/100 g dry weight). Lee et al., (2005), reported that Monascus-fermented soybeans (MFS) were more effective in antioxidant activity and scavenging ability on DPPH radicals with MFS-31499 and MFS-31527 more effective in reducing scavenging ability on DPPH radicals with MFS-31527 more effective in antioxidant activity and that Monascus-fermented soybeans (MFS) were more effective in antioxidant activity and scavenging ability on DPPH radicals with MFS-31499 and MFS-31527 more effective in reducing power and scavenging ability on hydroxyl radicals. Antioxidative activity was also reported in milk by many workers. Zulueta et al., (2009), found the antioxidant capacity of milk based on ORAC (Oxygen Radical Absorbance Capacity) method and showed casein as the major contributor to the total antioxidant capacity of whole milk, while albumin as the main contributor to the total antioxidant capacity of whey protein and vitamin C and uric acid as major contributors of total antioxidant capacity of deproteinized milk samples. Strawberries contain a wide range of antioxidants e.g. ascorbic acid, ellagic acid, and phenolic acids. In a similar study, Apostolidis et al., (2006) showed that strawberry yoghurt resulted in 52% inhibition of DPPH radicals. (Mendiola et al., 2008) reported 55% DPPH radical scavenged for yoghurt containing strawberry and 376 mg caffeic acid equivalent/L of sample. In another study, Jiménez et al., (2008) reported the TEAC activity of strawberry fortified yoghurt as 10 micromolar using ABTS method. Shah (2008),
evaluated the antioxidant potential of mango, pineapple, lemon, strawberry and blackberry based whey beverages based on ABTS, DPPH, FRAP and total phenolic content and found antioxidative activity of later two being 7-9 fold higher than rest. The differences in the antioxidant capacity by ABTS, FRAP and DPPH methods could be related to differences in reactivity of sample component. These results had revealed that fermented and unfermented soymilk and strawberry soymilk were free radical inhibitors or scavengers, acting possibly as primary antioxidants. They might have reacted with free radicals, particularly of the peroxy radicals, which are the major propagatous of the autooxidation chain of fat, thereby terminating the chain reaction (Gordon, 1990; Shahidi et al., 1992). The intracellular antioxidative peptides of the starter organism and their hydrogen-donating ability may also contribute to this increased reducing ability (Sung et al., 2000).

**CONCLUSIONS**

This study focused on enhanced total antioxidative potential of fermented soymilk supplemented with skim milk powder, inulin and strawberry pulp in comparison to unsupplemented fermented soymilk. Significant increase in antioxidative activity was noticed in soyghurt II by all the three methods judged against soyghurt I, which could be because of skim milk and inulin, both are beneficial for growth of both yoghurt and probiotic cultures. Additionally, strawberry pulp added to this

### Table 1: Antioxidative Capacity of Fermented Soymilk and Soyghurts

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<thead>
<tr>
<th>Samples</th>
<th>Total Antioxidative Capacity</th>
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<tr>
<td></td>
<td>% Inhibition</td>
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<tr>
<td>Soymilk</td>
<td>80.65 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Soymilk + La 195</td>
<td>90.97 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Soymilk + Yg 262</td>
<td>90.62 ± 0.54&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Soyghurt I</td>
<td>91.09 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Strawberry soymilk</td>
<td>87.05 ± 1.04&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Strawberry soymilk + La 195</td>
<td>91.16 ± 0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Strawberry soymilk + Yg 262</td>
<td>91.05 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Soyghurt II</td>
<td>92.34 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>F-ratio</td>
<td>46955.8***</td>
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<sup>a-d</sup> same column bearing different superscripts differ significantly, the level of significance was preset at α = 0.05 and significant ***p<0.001, ± standard error of three replicates

<sup>1</sup>Trolox Equivalent Antioxidative Capacity, studied at 750nm, <sup>2</sup>Free radical-scavenging activity (FRSA) studied at 517nm, <sup>3</sup>Ferric-reducing antioxidant power (FRAP) studied at 593nm
antioxidative effect of the product. Also the microplate adaptation of all the three methods is cost-effective and would be useful where a large number of samples are to be analyzed. In light of the numerous health benefits that have been linked to the consumption of soy products, the observation of increased antioxidant activity in soymghurt II is encouraging and need to be investigated in relation to other potential health-promoting activities of it.

REFERENCES


Deepika Yadav et al.


