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RESEARCH NOTE

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Antioxidative Activities of Soymilk Fermented with *Bacillus subtilis*

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Abstract To develop a dietary beverage with functionalities of *cheonggukjang*, soymilk was fermented with *Bacillus subtilis* and its antioxidant activities were examined. Antioxidative capacities of fermented soymilk (FS) with 5 different *B. subtilis* were significantly different. Among these, FS with 2829PNU015 revealed the greatest antioxidant activities. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, total antioxidant activity, and low density lipoprotein (LDL) oxidation inhibition of FS with 2829PNU015 were increased by 150, 140, and 240%, respectively, compared with those of unfermented soymilk ($p<0.05$). Further study for the improvement of sensory properties of FS with *B. subtilis* is required for a commercial production.

Keywords: *Bacillus subtilis,* fermentation, soymilk, low density lipoprotein (LDL) oxidation, Trolox equivalent antioxidant capacity (TEAC)

Introduction

Health promoting effects of soymilk products are well known to people with hypercholesterolemia, lactose-intolerance, or gluten sensitive as well as vegetarians. These beneficial effects of soymilk may be elevated by fermentation with probiotic bacteria (1) and lactic acid bacteria and/or bifido bacteria have been widely used for this purpose. The fermented soymilk (FS) with probiotic bacteria demonstrated antioxidative activities (2), cholesterol lowering effects (3), antimutagenic and anticarcinogenic actions (4), and immunogenic activities (5). Soymilk is also a suitable medium for the growth of *Bacillus* spp., however, this microorganism has never been used for the production of FS. There are continuing needs to improve the existing starter cultures or to select new strains for use in specific fermentation process in order to develop new products such as FS having functionalities of *cheonggukjang* (CKJ).

CKJ is a traditional Korean fermented soybean with *Bacillus subtilis* which is similar to *natto*. The functional properties of CKJ include antioxidant activity (6,7), anti-hypertensive effect (8), hypolipidemic effect (9,10), and anti-diabetic activity (11). These beneficial effects of CKJ might be elevated as daily intakes of CKJ increases. Therefore, various types of CKJ products such as CKJ powder or tablet form of CKJ have been introduced on the market for better application. However, a beverage is still needed to intake these products. Accordingly, there is a demand for beverage type CKJ for a convenient use. In order to develop a dietary beverage having CKJ functionalities, especially with antioxidative effects, we first tried to ferment soymilk with *B. subtilis* and then antioxidant activities of FS with 5 different strains were examined.

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Materials and Methods

Bacillus subtilis *B. subtilis* 2805PNU014, 2829PNU015, and 2825PNU016 were obtained from the Microbiology Laboratory at Pusan National University. *B. subtilis* MYCO10001 and KCCM11316 were purchased from Innobiz Myco Bio-Venture Co. and Korean Culture Center, respectively.

Fermentation of soymilk *B. subtilis* was activated through 2 successive transfers in nutrient broth (Becton, Dickinson & Co., Sparks, MD, USA) at 37°C for 24 hr using 2% inoculums. Soymilk (Donghwa Food Co., Ltd., Yangsan, Korea) containing 4.0% soy protein was dispensed into 500-mL bottles and 1%(w/v) glucose was added to it, and heat treated at 121°C for 15 min. Each bottle was then inoculated with each microorganism of total to be 10^{7-8}/mL before incubation at 40°C about 8 hr. Fermentation was terminated when the pH of the soymilk reached 5.5±0.2. Temperature and fermentation time were decided from the preliminary experiment (data not shown).

DPPH scavenging activity Antioxidant activities of fermented soymilk (FS) were studied with methanol extracts of FS. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was measured by the method of Hatano *et al.* (12).

Trolox equivalent antioxidant capacity (TEAC) TEAC was determined with a slight modification of Re *et al.* (14) to measure the total antioxidant activity of FS. 2,2'-Azinobis [3-ethylbezothiazoline-6-sulfonic acid (ABTS) radical cation ($ABTS^{\bullet+}$) was produced by reacting potassium persulfate and ABTS solution (7 mM) for 12-16 hr in the dark. Before experiment, the absorbance of $ABTS^{\bullet+}$ solution was adjusted at 734 nm to be 0.70±0.02 with ethanol. Fresh preparation of $ABTS^{\bullet+}$ solution is recommended although it is stable for 2 days when stored in the dark at room temperature. Methanol extracts of the FS were diluted with ethanol to produce the inhibition between 20-80%. TEAC of sample was determined by reading the absorbance at 30°C exactly 1 min after mixing 1.0 mL of

ABTS$^{\bullet+}$ and 20 μL of sample solution up to 6 min. Trolox standards of final concentration between 0-1.0 mM were used to prepare the standard curve. The best-fit line equation was determined by the standard curve. TEAC of the unknown sample was calculated by the formula; TEAC (mM)$=(T0_a-T6_a/T0_a)-(T0_b-T6_b/T0_b)$, where a refers the sample and b is the blank. TEAC was defined as the standard unit for the total antioxidant capacity of all samples.

Low density lipoprotein (LDL) oxidation inhibition Reaction mixture was prepared to be a final concentration of 0.1 mg LDL protein (Sigma-Aldrich, St. Louis, MO, USA), methanol extracts of the fermented soymilk (100 μg) in ethanol, and absolute ethanol (0.5%). LDL oxidation was initiated by adding Cu^{2+} solution (final concentration to be 5 μM) followed by incubation for 4 hr in the water bath temperature adjusted at 40°C. LDL was prepared in phosphate buffered saline (PBS, pH 7.4) and methanol extracts were prepared in ethanol. The extent of LDL oxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS) generated during the oxidation. In brief, 1 mL of oxidized LDL medium was reacted with 1 mL of TBARS solution (mixture of 0.4% thiobarbituric acid, 15% trichloroacetic acid, and 2.5% HCl) in a screw-capped tube at 95-100°C for 20 min to prevent evaporation while boiling. The reaction mixture was stirred vigorously followed immediate cooling on the ice. The optical density of supernatant obtained from centrifugation at 500×g for 5 min at 4°C was measured at 532 nm. The amount of TBARS generated during LDL oxidation was expressed as malondialdehyde (MDA) concentration. 1,3-Propanedialdehyde (Sigma-Aldrich) was used for the preparation of standard curve.

Statistical analysis Analysis of one-way variance (ANOVA) was followed by Duncan's multiple rage test in order to determine the statistical significance of measurements between groups, using the SAS software ($p<0.05$).

Results and Discussion

DPPH scavenging activity of the fermented soymilk IC_{50} for the DPPH scavenging activities of FSs were lowered significantly compared to that of unfermented soymilk ($p<0.05$), demonstrating that fermentation process increases DPPH scavenging activity (Table 1). In this study, approximately 140% elevation in DPPH scavenging activity was observed by the fermentation of soymilk with *B. subtilis* 2829PNU015, which was the highest among samples. IC_{50} for DPPH scavenging activity of FS with 2829PNU015 account for 31% activity of CKJ ($IC_{50,}$ 238.1 μg/mL) used for isolating this strain (6).

Total antioxidant activity of the fermented soymilk TEAC for FSs were significantly higher than that of unfermented soymilk. TEAC for FS with 2829PNU015 was 0.55 Trolox mM which is 275% higher than that of unfermented soymilk (0.39 Trolox mM). TEAC for FS with 2829PNU015 exhibited approximately 55% activity of vitamin C (0.99±0.04 Trolox mM) and vitamin E (0.97±0.01 Trolox mM) (16), or 16% of blackbean (3.43±0.01 Trolox mM) (17). Ascorbic acid autoxidation was significantly inhibited by the FS with lactic acid bacteria and its inhibition rate was increased by 11.92-16.38% than soymilk depends on the bacterial strain used (2).

Table 1. DPPH scavenging activity and total antioxidant activity of the soymilk fermented with *Bacillus subtilis*

B. subtilis strain used[1)]	DPPH scavenging activity	TEAC
	IC_{50} (μg/mL)	Trolox equiv (mM)
Unfermented	1,153.25[a2)]	0.39±0.00[d]
FS-PNU014	947.97[d]	0.46±0.02[b]
FS-PNU015	769.23[e]	0.55±0.03[a]
FS-PNU016	919.08[d]	0.44±0.04[bc]
FS-Myco	1,022.97[c]	0.44±0.01[bc]
FS-KCCM	1,077.19[b]	0.42±0.01[cd]

[1)]FS-PNU014, fermented soymilk (FS) with *B. subtilis* 2805PNU014; FS-PNU015, FS with *B. subtilis* 2829PNU015; FS-PNU016, FS with *B. subtilis* 2825PNU016; FS-KCCM, FS with *B. subtilis* KCCM11316; FS-Myco, FS with *B. subtilis* Myco10001.
[2)]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

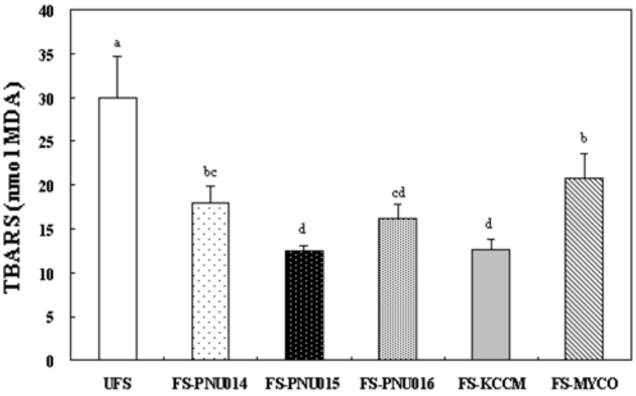

Fig. 1. Inhibition of LDL oxidation expressed as TBARS of soymilk fermented with *Bacillus subtilis*. UFS; un-fermented soymilk. FS-PNU014, fermented soymilk (FS) with *B. subtilis* 2805PNU014; FS-PNU015, FS with *B. subtilis* 2829PNU015; FS-PNU016, FS with *B. subtilis* 2825PNU016; FS-KCCM, FS with *B. subtilis* KCCM11316; FS-MYCO, FS with *B. subtilis* Myco10001. [a-d]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

LDL oxidation inhibition activity of the fermented soymilk TBARS concentration of unfermented soymilk was higher than that of FSs (Fig. 1), indicating that FS has greater effect on LDL oxidation inhibition. LDL susceptibility to oxidation is strongly associated with the development of atherosclerosis. Approximately, 30 to 60% LDL oxidation inhibition of FS was observed when different strain was used. Among FSs, FS with *B. subtilis* 2829PNU015 and KCCM11316 showed higher LDL oxidation inhibition activity. *Natto*, Japanese soybean product fermented with *B. subtilis natto* revealed anti-atheorgenic activity via inhibiting LDL oxidation in rats fed cholesterol (15).

In this study, we have succeeded in fermenting the soymilk with *B. subtilis*. FS with *B. subtilis* was opaque fluid having a weak smell of CKJ. The inoculums size of $10^{7\text{-}8}$/mL seems to be sufficient. Calcium-fortified soymilk with *lactobacillus* was fermented with 10^8/mL (16) to maintain viability above 10^7/mL during refrigerated storage. A minimum of 10^6/mL probiotic bacteria is required to effectively modulate the intestinal microbial balance and provide therapeutic dose of soymilk fermented with lactic acid bacteria (17).

The FS with *B. subtilis* has higher antioxidant activity than soymilk in terms of DPPH scavenging activity, total antioxidant activity, and LDL oxidation inhibition. And these effects were varied with the bacterial strain used. The results of the current study are in good line with the report from the FS with lactic acid bacteria and bifidobacteria, of which antioxidant activities in terms of ascorbate autoxiation inhibition, superoxide anion radical and hydrogen peroxides scavenging activities were increased during fermentation, but varied with the different starter organism used (2). The antioxidant effects of the FS may be attributed to the isoflavones and tocopherol, the main phenols in soybean (18). LDL susceptibility to oxidation is strongly associated with the development of atherosclerosis. A large body of evidence supports the key role of oxLDL in the early inflammatory (19) and more advanced stages of the atherosclerosis lesions. In this study, we have found that the soymilk fermented with *B. subtilis* seems to have pro or pre-biotic activity, like the one fermented with lactic acid and/or bifido bacteria and their activities will be varied with strain used. Soymilk fermented with *B. subtilis* 2829PNU015 (registered as KCCM42923) demonstrated the highest antioxidant activities.

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