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Profiles of Isoflavone and Fatty Acids in Soymilk Fermented with *Lactobacilli*, *Bifidobacteria*, or *Streptococci*

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Abstract Distribution of isoflavones and fatty acids in soymilk fermented with 7 *Lactobacilli* (L-type), 7 *Bifidobacteria* (B-type), or 5 *Streptococci* (S-type) were monitored. Total isoflavones in fermented soymilk ranged from 5.24 to 8.59 μmol/g dry basis while those in unfermented soymilk were 8.06 μmol/g dry basis. Depending on the types of inoculated microorganisms, fermented soymilk showed different profiles in isoflavones, especially aglycones and β-glucosides. Four L-type fermented soymilk had significantly higher aglycone content (33.69-46.21%) and low β-glucosides compared to control ($p<0.05$). All B-type fermented soymilk showed significantly high aglycone levels ($p<0.05$). Out of 5 *Streptococci*, 4 strains produced over 82.2% aglycones. Lipid content ranged from 162 to 224 mg/g and linoleic acid was the highest, followed by oleic, linolenic, palmitic, and stearic acids. Average ratio of unsaturated to saturated fatty acids in control, L-, B-, and S-type fermented soymilks was 6.30, 6.09, 6.30, and 5.94, respectively. This study can help to develop a fermented soymilk containing high isoflavone aglycones and low fat content.

Keywords: fermented soymilk, isoflavone, fatty acid, *Lactobacilli, Bifidobacteria, Streptococci*

Introduction

Recently, consumption of soy products has gained popularity in both Oriental and Western countries partly due to the increase of awareness on the health beneficial compounds in soy foods. Especially soy isoflavones are regarded as phytoestrogens because of their structural similarity to human estrogen and are associated with reducing the risks of heart disease, cancers, or osteoporosis (1,2). Three soy isoflavone aglycones (daidzein, genistein, and glycitein) and their conjugated β-glucosides, 6-*O*″-acetyl-β-glucosides, or 6-*O*″-malonyl-β-glucosides are found in soybeans and soy foods. Distribution and profiles of isoflavones in soy foods are greatly influenced by many factors such as processing types, temperature, duration of heating, moisture content, and fermentation (3-5).

Aglycones of isoflavones absorbed faster than their conjugated forms in gastro-intestinal track (6). Many researchers have tried to increase the concentration of isoflavone aglycones in soy foods using natural products like almond (7), microorganisms possessing high β-glucosidase activity (8,9), or commercially available β-glucosidase enzymes (10). Zhang *et al.* (11) used almond powder to produce soy bread enriched with high isoflavone aglycones.

Soymilk is a water extract of soybeans and does not possess cholesterol and lactose. Although soymilk has many nutritive advantages, peculiar beany off-flavor, indigestible galactosyl oligosaccharides, or flatulence factors play important roles in decreasing consumer acceptance on soymilk (12). Introducing fermentation process may enhance aglycone content and the sensory quality of soymilk by changing profiles of lipids (13).

Bifidobacteria and *Lactobacillus* are natural inhabitants in the gastro-intestinal track and are classified to probiotics showing health beneficial effects including activation of immune system, inhibition of the growth of potential pathogens, and reduction of cholesterol (14). Fermentation of soymilk with lactic acid bacteria has been introduced to change isoflavone profiles and to increase aglycone levels. Wei *et al.* (13) reported that soymilk fermented with *Lactobacillus* or *Bifidobacterium* possessed 62-96% of aglycones. Chien *et al.* (14) monitored isoflavone distribution and β-glucosidase activity in soymilk inoculated with *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*.

Fat content and profiles of fatty acids in soymilk can affect the oxidative stability and the sensory quality. Especially, beany off-flavor and oxidized volatiles can be formed from unsaturated fatty acids during processing and storage, which may play an important role in the decreases of consumers' acceptance. Also, high fat content may influence the consumers when body weight is concerned. However, studies on the distribution of both isoflavones and fatty acids in fermented soymilk are rare in the literature.

The objective of this study was to monitor the distribution of isoflavones and fatty acid compositions in fermented soymilk using 7 *Lactobacilli*, 7 *Bifidobacteria*, or 5 *Streptococci* to develop a fermented soymilk containing high isoflavone aglycone and low fat content.

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Materials and Methods

Materials Boron trifluoride solution (14% methanolic), mixtures of standard fatty acids, daidzein, genistein, and genistin were purchased from Sigma-Aldrich. (St. Louis, MO, USA), and 6 standard compounds including glycitein, glycitin, 6-*O*″-acetyl-β-daidzin, 6-*O*″-acetyl-β-genistin, 6-*O*″-acetyl-β-glycitin, and 6-*O*″-malonyl-β-genistin were purchased from LC Laboratories (Woburn, MA, USA). High performance liquid chromatography (HPLC)-grade methanol and acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Sample preparation Soymilk and fermented soymilk were produced by Maeil Dairy Industry Co. (Gyeonggi, Korea). Briefly, soybeans harvested in the year of 2005 at Taebak area in Korea were washed and soaked with 3 times of distilled water for 30 min at 95°C in a water bath (Jisico, Seoul, Korea). Cotyledon and embryo of soybeans were removed and 4 times of distilled water added. Crude soymilk was produced from a mixture of soybean using a soymilk producer (Hyundae Machine, Incheon, Korea). After homogenization process, soymilk was autoclaved at 121°C for 15 min (Eyela, Tokyo, Japan) and then stored at 4°C. Microorganisms were selected based on preliminary screening studies for the high α-galactosidase or β-glucosidase activity (data not shown). Soymilk was inoculated with about 10^6 CFU/mL of 7 *Lactobacilli* (L-type), 7 *Bifidobacteria* (B-type), or 5 *Streptococci* (S-type), respectively, and incubated for 24 hr at 37°C. Microorganisms and their sources used in this study are listed in Table 1. Six out of 19 lactic acid bacteria were separated from feces of Korean adults and the others are commercially available. Soymilk before fermentation was regarded as control samples. Soymilk with or without fermentation were freeze dried for the isoflavone and fatty acid analysis.

Table 1. Microorganisms and their sources used in this study

Sample	Microorganism	Source
L-6	*Lactobacillus acidophilus*	Commercial
L-14	*Lactobacillus bulgaricus* CS209	Commercial
L-22	*Lactobacillus casei* LC911	Commercial
L-23	*Lactobacillus casei* LC939	Commercial
L-58	*Lactobacillus acidophilus* NCFM	Commercial
L-69	*Lactobacillus* sp.Y05	Adult feces
L-73	*Lactobacillus* sp.Y22	Adult feces
B-12	*Bifidobacterium longum* BB46	Adult feces
B-18	*Bifidobacterium longum* BB730	Commercial
B-19	*Bifidobacterium longum* BL720	Commercial
B-21	*Bifidobacterium longum* BL-730	Commercial
B-22	*Bifidobacterium* sp. RD001	Adult feces
B-34	*Bifidobacterium* sp. RD062	Adult feces
B-62	*Bifidobacterium* sp. RD121	Adult feces
S-5	*Streptococcus thermophilus* TH-3	Commercial
S-8	*Streptococcus thermophilus* St-body-1	Commercial
S-11	*Streptococcus thermophilus* ST-1	Commercial
S-12	*Streptococcus thermophilus* TA052	Commercial
S-16	*Streptococcus thermophilus* A	Commercial

Isoflavone analysis Isoflavone analysis method from soymilk was adapted from Yang *et al.* (7). Briefly, 12 mL solvent mixture was added to 1 g of sample and vortex-mixed for 2 hr. Samples were centrifuged at 2,208×g for 10 min (Hanil, Incheon, Korea) and aliquot of 1 mL of supernatant was dried under nitrogen flow. Samples with the addition of 1 mL of methanol were filtered with a 0.2-μm syringe filter (Alltech Associates Inc., Deerfield, IL, USA) and then injected to HPLC (Hitachi, Tokyo, Japan).

Isoflavones were separated using a HPLC equipped with an ultraviolet (UV) detector, a 4-μm Waters Novapak C_{18} reversed-phase HPLC column (150×3.9 mm i.d.), a Novapak C_{18} guard column, and a 0.5-μm precolumn filter. Mobile phase was mixture of 1%(v/v) acetic acid in water (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 0.6 mL/min with following gradient condition: 15% solvent B from 0 to 5 min, increase of solvent B up to 35% from 5 to 44 min, decrease of solvent B up to 15% from 44 to 45 min, and then reequilibration of solvent B at 15% for 5 min. Injection volume was 10 μL and isoflavones in eluent were detected at 254 nm.

Quantification of each isoflavone was achieved by a combination of peak areas from HPLC analysis and of absorbance from an UV-Vis spectrophotometer according to the previous report (7).

Fatty acid analysis Lipid in samples was extracted using *n*-hexane in a Soxhlet apparatus. Fatty acid methyl ester (FAME) was prepared by a modified method using BF_3/MeOH (14% boron trifluoride) according to the AOAC (15).

Gas chromatography (GC) condition for fatty acid analysis GC Hewlett-Packard 5890-II gas chromatograph equipped with a flame ionization detector (FID), and a DB-23 (60 m×0.32 mm i.d., 0.25 μm film) from J&W Scientific (Folsom, CA, USA) was used for fatty acid analysis. The oven temperature was held at 100°C for 1 min, increased from 100 to 195°C at 15°C/min, from 195 to 210°C at 1°C/min, and from 210 to 240°C at 5°C/min, and held at 240°C for 7.5 min. The temperatures of both injector and detector were 260°C. The flow rate of helium carrier gas was 1.1 mL/min, and the split ratio was 1:50.

Statistical analysis Data were analyzed statistically by analysis of variance (ANOVA) and Duncan's multiple-range test using SPSS software program (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered significant.

Results and Discussion

Isoflavone distribution in fermented soymilk Isoflavone distribution in soymilk with or without fermentation is shown in Table 2. Distribution of isoflavones in soymilk was greatly dependent on the types of an inoculated starter culture, which agrees with previous reports on the fermented soy foods (13,16). Total isoflavones (TI) in fermented soymilk ranged from 5.24 to 8.59 μmol/g dry basis while those in control were 8.06 μmol/g. Average TI from 7 L-, 7 B-, and 5 S-type fermented samples were 7.74, 7.98, and 6.55 μmol/g, respectively, which implies that metabolisms using isoflavones occurred in the inoculated microorganisms

Table 2. Distribution of isoflavones in soymilk fermented with *Lactobacilli* (L), *Bifidobacteria* (B), or *Streptococci* (S)

	μmol/g Isoflavone/g dry base soy												
Sample[1)]	DE	DI	ADI	MDI	GE	GI	AGI	MGI	GY	GYI	AGTI	MGYI	TI
Control	0.165[2)]	4.287	0.041	0.077	0.216	2.321	0.312	0.300	0.046	0.000	0.248	0.050	8.06gh
L-6	0.541	2.806	0.056	0.254	2.187	0.987	0.024	0.627	0.064	0.578	0.055	0.107	8.29hi
L-14	0.170	3.354	0.136	0.101	0.241	2.147	0.198	0.160	0.047	0.634	0.050	0.079	7.32de
L-22	0.189	3.319	0.135	0.105	0.281	2.211	0.208	0.168	0.049	0.627	0.046	0.076	7.42e
L-23	0.184	3.452	0.152	0.106	0.262	2.266	0.210	0.163	0.047	0.645	0.051	0.078	7.62ef
L-58	0.594	2.665	0.058	0.269	2.396	0.880	0.024	0.652	0.069	0.565	0.069	0.104	8.34hi
L-69	1.516	1.506	0.121	0.064	1.637	1.526	0.195	0.129	0.070	0.602	0.042	0.051	7.46e
L-73	1.315	1.557	0.133	0.223	1.800	1.240	0.203	0.566	0.071	0.551	0.052	0.083	7.79fg
B-12	0.768	2.647	0.078	0.126	2.248	1.215	0.073	0.181	0.064	ND[3)]	ND	ND	7.40c
B-18	2.377	0.387	0.093	0.244	3.903	0.137	0.113	0.647	0.445	0.096	0.050	0.089	8.58i
B-19	0.939	1.926	ND	ND	2.773	1.000	0.069	0.293	0.069	0.410	ND	ND	7.48e
B-21	0.722	2.223	0.162	0.130	0.957	2.078	0.294	0.288	0.038	0.428	0.030	0.049	7.40e
B-22	2.299	0.657	ND	ND	4.694	0.064	0.027	0.096	0.300	0.140	ND	ND	8.28hi
B-34	2.869	ND	ND	ND	4.758	ND	ND	ND	0.548	ND	ND	ND	8.17h
B-62	2.360	0.966	ND	ND	4.815	ND	ND	0.110	0.336	ND	ND	ND	8.59i
S-5	1.969	0.194	0.065	0.137	3.408	0.077	0.122	0.100	0.358	0.054	0.084	0.055	6.62b
S-8	1.979	0.451	0.072	0.120	3.411	0.028	0.200	0.113	0.367	0.077	0.131	0.055	7.00c
S-11	2.092	0.222	0.032	0.110	3.652	0.070	0.426	0.086	0.018	0.056	0.068	0.025	6.86bc
S-12	2.034	0.392	0.075	0.109	3.570	0.036	0.025	0.102	0.479	0.076	0.094	0.039	7.03cd
S16	0.063	2.453	0.062	0.053	0.120	1.761	0.024	0.080	0.066	0.461	0.039	0.063	5.24a

[1)]DE, Daidzein; DI, daidzin; ADI, acetyl-β-daidzin; MDI, malonyl-β-daidzin; GE, genistein; GI, genistin; AGI, acetyl-β-genistin; MGI, malonyl-β-genistin; GY, glycitein ; GYI, glycitin; AGYI, acetyl-β-glycitin; MGYI, malonyl-β-glycitin; TI, total isoflavones.
[2)]Average of triplicates (n=3).
[3)]Not detected.

during fermentation with various degrees. Some chemical forms of isoflavones were not detected in B-type soymilk samples while L- and S-type samples showed all 12 chemical forms of isoflavones. Soymilk fermented with L-58, B-62, or S-12 showed the highest TI as 8.34, 8.59, or 7.03 μmol/g, respectively, among the same genus of species (Table 2).

Relative percentages (%) of isoflavones in fermented soymilk are shown in Table 3. Relative concentration of aglycones, β-glycosides, 6-*O*″-acetyl-β-glycosides, and 6-*O*″-malonyl-β-glycosides in control were 5.28, 81.96, 7.46, and 5.30%, respectively (Table 3), which indicates that large portions of malonyl derivatives in raw soybeans were converted into β-glycosides during soaking and autoclaving process. Depending on the types of microorganisms, fermented soymilk showed different profiles in isoflavones, especially aglycones and β-glucosides. Four L-type fermented soymilk had significantly higher aglycones (33.69-46.21%) than control ($p<0.05$) while aglycones of 2 L-type (L-14 and L-23) fermented soymilk were not significantly different ($p>0.05$), which implies that isoflavone distribution in soymilk is greatly diverse even in the same *Lactobacillus* species. All B-type fermented soymilk showed significantly high aglycone content ($p<0.05$). Fermented soymilk with B-34 had 100% aglycones and some unidentified new peaks in HPLC chromatograms, which could be metabolites from isoflavones (data not shown). Out of 5 *Streptococci*, 4 species produced over 82.2% aglycones.

Fermented soymilk with high aglycone content had significantly lower β-glucosides than control ($p<0.05$), which implies that aglycones were converted from β-glucosides. According to the reports of Wei *et al.* (13) and Chien *et al.* (14), soymilk fermented with *Lactobacillus paracasei*, *L. acidophilus, Bifidobacterium logum*, or *Streptococcus thermophilus* showed significant increase in the levels of aglycones. Otieno *et al.* (17) reported biotransformation of isoflavones in soymilk using *Bifidobacterium animalis*, 3 strains of *L. acidophilus*, and 2 of *L. casei* possessing high β-glucosidase activity. Authors observed that aglycones in soymilk increased from 8.0 to 60.3-76.9% and levels of β-glucosides decreased from 83 to 8.3-14.5% after fermentation. Tsangalis *et al.* (18) tested 5 strains of *Bifidobacterium* for the biotransformation of isoflavones and found *B. animalis*, *B. longum*-a, and *B. pseudolongum* could hydrolyze conjugated forms of isoflavones into aglycones and transform daidzein into equol in soymilk. Our study confirmed the increases of aglycones in fermented soymilk inoculated with selected lactic acid bacteria.

Fatty acid profiles in fermented soymilk Lipid content and distribution of fatty acids in fermented soymilk are shown in Table 4. Lipid content ranged from 162 to 224 mg/g in fermented soymilk and control had 197 mg/g. Average concentration of lipid in L-, B-, and S-types was 188.5, 190.1, or 164.4 mg/g, respectively. Generally, S-type samples had lower lipid content than control, L-type, and B-type samples.

Linoleic acid was the highest, followed by oleic, linolenic, palmitic, and stearic acids in all fermented soymilk

Table 3. Relative percentage (%) of isoflavone derivatives in soymilk fermented with *Lactobacilli* (L), *Bifidobacteria* (B), or *Streptococci* (S)

Sample	Aglycone	β-Glucoside	Acetyl-β-glucoside	Malonyl-β-glucoside
Control	5.28ab[1)]	81.96i	7.46l	5.30e
L-6	33.69e	52.75g	1.64c	11.92gh
L-14	6.27abc	83.84j	5.25i	4.64de
L-22	7.01c	83.03j	5.25i	4.70de
L-23	6.48bc	83.55j	5.42j	4.55de
L-58	36.66f	49.25f	1.80c	12.29h
L-69	43.21h	48.73f	4.79h	3.27bc
L-73	40.88g	42.95d	4.97i	11.19g
B-12	41.61g	52.20g	2.05cd	4.15bcd
B-18	78.37j	7.22b	2.98f	11.42gh
B-19	50.55i	44.61e	0.92b	3.92bcd
B-21	23.21d	63.93h	6.57k	6.30e
B-22	88.12m	10.40c	0.32a	1.16a
B-34	100.00n	ND[2)]	ND	ND
B-62	87.48m	11.25c	ND	1.28a
S-5	86.58m	4.90a	4.11g	4.41cde
S-8	82.20k	7.94b	5.75j	4.11bcd
S-11	84.02l	5.07a	7.67l	3.23b
S-12	86.49m	7.17b	2.77ef	3.57bcd
S-16	4.73a	89.12k	2.40de	3.74bcd

[1)]Different letters are significant among the same chemical forms of isoflavones at $p<0.05$.
[2)]Not detected.

irrespective of the types of microorganisms. Fatty acid compositions in fermented soymilk changed depending on the types of inoculated starter cultures. Average levels of linoleic acid (C18:2) in L-, B-, and S-type fermented soymilks was 54.25, 53.70, and 53.33%, respectively, and those of linolenic acid (C18:3) were 9.98, 9.43, and 9.12%, respectively. However, average content of oleic acid (C18:1) in L-, B-, and S-type samples was 21.87, 22.60, and 22.90 %, respectively, and that of stearic acid (C18:0) was 3.27, 3.54, and 3.78%, respectively. Average ratio of unsaturated to saturated fatty acids in control, L-, B-, and S-type fermented soymilks was 6.30, 6.09, 6.30, and 5.94, respectively. Fermented soymilk with *Lactobacilli* had relatively high linoleic and linolenic acids and low oleic and stearic acids whereas S-type samples had relatively low linoleic and linolenic acids and high oleic and stearic acids compared to control (Table 4). Especially, fermented soymilk with S-11 (*S. thermophilus* ST-1) showed the lowest linoleic acid content and ratio of unsaturated to saturated fatty acids (Table 4).

Yuan and Chang (19) reported that level of linoleic acid was positively correlated with hexanal content, a typical volatile for beany and grassy off-flavor in soymilk (r=0.93). Also, it is well-documented that the degree of unsaturated fatty acids is positively correlated with the rates of oxidation. The relative reaction ratio of triplet oxygen with oleic, linoleic, and linolenic acid are 1:12:25 (20). Therefore, low levels of linoleic acid and low ratio of unsaturated to saturated fatty acids may enhance the sensory quality and oxidative stability of lipids in fermented soymilk during storage.

Table 4. Lipid contents and fatty acid compositions in soymilk fermented with *Lactobacilli* (L), *Bifidobacteria* (B), or *Streptococci* (S) [1)]

Control	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	USFA	SFA	U/S	mg Fatty acid/g sample
Control	9.66a	3.30a	21.85a	54.30k	9.93j	0.31a	0.23a	0.43a	86.3	13.7	6.3l[2)]	197
L-6	9.69bc	3.29c	22.00g	54.18c	9.93c	0.30ab	0.18a	0.43a	86.3	13.7	6.3kl	173
L-14	9.71c	3.24a	21.74b	54.36f	10.06f	0.30a	0.18a	0.43a	86.3	13.7	6.3mn	189
L-22	9.70c	3.24a	21.67a	54.40g	10.08g	0.29a	0.18a	0.43a	86.3	13.7	6.3n	194
L-23	9.70bc	3.27b	21.78c	54.30e	10.05f	0.30ab	0.18a	0.43a	86.3	13.7	6.3kl	169
L-58	9.67abc	3.25a	21.88e	54.29e	10.01e	0.29a	0.18a	0.43a	86.4	13.6	6.3o	172
L-69	9.66ab	3.27b	21.92f	54.22d	10.00e	0.30ab	0.18a	0.43a	86.3	13.7	6.3mn	224
L-73	9.70bc	3.35e	22.17h	54.01a	9.84b	0.31b	0.18a	0.46b	86.2	13.8	6.2j	199
B-12	9.81c	3.61b	22.62b	53.59j	9.37i	0.33b	0.23a	0.44b	85.8	14.2	6.0h	190
B-18	9.65a	3.30cd	22.21i	54.09b	9.84b	0.30ab	0.19a	0.43a	86.3	13.7	6.3m	204
B-19	9.80c	3.64c	22.69de	53.51h	9.34h	0.34c	0.23a	0.45c	85.8	14.2	6.0g	202
B-21	9.78b	3.61b	22.71f	53.56i	9.34h	0.33b	0.22a	0.45c	85.8	14.2	6.1i	205
B-22	9.82d	3.85i	23.51k	53.06a	8.76a	0.34c	0.22a	0.44b	85.6	14.4	5.9b	165
B-34	9.67ab	3.30d	21.85d	54.31e	9.95d	0.30ab	0.18a	0.43a	86.3	13.7	6.3kl	190
B-62	9.78b	3.80h	23.34j	53.23c	8.86b	0.34bc	0.22a	0.44	85.6	14.4	6.0f	175
S-5	9.92e	3.71e	22.71de	53.41f	9.25g	0.33bc	0.22a	0.45c	85.6	14.4	5.9c	162
S-8	9.83d	3.80h	23.08h	53.27d	9.01d	0.34c	0.22a	0.45c	85.6	14.4	5.9c	172
S-11	9.82d	3.79g	23.29i	53.17b	8.93c	0.34c	0.21a	0.44b	85.6	14.4	5.9d	162
S-12	9.95f	3.75f	22.77g	53.33e	9.19e	0.34c	0.22a	0.45c	85.5	14.5	5.9a	167
S-16	9.91e	3.69d	22.69c	53.48g	9.23f	0.33b	0.22a	0.44b	85.6	14.4	6.0e	159

[1)]USFA, Unsaturated fatty acid; SFA, saturated fatty acid; U/S, ratio of USFA to SFA.
[2)]Different letters are significant among treatment at $p<0.05$.

This study can help to produce a fermented soymilk containing high content of aglycones and low unsaturated fatty acids. It will be advisable to use a combination of *Bifidobacterium* and *Streptococus* as starter cultures for soymilk fermentation to increase isoflavone aglycones and the oxidative stability by reducing the ratio of unsaturated to saturated fatty acids.

Isoflavone profiles and fatty acid compositions were analyzed in soymilk fermented with 7 *Lactobacilli*, 7 *Bifidobacteria*, or 5 *Streptococci*. Significant changes of isoflavone aglycones and β-glucosides were observed in fermented soymilk depending on the types of inoculated microorganisms. Total isoflavones in fermented soymilk were different, which implies that metabolisms using isoflavones occurred with various degrees. The ratios of unsaturated to saturated fatty acids in some fermented soymilk were significantly lower than those of non-fermented control samples.

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