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Original Article

Time course effects of fermentation on fatty acid and volatile compound profiles of *Cheonggukjang* using new soybean cultivars

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ABSTRACT

In this study, we investigated the effects of the potential probiotic *Bacillus subtilis* CSY191 on the fatty acid profiles of *Cheonggukjang*, a fermented soybean paste, prepared using new Korean brown soybean cultivars, protein-rich cultivar (*Saedanbaek*), and oil-rich cultivar (*Neulchan*). Twelve fatty acids were identified in the sample set—myristic, palmitic, palmitoleic, stearic, oleic, vaccenic, linoleic, α-linolenic, arachidic, gondoic, behenic, and lignoceric acids—yet, no specific changes driven by fermentation were noted in the fatty acid profiles. To further explore the effects of fermentation of *B. subtilis* CSY191, complete profiles of volatiles were monitored. In total, 121, 136, and 127 volatile compounds were detected in the *Saedanbaek*, *Daewon* (control cultivar), and *Neulchan* samples, respectively. Interestingly, the content of pyrazines—compounds responsible for pungent and unpleasant *Cheonggukjang* flavors—was significantly higher in *Neulchan* compared to that in *Saedanbaek*. Although the fermentation period was not a strong factor affecting the observed changes in fatty acid profiles, we noted that profiles of volatiles in *Cheonggukjang* changed significantly over time, and different cultivars represented specific volatile profiles. Thus, further sensory evaluation might be needed to determine if such differences influence consumers' preferences. Furthermore, additional studies to elucidate the associations between *B. subtilis* CSY191 fermentation and other nutritional components (e.g., amino acids) and their health-promoting potential are warranted.

Abbreviations: FAME, fatty acid methyl ester; FID, flame ionization detector; IV, iodine value; IS, internal standard; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; NIST, National Institute of Standards and Technology; PUFA, polyunsaturated fatty acids; O/L, ratio of oleic to linoleic acids; RSD, relative standard deviation; SRM, standard reference material.

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1. Introduction

Soybeans have been an important dietary item in Asian countries including China, Korea, and Japan owing to their high protein and oil contents (approximately 40% and 20% of their dry weights, respectively) [1]. In addition, a number of studies have investigated the health-promoting effects of soybeans and soybean products, particularly their effects against cancers, cardiovascular diseases, and other chronic diseases, making this an important crop in the food industry [2,3].

In South Korea, fermented soybean foods are very common daily staples; commonly consumed fermented soybean foods include soybean paste (*Doenjang*), soy sauce, and *Cheonggukjang* (unsalted soybean paste). In particular, *Cheonggukjang* is characterized by excellent nutritional components and fast digestion. *Cheonggukjang* is made from steamed soybeans fermented by *Bacillus subtilis*. Fermentation by *B. subtilis* produces diverse metabolites including amino acids, organic acids, and fatty acids [4]. According to previous findings in the literature, intake of *Cheonggukjang* may improve beneficial immune activity [5] and asthma [6], control lipid metabolism [7], and attenuate neurodegenerative diseases [8].

As previously mentioned, although many studies have investigated the health-promoting effects of soybean products and their bioactive constituents, potentially enriched through fermentation [9], few reports have outlined the time course effects of fermentation with regard to changes in the nutritional characteristics of soybeans. Furthermore, even fewer studies have compared the nutritional characteristics of soybean cultivars throughout the fermentation processes. To fill the information gap, the authors analyzed the complete profiles of fatty acids and volatile compounds in *Cheonggukjang* and their changes in response to fermentation using the potential probiotic *B. subtilis* CSY191. In the present study, three Korean brown soybean cultivars—*Daewon* (normal), *Saedanbaek* (protein-rich), and *Neulchan* (oil-rich)—were selected to make comparisons and determine if different cultivars are responsible for changes in fatty acid and volatile compound profiles during fermentation.

2. Materials and methods

2.1. Materials

Three Korean brown soybean cultivars (*Saedanbaek*, *Daewon*, and *Neulchan*) were provided by the National Institute of Crop Science of the Rural Development Administration (Miryang, South Korea). The probiotic *B. subtilis* CSY191 was isolated from the Korean traditional soybean paste (*Doenjang*) as described previously [10] and used as the starter organism. High performance liquid chromatography-grade methanol, chloroform, hexane, anhydrous sodium sulfate, sodium chloride, and American Chemical Society-grade boron trifluoride in methanol were purchased from Fisher Scientific Company (Suwanee, GA, USA). Heptadecanoic acid and a variety of fatty acid methyl esters (37 FAMEs) were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Preparation of Cheonggukjang

Soybean samples (1 kg) were washed and soaked with three volumes of tap water at 20 ± 2°C for 12 hours and steamed for 15 minutes at 121 ± 1°C. The steamed soybeans were cooled at 40°C for 1 hour and then inoculated with 5% (w/w) *B. subtilis* CSY191 (7.65 log CFU/mL), followed by fermentation for up to 48 hours at 37 ± 2°C in an incubator. Samples were obtained after 0 hour, 12 hours, 24 hours, and 48 hours of fermentation. After the 24-hour fermentation period, we observed that more diverse volatile compound profiles were demonstrated than at the time point of 24 hours. On the basis of the literature including our previous research, 48-hour fermentation of soybeans is a widely accepted condition. Each of the *Cheonggukjang* samples were freeze-dried, ground to a powder, and stored at −80°C until analysis.

2.3. Lipid extraction

Total lipids of *Cheonggukjang* samples were extracted according to the classical Bligh–Dyer method [11]. Briefly, 10 g of the *Cheonggukjang* powder was extracted with a mixture of 20 mL deionized water, 50 mL methanol, 25 mL chloroform, and 10 mg hydroquinone. The contents were then blended on a shaker (3000*g*) for 2 minutes. The slurry was filtered through a Whatman No. 1 filter paper (GE Healthcare, Little Chalfont, UK). Sodium chloride (NaCl, 1 g) was added to the filtrate to facilitate phase separation and then placed at room temperature overnight for separation. Next, the chloroform phase was filtered again and completely evaporated. Extracted samples were flushed with nitrogen to prevent further oxidation and stored at −80°C until further analysis.

2.4. FAME and gas chromatography analysis

In order to analyze the fatty acid profile of extracted lipids from *Cheonggukjang*, FAME samples were prepared according to Ngeh-Ngwainbi's method with slight modifications [12]. Heptadecanoic acid (C17:0, 1 mg/mL in hexane, 1 mL) was used as the internal standard (IS) for the analysis. Extracted lipids (100 mg) were mixed with 1 mL of 0.5N sodium hydroxide in methanol (w/v). The mixtures were heated to 100°C for 5 minutes in a heating block (Thermo Fisher Scientific, Rockford, IL, USA). After cooling to room temperature, 2 mL boron trifluoride in methanol (14%, w/v) was added, and the mixture was heated to 100°C for 30 minutes for methylation. Each FAME was then extracted three times with 1.5 mL of hexane.

A gas chromatography (GC) system (Agilent Technologies 7890A) interfaced with a flame ionization detector (FID) was used for analyzing the fatty acid profiles. The column was a SP-2560 capillary column (100 m × 0.25 mm i.d., 0.25 μm film thickness), and the oven program was set as follows: initial temperature, 140°C; ramping up at 4°C/min to 230°C; maintaining time, 35 minutes at 230°C. Detailed GC analysis conditions have been described in our previous work [13].

2.5. Fatty acid quantification

A relative response factor was calculated for each FAME using the IS as described previously [13]. Each FAME had a different response factor, calculated as follows:

$$R_i = (P_{si} \times Ws_{C17:0})/(Ps_{C17:0} \times Ws_{is})$$

where R_i refers to each relative response factor for fatty acid i, P_{si} is the peak area of each FAME i in the FAME standard solution, $Ws_{C17:0}$ is the mass (mg) of the C17:0 FAME, $Ps_{C17:0}$ is the peak area of C17:0 FAME, and Ws_{is} is the mass (mg) of the individual FAME i in the injected FAME standard solution.

Each fatty acid was identified by being compared to the standard FAME values using its retention time.

2.6. Characterization of fatty acids

The oleic acid/linoleic acid (O/L) ratio and iodine value (IV) were calculated according to the following formulae [14]:

$$O/L = \% \text{ oleic acid}/\% \text{ linoleic acid}$$

$$IV = (0.8601 \times \% \text{ oleic acid}) + (1.7321 \times \% \text{ linoleic acid}) + (0.7854 \times \% \text{ gondoic acid})$$

2.7. Method validation for fatty acid analysis

Accuracy and interday precision, i.e., relative repeatability standard deviation and % relative standard deviation (RSD), of the results obtained for the analysis of fatty acids in *Cheonggukjang* lipid extracts were determined using the Standard Reference Material (SRM) 1849a, National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). Each assay was analyzed five times, and fatty acid data were compared against the certified values provided by NIST. % RSD, bias, and % accepted value were determined as follows:

$$\% \text{ RSD} = (\text{standard deviation} \times 100)/\text{mean}$$

$$\text{Bias} = \text{Accepted value provided from NIST} - \text{Analytical value obtained from experiments in this study}$$

$$\% \text{ Accepted value} = (\text{Analytical value obtained from experiments in this study} \times 100)/\text{Accepted value provided from NIST}$$

2.8. Analysis of volatile compounds

Extraction of the volatile compounds of *Cheonggukjang* using a simultaneous steam distillation and extraction method (SDE) and subsequent GC with mass spectrometry (GC-MS) analysis were carried out as we have previously reported [13]. In brief, 10 g of the sample was hydrolyzed with 1 L distilled water to liberate volatile compounds from the sample. Pentadecane (1 mg/mL in hexane, 1 mL) was added as an IS. The sample mixture was transferred to a 1 L round flask SDE apparatus and was heated to 110°C. To collect the volatile compounds liberated by heating, 100 mL of a mixture of *n*-pentane and diethyl ether (1:1, v/v) was also heated separately in the other vessel in the SDE system and redistilled prior to use. After the mixture was heated for 3 hours at 110°C, the organic solvent phase was collected and stored at 110°C overnight, and the mixture was then eluted with 10 g of anhydrous sodium sulfate on a No. 1 filter paper to remove moisture, and dried to a volume of 1 mL under a flow of nitrogen gas. Volatile compounds in the samples were analyzed using GC-MS. An HP-5MS capillary column (30 cm × 0.25 mm, i.d. 0.25 μm) was used, and the mass range (*m*/z) of 30–550 amu was scanned. The initial oven temperature was set at 40°C and held for 5 minutes prior to ramping up at 5°C/min to 200°C. Detected peaks in total ion chromatograms were identified and confirmed using the NIST database and fragmentation patterns. Finally, respective retention indices (RIs) were further compared to identify volatile compounds as follows [15]:

$$RI_x = 100n + 100((t_{Rx} - t_{Rn})/(t_{Rn+1} - t_{Rn}))$$

where RI_x is the RI of the observed compound, t_{Rx} is the retention time of the observed compound, t_{Rn} is the retention time of *n*-alkane, and t_{Rn+1} is the retention time of the next *n*-alkane.

Each volatile compound was quantified from the area of the IS to the area of each volatile compound as follows [16]:

$$\text{Quantification} = (PA_x/PA_i) \times \text{mass of the IS}$$

where PA_x is the peak area of observed compound and PA_i is the peak area of the IS.

For the identification of each compound, this study used two identification procedures: one is matching between observed peak and standard fragmentation provided by NIST library (general identification procedure), and the other was by matching the RI of each compound.

If comparison between the observed peaks and standards in the NIST library shows more than 75% conformity, the RI value of each compound was checked against reference data [11].

2.9. Statistical analysis

All data were reported as mean ± standard deviation. Differences in means for each cultivar were determined using Tukey's multiple range test at $p < 0.05$ using the Statistical Analysis System (SAS) software (ver. 9.1; SAS institute, Cary, NC, USA). Associations between fatty acids were also examined using the Pearson correlation coefficients and SAS.

3. Results and discussion

Three cultivars—*Daewon*, *Saedanbaek*, and *Neulchan*—were chosen for this study. *Daewon* is a conventional soybean cultivar harvested in South Korea for producing soybean products such as soybean paste or soybean sauce. According to the literature, *Daewon* cultivar has about 40% protein

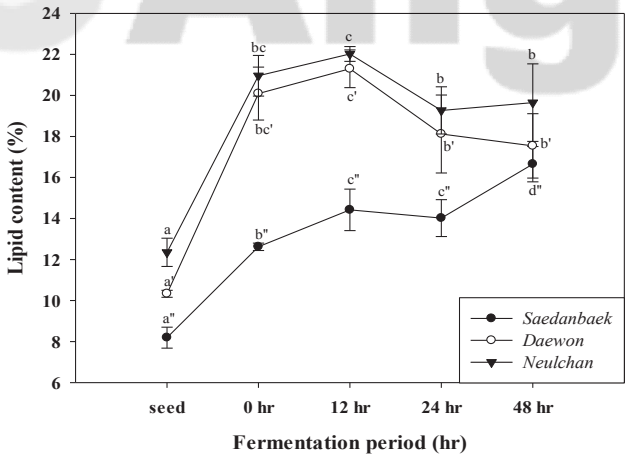

Figure 1 – Lipid contents of novel soybean cultivars at various fermentation times. Different letters correspond to the significant differences relating to the fermentation period using Tukey's multiple test ($p < 0.05$).

content and 18% lipid content; *Saedanbaek* cultivar, harvested as a protein-rich cultivar for producing tofu, has 48% protein content and 16% lipid content; and *Neulchan* cultivar, used for producing soybean milk products, has more than 20% lipid content [17]. *Daewon* cultivar is a control sample for the normal cultivar, *Saedanbaek* cultivar is known for its for high protein content due to producing volatile compounds released from decomposition of protein, and *Neulchan* cultivar is considered as the change of fatty acid profiles as oil-rich cultivar.

To characterize the soybean cultivars (i.e., *Saedanbaek*, *Daewon*, and *Neulchan*), total lipid contents were analyzed throughout the fermentation process (Figure 1). In general, the lipid contents of all cultivars increased over time to varying extents. As a result, no difference in lipid content was noted among the three cultivars at the end of fermentation, 48 hours after inoculation [18]. Fermented soy foods such as *Cheonggukjang* undergo deglycosylation by microorganisms during the fermentation period. Owing to the deglycosylation, various beneficial components are produced in fermented soyfoods. In addition, Wang et al [19] reported the hydrolysis of carbohydrates in soybean during the fermentation period, resulting in the production of free fatty acids. This study also noted that fermentation is positively related to the lipid contents of samples mainly containing fatty acids (Figure 1). In addition, fermentation involves a heating procedure with hydration by which water can catalyze liberated lipids containing fatty acids. Therefore, the efficiency of lipid extraction can be increased between raw soybean and fermented *Cheonggukjang*.

The accuracy and interday precision of the fatty acid analysis method were determined using the SRM 1849a (Table 1). Representative GC chromatograms of *Cheonggukjang* made from three cultivars are also provided in Figure 2. Table 1 indicates the accuracy and interday precision (i.e., %RSD) for the method of fatty acid analysis. The accuracy value was calculated based on the percentage of the certified fatty acid content of SRM 1849a. As represented, the accuracy ranged from 92.89 ± 0.09% to 103.60 ± 0.40%, whereas the reproducibility of the method, represented by the RSD, was less than 10% for all fatty acids.

The complete fatty acid profiles of soybean cultivars and time course effects of *Cheonggukjang* fermentation by *B. subtilis* CSY191 (i.e., 0 hour, 12 hours, 24 hours, and 48 hours after inoculation of *B. subtilis* CSY191) are presented in Table 2. Ten fatty acids were identified in the sample set—palmitic (C16:0), stearic (C18:0), oleic (C18:1 ω-9), vaccenic (C18:1 ω-7), linoleic (C18:2 ω6), α-linolenic (C18:3 ω3), arachidic (C20:0), gondoic (C20:1 ω-9), behenic (C22:0), and lignoceric (C24:0) acids—by GC-FID. In all samples analyzed, myristic (C14:0) and palmitoleic (C16:1 ω-7) acids were detected in trace level (less than

Table 1 – Accuracy (% of accepted value) and interday precision (%RSD) determined through analysis of lipid extracted from SRM 1849.[a]

Fatty acids	Weight percentage (%)			% of accepted value[d]	% RSD[e]
	Accepted value[a]	Analytical value[b]	Bias[c]		
C14:0	4.79 ± 0.16	4.64 ± 0.14	0.15	96.82 ± 0.18	3.02
C16:0	9.85 ± 1.10	9.68 ± 0.24	0.16	98.34 ± 0.78	2.48
C16:1 ω-7	0.11 ± 0.01	0.10 ± 0.01	0.00	95.98 ± 0.01	9.53
C18:0	4.13 ± 0.09	4.24 ± 0.06	−0.10	102.46 ± 0.06	1.30
C18:1 ω-9	50.46 ± 5.50	51.36 ± 2.75	−0.04	101.77 ± 3.60	5.35
C18:1 ω-7	1.01 ± 0.03	1.04 ± 0.04	−0.89	103.60 ± 0.40	4.22
C18:2 ω-6	25.92 ± 2.10	25.31 ± 1.12	0.61	97.63 ± 2.63	4.42
C18:3 ω-3	0.40 ± 0.01	0.38 ± 0.02	0.02	96.25 ± 0.12	5.20
C20:0	0.29 ± 0.02	0.28 ± 0.01	0.01	96.42 ± 0.10	3.56
C20:1 ω-9	2.55 ± 0.25	2.51 ± 0.08	0.04	98.30 ± 0.43	3.19
C22:0	0.32 ± 0.01	0.30 ± 0.01	0.02	94.68 ± 0.17	3.33
C24:0	0.16 ± 0.01	0.15 ± 0.01	0.01	92.89 ± 0.09	3.27

SD = standard deviation; SRM = standard reference material.
[a] The accepted value of the *Cheonggukjang* lipid is calculated from the certified fatty acid content of SRM 1849a based on the weight percentage.
[b] Data represents the mean ± SD ($n = 3$).
[c] Bias = accepted value − analytical value.
[d] The ratio of the analytical value to accepted value expressed as a percentage.
[e] RSD indicates interday relative standard deviation (SD × 100/mean).

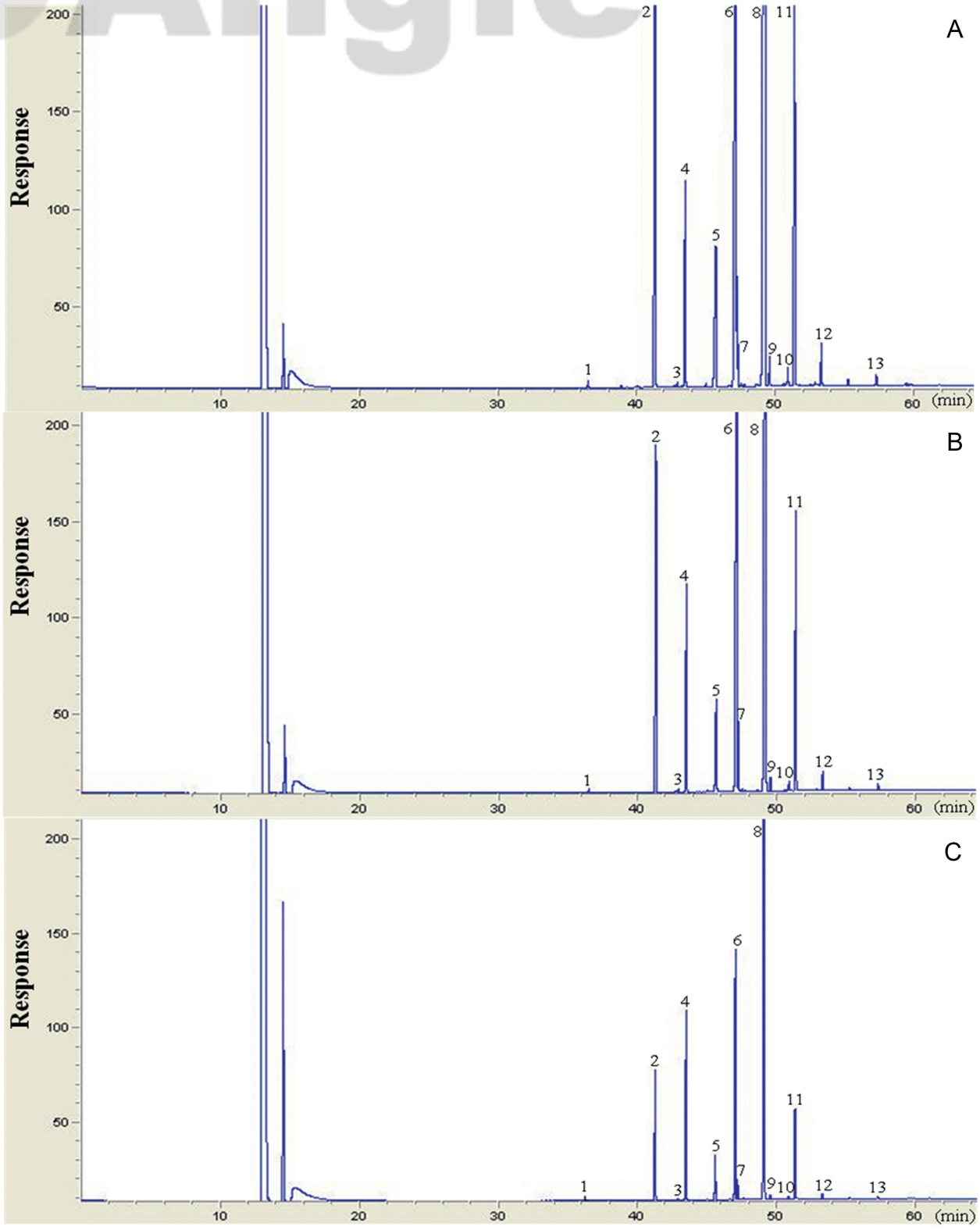

Figure 2 – Representative GC-FID chromatograms of (A) *Saedanbaek*, (B) *Daewon*, and (C) *Neulchan*. [Peaks were assigned as follows. 1 = myristic acid (C14:0); 2 = palmitic acid (C16:0); 3 = palmitoleic acid (C16:1 ω-7); 4 = internal standard (IS, C17:0); 5 = stearic acid (C18:0); 6 = oleic acid (C18:1 ω-9); 7 = vaccenic acid (C18:1 ω-7); 8 = linoleic acid (C18:2 ω-6); 9 = linolenic acid (C18:3 ω-3); 10 = arachidic acid (C20:0); 11 = gondoic acid (C20:1 ω-9); 12 = behenic acid (C22:0); 13 = lignoceric acid (C24:0).] GC-FID = gas chromatography-flame ionization detector.

Table 2 – Changes in fatty acid profiles of *Saedanbaek*, *Daewon*, and *Neulchan* cultivars during *Cheonggukjang* fermentation by *B. subtilis* CSY191 over time.

Fatty acids	Soybean seed	Fermentation time of *Saedanbaek* cultivar			
		0 h	12 h	24 h	48 h
C14:0	TR	TR	TR	TR	TR
C16:0	12.07 ± 0.36	12.01 ± 0.38	11.99 ± 0.31	12.07 ± 0.35	12.07 ± 0.39
C16:1 ω-7	TR	TR	TR	TR	TR
C18:0	3.62 ± 0.12	3.61 ± 0.13	3.66 ± 0.14	3.76 ± 0.13	3.78 ± 0.15
C18:1 ω-9	22.51 ± 1.21	23.12 ± 1.08	23.38 ± 1.32	22.02 ± 1.15	22.06 ± 1.20
C18:1 ω-7	1.13 ± 0.06	1.29 ± 0.07	1.26 ± 0.04	1.22 ± 0.05	1.26 ± 0.06
C18:2 ω-6	50.84 ± 3.27	50.34 ± 3.33	49.76 ± 3.11	50.58 ± 3.36	50.81 ± 3.17
C18:3 ω-3	8.27 ± 0.26	7.99 ± 0.25	8.32 ± 0.31	8.72 ± 0.29	8.36 ± 0.30
C20:0	0.41 ± 0.03	0.46 ± 0.03	0.45 ± 0.02	0.45 ± 0.04	0.46 ± 0.04
C20:1 ω-9	0.23 ± 0.02	0.25 ± 0.02	0.24 ± 0.03	0.24 ± 0.03	0.24 ± 0.02
C22:0	0.64 ± 0.05	0.66 ± 0.05	0.67 ± 0.06	0.67 ± 0.08	0.67 ± 0.07
C24:0	0.18 ± 0.02	0.17 ± 0.01	0.18 ± 0.02	0.16 ± 0.03	0.18 ± 0.02
SFA	16.93 ± 0.56	16.92 ± 0.63	16.95 ± 0.54	17.13 ± 0.57	17.16 ± 0.60
MUFA	23.87 ± 1.21	24.67 ± 1.32	24.88 ± 1.09	23.47 ± 1.37	23.56 ± 1.36
PUFA	59.11 ± 2.88	58.32 ± 2.91	58.08 ± 2.79	59.30 ± 2.93	59.18 ± 2.85
IV	130.21 ± 4.38	129.28 ± 4.29	129.33 ± 4.61	130.59 ± 4.53	130.14 ± 4.65
O/L	0.47 ± 0.04	0.49 ± 0.03	0.50 ± 0.04	0.46 ± 0.04	0.46 ± 0.05
Fatty acids	Soybean seed	Fermentation time of *Daewon* cultivar			
		0 h	12 h	24 h	48 h
C14:0	TR	TR	TR	TR	TR
C16:0	10.90 ± 0.39	10.63 ± 0.41	10.50 ± 0.38	10.59 ± 0.42	11.39 ± 0.46
C16:1 ω-7	TR	TR	TR	TR	TR
C18:0	3.41 ± 0.14	3.31 ± 0.13	3.31 ± 0.16	3.30 ± 0.17	3.41 ± 0.15
C18:1 ω-9	21.38 ± 1.31	21.82 ± 1.27	22.24 ± 1.33	21.01 ± 1.38	21.19 ± 1.34
C18:1 ω-7	1.48 ± 0.05	1.38 ± 0.06	1.36 ± 0.08	1.47 ± 0.09	1.47 ± 0.08
C18:2 ω-6	54.60 ± 3.43	54.70 ± 3.32	54.59 ± 3.35	55.25 ± 3.41	54.61 ± 3.45
C18:3 ω-3	6.88 ± 0.28	6.88 ± 0.31	6.72 ± 0.32	7.06 ± 0.33	6.61 ± 0.27
C20:0	0.38 ± 0.04	0.34 ± 0.03	0.35 ± 0.04	0.37 ± 0.05	0.35 ± 0.04
C20:1 ω-9	0.22 ± 0.03	0.22 ± 0.03	0.22 ± 0.02	0.22 ± 0.03	0.22 ± 0.02
C22:0	0.49 ± 0.05	0.47 ± 0.06	0.48 ± 0.05	0.48 ± 0.04	0.49 ± 0.06
C24:0	0.18 ± 0.03	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.03	0.19 ± 0.04
SFA	15.36 ± 0.58	14.92 ± 0.61	14.80 ± 0.56	14.91 ± 0.53	15.83 ± 0.62
MUFA	23.09 ± 1.32	23.42 ± 1.27	23.82 ± 1.30	22.70 ± 1.28	22.87 ± 1.27
PUFA	61.48 ± 3.27	61.58 ± 3.13	61.31 ± 3.24	62.31 ± 3.22	61.22 ± 3.30
IV	132.42 ± 4.47	132.88 ± 4.52	132.61 ± 4.66	133.68 ± 4.35	131.53 ± 4.28
O/L	0.42 ± 0.04	0.42 ± 0.03	0.43 ± 0.05	0.41 ± 0.04	0.41 ± 0.04
Fatty acids	Soybean seed	Fermentation time of *Neulchan* cultivar			
		0 h	12 h	24 h	48 h
C14:0	TR	TR	TR	TR	TR
C16:0	10.60 ± 0.42	10.53 ± 0.37	10.51 ± 0.41	10.46 ± 0.45	10.33 ± 0.44
C16:1 ω-7	TR	TR	TR	TR	TR
C18:0	3.72 ± 0.15	3.60 ± 0.14	3.74 ± 0.16	3.64 ± 0.18	3.61 ± 0.15
C18:1 ω-9	21.66 ± 1.26	21.89 ± 1.21	21.37 ± 1.36	21.02 ± 1.39	21.00 ± 1.24
C18:1 ω-7	1.45 ± 0.07	1.47 ± 0.06	1.45 ± 0.05	1.45 ± 0.07	1.44 ± 0.08
C18:2 ω-6	55.06 ± 3.41	54.91 ± 3.29	55.24 ± 3.84	55.77 ± 3.76	55.93 ± 3.52
C18:3 ω-3	6.63 ± 0.29	6.74 ± 0.33	6.81 ± 0.32	6.79 ± 0.28	6.90 ± 0.31
C20:0	0.34 ± 0.04	0.31 ± 0.03	0.33 ± 0.05	0.32 ± 0.04	0.31 ± 0.05
C20:1 ω-9	0.19 ± 0.02	0.19 ± 0.03	0.19 ± 0.02	0.19 ± 0.02	0.18 ± 0.03
C22:0	0.36 ± 0.05	0.36 ± 0.05	0.36 ± 0.04	0.36 ± 0.04	0.31 ± 0.04
C24:0	0.17 ± 0.01	0.15 ± 0.01	0.19 ± 0.02	0.17 ± 0.03	0.18 ± 0.02
SFA	15.02 ± 0.61	14.80 ± 0.53	14.94 ± 0.55	14.78 ± 0.52	14.55 ± 0.56
MUFA	23.30 ± 1.31	23.55 ± 1.34	23.01 ± 1.29	22.66 ± 1.32	22.62 ± 1.37
PUFA	61.68 ± 3.16	61.65 ± 3.18	62.05 ± 3.23	62.56 ± 3.29	62.82 ± 3.26
IV	132.72 ± 4.78	132.98 ± 4.52	133.27 ± 4.37	133.83 ± 4.43	134.36 ± 4.50
O/L	0.42 ± 0.03	0.43 ± 0.04	0.41 ± 0.04	0.40 ± 0.05	0.40 ± 0.05

Data represents the mean ± SD ($n = 3$).
IV = iodine value; MUFA = monounsaturated fatty acid; O/L = oleic acid/linoleic acid ratio; PUFA = polyunsaturated fatty acid; SD = standard deviation; SFA = saturated fatty acid; SRM = standard reference material; TR = trace amount (<0.1%).

1%), whereas C18:2 ω-6 and C18:1 ω-9 were the most prevalent acids regardless of fermentation time. Specifically, after 48 hours of fermentation by *B. subtilis* CSY191, the percentages of C18:1 ω9 and C18:2 ω6 in *Saedanbaek* were 22.06 ± 1.20% and 50.81 ± 3.17%, respectively. Not surprisingly, significant changes in lipid characteristics (e.g., IV and O/L) were not observed upon fermentation by *B. subtilis* CSY191 (Table 2). Similarly, the trace levels of C14:0 and C16:1 ω-7 were detected in the *Daewon* cultivar; yet, C18:2 ω-6 was the most abundant fatty acid (54.60 ± 3.43), followed by C18:1 ω-9 (21.38 ± 1.31), and C16:0 (10.90 ± 0.39). The % weights of C18:2 ω6, C18:1 ω9, and C16:0 were not affected by the fermentation time by *B. subtilis* CSY191 (Table 2). Lastly, in *Neulchan*, C18:2 ω-6 was the most abundant fatty acid (55.06 ± 3.41, % weight), relative to other cultivars, followed by C18:1 ω-9 and C16:0, respectively. In addition, the trace levels of C14:0 and C16:1 ω-7 were detected in the *Neulchan* cultivar, yet no significant change was observed after fermentation as found in the other cultivars. Of note, however, a slight difference in the fatty acid compositions of different cultivars was observed. For instance, the C18:2 ω-6 content ranged from 50.84% to 55.06%, and was the highest fatty acid content in the soybean cultivars investigated in the study. Overall, the results of the fatty acid analysis were in line with previous studies, including the recent study of Zhang and coworkers [20], who investigated 13 commercial soybean cultivars. Previously, Kim et al [21] investigated the effects of fermentation on metabolic changes in *Cheonggukjang*. In their study, the metabolites of fermented *Cheonggukjang* were significantly influenced by fermentation time (up to 72 hours) and not by the *Bacillus* strains. This may be because of nonspecific microbial enzymatic activities in reference to soybean protein. However, in the current study, the changes in fatty acid profiles were not as pronounced as those demonstrated in amino acid metabolites [21].

Associations between fatty acids detected in *Cheonggukjang* were further examined using the Pearson correlation analysis (Table 3). For instance, we noted that IV was positively correlated with PUFA ($r = 0.99$) while negatively correlated with SFA ($r = -0.95$), which is expected given that it has been utilized as an indication of degree of unsaturation of fatty acids elsewhere [22,23]. In addition, it was also observed that C18:2 ω-6 is negatively correlated with C18:1 ω9 ($r = -0.84$; $p < 0.05$), which is biologically plausible considering the catalytic activity of oleoyl-phosphatidylcholinedesaturase; this microsomal enzyme introduces a carbon double bond to produce C18:2 ω-6 from C18:1 ω-9 [24]. This negative association between two fatty acids (i.e., C18:2 ω-6 and C18:1 ω-9) has been also noted in other studies [25].

To further explore the effects of fermentation of *B. subtilis* CSY191, complete profiles of volatile compounds were monitored (Tables 4, 5, and 6 for *Saedanbaek*, *Daewon*, and *Neulchan*, respectively). To our knowledge, this is the first study to analyze volatile compounds in *Cheonggukjang* prepared by the *Saedanbaek* and *Neulchan* cultivars and monitor the time course effects of *B. subtilis* CSY191 fermentation. Intuitively, it is clear that fermentation of *B. subtilis* CSY191 produced diverse volatiles, regardless of soybean cultivars. Specifically, following fermentation, 121, 136, and 127 volatile compounds were detected in the *Saedanbaek*, *Daewon*, and *Neulchan* samples, respectively. First, we noted that levels of many ketones in *Cheonggukjang* were elevated after 12 hours of fermentation. Specifically, 13 different ketones were detected in the *Neulchan* cultivar after 48 hours of fermentation; the most prevalent volatile ketones included acetone, 2,3-butanedione, and 3-hydroxy-3-methyl-2-butanone (Table 6). In contrast, in the *Saedanbaek* cultivar, only seven ketones were shown with reduced abundance of peak areas (Table 4). Such differences between cultivars are likely driven by their lipid contents because ketones can be produced from fatty acid β oxidation via fermentation processes [26,27]. Throughout the tested cultivars, volatile acids and alcohols were mostly minor even though some alcohols, including ethanol were still noticeably high at the end of the fermentation period. Of note, it was demonstrated that the production of one alcohol—2,3-butanediol—was significantly increased after 12 hours of

Table 3 – Pearson correlation coefficients (*r*) between fatty acids of *Cheonggukjang*.

	C16:0	C18:0	C18:1 ω-9	C18:1 ω-7	C18:2 ω-6	C18:3 ω-3	C20:1 ω-9	C22:0	C22:0	C24:0	SFA	MUFA	PUFA	IV
C18:0	0.40													
C18:1 ω-9	0.69	0.23												
C18:1 ω-7	−0.84*	−0.38	−0.73											
C18:2 ω-6	−0.96*	−0.42	−0.84*	0.89*										
C18:3 ω-3	0.90*	0.50	0.67	−0.90*	−0.94*									
C20:0	0.92*	0.32	0.72	−0.78	−0.95*	0.91*								
C20:1 ω-9	0.82*	−0.11	0.67	−0.71	−0.82*	0.74	0.89*							
C22:0	0.94*	0.17	0.74	−0.84*	−0.94*	0.89*	0.96*	0.95*						
C24:0	0.66	−0.38	0.46	−0.53	−0.61	0.52	0.71	0.93*	0.82*					
SFA	0.98*	0.46	0.70	−0.84*	−0.97*	0.92*	0.94*	0.81*	0.94*	0.63				
MUFA	0.64	0.18	0.99*	−0.65	−0.80*	0.60	0.68	0.65	0.70	0.44	0.65			
PUFA	−0.94*	−0.37	−0.89*	0.84*	0.99*	−0.88*	−0.92*	−0.83*	−0.93*	−0.62	−0.94*	−0.86*		
IV	−0.95*	−0.34	−0.84*	0.79	0.96*	−0.82*	−0.91*	−0.83*	−0.92*	−0.66	−0.95*	−0.82*	0.99*	
O/L	0.87*	0.35	0.95*	−0.83*	−0.97*	0.85*	0.88*	0.78	0.88*	0.55	0.88*	0.93*	−0.98*	−0.95*

A total of 15 samples were extracted for analysis ($n = 5$ per cultivar).
IV = iodine value; MUFA = monounsaturated fatty acid; O/L = oleic acid/linoleic acid ratio; PUFA = polyunsaturated fatty acid; SFA = saturated fatty acid.
* Significant at $p < 0.05$.

Table 4 – Volatile compounds present in the *Saedanbaek* cultivar.

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
Acids							
Acetic acid	7.58	625	0.31	ND	ND	ND	ND
3-Methyl butanoic acid	18.55	859	0.15	ND	ND	ND	ND
Benzoic acid	31.59	1179	0.83	ND	ND	ND	ND
Alcohols							
Methanol	3.34	<500	18.57	1.08	0.94	0.70	0.59
Ethanol	3.83	<500	54.49	93.52	7.07	3.34	2.81
Isopropyl alcohol	4.29	505	25.95	ND	ND	ND	ND
2-Methyl-2-propanol	4.77	529	ND	2.72	ND	ND	ND
1-Propanol	5.59	564	1.20	ND	ND	ND	ND
2-Methyl-1-propanol	8.18	637	0.23	ND	0.21	0.22	0.21
2-Butanol	6.84	607	0.60	ND	ND	ND	ND
1-Butanol	10.45	679	0.50	ND	0.09	ND	ND
1-Pentanol	15.57	781	0.24	ND	ND	ND	ND
2-Methyl-1-butanol	14.29	755	0.26	ND	0.10	ND	0.08
2,2-Dimethyl-1-propanol	16.07	790	ND	ND	21.21	ND	ND
2,3-Butanediol	16.28	794	ND	ND	27.70	7.64	0.42
1-Hexanol	19.16	876	0.23	ND	ND	ND	ND
5-Methyl-2-(1-methylethyl)-1-hexanol	38.60	1411	0.10	ND	ND	ND	ND
Aldehydes							
Formaldehyde	2.98	<500	ND	ND	ND	ND	2.25
Acetaldehyde	3.28	<500	2.08	0.79	1.09	1.50	1.71
2-Methyl propanal	5.18	547	0.13	0.18	0.04	0.04	0.04
Butanal	5.99	579	0.42	ND	ND	ND	ND
3-Methyl butanal	8.57	645	0.10	0.19	ND	ND	ND
2-Methyl butanal	9.14	656	ND	0.09	ND	ND	ND
n-Pentanal	11.10	689	0.47	0.12	0.14	ND	ND
Hexanal	16.13	791	1.77	0.45	ND	ND	0.31
n-Heptanal	19.69	891	1.03	ND	ND	ND	ND
2,4-Dimethyl pentanal	21.40	934	0.14	ND	ND	ND	ND
Benzaldehyde	21.71	942	1.01	ND	ND	ND	ND
2,4-Nonadienal	22.85	968	ND	0.35	0.37	0.27	ND
Octanal	22.86	968	0.59	ND	ND	ND	1.22
Nonanal	26.84	1064	1.15	ND	ND	ND	ND
n-Decanal	32.31	1196	1.53	ND	ND	ND	ND
Undecanal	35.66	1298	0.30	ND	ND	ND	ND
Esters							
Acetic acid, methyl ester	4.76	528	1.37	ND	ND	ND	ND
Acetic acid, ethyl ester	7.15	614	9.54	2.41	3.05	2.84	1.63
Propanoic acid, 2-methyl-, methyl ester	11.19	690	ND	ND	0.23	0.40	0.59
2- Bromopropionic acid, pentyl ester	15.54	780	0.29	ND	ND	ND	ND
Butanoic acid, 3-methyl-, methyl ester	15.64	782	ND	ND	ND	0.12	0.14
Butanoic acid, 2-methyl-, methyl ester	15.73	784	ND	ND	ND	0.14	0.22
Sulfurous acid, decylpentyl ester	23.38	980	0.11	ND	ND	0.06	ND
4-Bromobenzoic acid, 2-butyl ester	35.07	1280	0.65	ND	ND	ND	ND
Hydrocarbons							
1,1-Dimethylcyclopropane	4.62	522	ND	0.02	0.05	ND	0.08
Dichloromethane	4.72	526	0.71	0.35	0.35	0.44	1.19
Cyclopentene	5.41	557	ND	ND	0.04	0.38	0.03
2-Methyl pentane	5.66	567	ND	0.32	0.38	ND	0.46
2-Methyl-1-pentene	6.24	589	ND	0.21	ND	ND	0.01
n-Hexane	6.57	600	1.00	2.53	2.16	1.58	1.65
Benzene	9.32	659	4.96	3.82	4.55	4.62	4.98
Cyclohexane	9.52	663	ND	0.38	0.19	0.24	ND
2,2,4,4-Tetramethyl pentane	11.36	693	ND	ND	0.11	ND	0.17
1-Octene	16.21	792	0.11	ND	ND	ND	0.16
2,4-Dimethyl hexane	16.60	799	0.19	0.37	0.36	1.01	ND
n-Octane	16.63	800	ND	0.41	ND	ND	0.46
2,3,4-Trimethyl hexane	17.57	829	0.06	0.11	ND	0.26	0.32
2,4-Dimethyl-1-heptene	18.23	849	0.07	0.60	0.39	0.96	0.53
Ethyl benzene	18.75	864	0.25	0.14	0.15	0.13	0.15
1,2-Dimethyl benzene	19.01	872	0.48	0.38	0.40	0.40	0.37

Table 4 – (continued)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
1-Octene	19.74	892	ND	ND	ND	0.31	1.00
1,3-Dimethyl benzene	19.80	894	0.31	0.27	0.13	0.24	0.20
n-Nonane	20.04	900	0.22	0.18	0.19	0.13	0.15
4-Methyl nonane	21.94	947	ND	0.09	ND	0.21	ND
2,3,4-Trimethyl heptane	21.95	947	ND	ND	ND	0.08	0.15
2,2,6-Trimethyl octane	22.07	950	0.91	0.61	0.82	0.73	0.79
3-Methyl undecane	23.13	974	1.40	1.69	1.79	2.06	1.83
3,3-Dimethyl undecane	23.39	980	ND	0.10	0.10	ND	0.33
2,2,5-Trimethyl heptane	23.58	984	ND	0.10	ND	0.13	0.14
3-Ethyl-3-methyl heptane	23.72	987	0.09	0.13	ND	0.20	0.14
4,5-Dimethyl nonane	23.73	988	ND	ND	0.11	ND	0.17
2,2,3-Trimethyl nonane	23.88	991	0.40	0.27	0.37	0.26	0.26
2-Bromo-octane	23.98	993	0.09	ND	ND	ND	ND
2,8,8-Trimethyl decane	24.00	994	ND	0.06	0.10	0.05	0.04
2,2-Dimethyl decane	24.15	997	2.13	1.69	2.23	1.61	1.67
2,2,4-Trimethyl decane	24.48	1005	0.96	0.79	0.99	0.72	0.76
Butyl cyclohexane	24.58	1007	0.15	0.15	ND	0.13	0.10
5,5-Dimethyl undecane	24.79	1013	4.35	3.15	4.20	3.07	3.19
3,4,5-Trimethyl heptane	25.04	1019	0.15	0.10	0.14	0.14	0.14
3-Methyl decane	25.28	1026	0.22	0.20	0.21	0.17	0.18
2,6-Dimethyl octane	25.42	1029	0.81	0.71	0.85	0.63	0.70
2,2,6-Trimethyl decane	25.60	1034	3.49	2.53	2.84	2.30	2.30
2,2,9-Trimethyl nonane	25.75	1038	3.19	2.61	3.06	2.37	2.33
2,2,3,4,6,6-Hexamethyl heptane	25.87	1041	3.24	3.07	0.02	0.01	0.02
2,2-Dimethyl-3-decene	26.09	1046	ND	0.21	0.23	0.17	0.17
2,2,4,6,6-Pentamethyl heptane	26.26	1050	0.16	0.12	0.12	0.07	0.16
4-Methyl dodecane	26.47	1055	2.49	ND	2.33	1.80	1.76
2,2,7,7-Tetramethyl octane	26.66	1060	0.12	0.28	0.26	0.19	ND
2,2,6,6-Tetramethyl octane	26.67	1060	ND	ND	ND	ND	0.23
2,3,4-Trimethyl decane	26.90	1066	ND	0.37	0.37	0.28	0.74
5-(2-Methylpropyl)-nonane	27.04	1069	ND	ND	1.02	0.61	0.98
5-Butyl nonane	27.05	1070	0.77	0.89	ND	ND	ND
5-Methyl-5-propyl nonane	27.37	1077	0.79	0.69	0.71	0.59	0.51
2,4-Dimethyl undecane	27.71	1085	0.15	ND	ND	0.09	0.05
2,2,3,4-Tetramethyl pentane	28.00	1092	0.08	ND	ND	ND	ND
3,7-Dimethyl nonane	28.15	1095	0.17	0.05	0.12	0.09	0.07
9-Methyl-2-undecene	28.27	1098	ND	ND	ND	0.11	0.16
3-Methyl-5-undecene	28.29	1099	0.20	0.08	ND	ND	ND
3-Methyl-2-undecene	28.30	1099	ND	ND	0.15	0.14	0.12
2,5,5-Trimethyl heptane	28.48	1103	0.12	ND	ND	ND	ND
4-Ethyl-2,2,6,6-tetramethyl heptane	28.63	1107	ND	ND	ND	ND	0.13
2,2,4-Trimethyl decane	28.64	1107	0.17	0.17	0.19	ND	ND
2,2-Dimethyl octane	28.65	1108	ND	ND	ND	0.11	ND
Dodecane	32.49	1200	0.58	0.80	1.01	0.19	ND
1,5-Diethyl-2,3-dimethyl cyclohexane	32.59	1203	0.19	ND	ND	ND	ND
1,4-Dicyclohexyl butane	32.61	1204	ND	0.28	0.45	0.20	ND
Ketones							
Acetone	4.10	<500	38.78	14.97	6.32	5.09	16.11
2,3-Butanedione	5.88	575	0.93	ND	18.20	21.88	8.99
2-Butanone	6.30	591	1.00	0.22	0.78	0.32	0.50
3-Methyl-2-butanone	9.15	656	ND	ND	ND	0.30	0.82
2-Pentanone	10.91	686	0.06	0.10	0.18	0.12	0.15
3-Pentanone	11.01	688	ND	ND	0.13	0.11	ND
4-Methyl-2-pentanone	13.80	745	ND	ND	ND	ND	0.17
3-Methyl-2-pentanone	14.42	758	ND	ND	ND	ND	1.12
Cyclopentanone	15.68	783	0.16	ND	ND	ND	ND
Cyclohexanone	19.51	886	0.27	ND	ND	ND	ND
3-Methyl-2-hexanone	20.15	903	0.17	0.14	0.14	0.12	ND
6-Methyl-5-hepten-2-one	22.40	958	0.20	ND	ND	ND	ND
Miscellaneous							
Dimethyl sulfide	4.52	517	11.82	0.13	0.11	0.16	ND

(continued on next page)

Table 4 – (*continued*)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
2,5-Dihydro-furan	5.48	560	0.01	ND	ND	ND	ND
Dimethyl disulfide	13.70	743	ND	ND	0.24	0.22	0.22
2,5-Dimethyl pyrazine	20.52	912	ND	ND	ND	ND	0.39
Benzothiazole	33.84	1243	0.27	ND	ND	ND	ND
1,3-Isobenzofurandione	35.97	1310	0.38	ND	ND	ND	ND

Volatiles were collected at various fermentation time points and represented as peak area. The data represents the means of duplicates. The gas chromatographic retention data and mass spectral data were compared to those of authentic samples and library compounds, respectively. ND = not detected.

Table 5 – Volatile compounds present in the *Daewon* cultivar.

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
Acids							
Acetic acid	7.54	623	0.95	ND	0.23	ND	ND
2-Ethyl butanoic acid	18.00	842	ND	ND	0.07	0.16	0.22
Alcohols							
Methanol	3.34	<500	10.12	1.50	ND	ND	1.77
Ethanol	3.83	<500	86.51	84.82	3.30	2.63	1.93
Isopropyl alcohol	4.29	501	14.08	ND	ND	ND	ND
1-Propanol	5.59	563	2.79	ND	ND	ND	ND
2-Butanol	6.84	607	1.29	ND	ND	ND	ND
2-Methyl-1-propanol	8.17	637	1.31	ND	0.12	0.16	0.14
1-Butanol	10.53	679	0.25	0.07	0.13	0.08	ND
1-(1-Methylethoxy)-2-propanol	11.16	689	ND	ND	ND	ND	0.83
3-Pentanol	13.53	738	ND	ND	ND	ND	0.09
3-Methyl-3-buten-1-ol	13.96	748	ND	ND	ND	ND	0.09
3-Methyl-1-butanol	14.18	752	0.45	ND	ND	ND	0.12
2-Methyl-1-butanol	14.26	754	1.12	ND	ND	ND	0.10
1-Pentanol	15.62	781	0.25	ND	ND	ND	ND
5-Methyl-2-heptanol	16.22	793	ND	ND	ND	ND	0.30
2,3-Butanediol	16.38	795	0.37	ND	24.84	9.22	0.18
3-Methyl-2,4-pentanediol	16.47	797	ND	ND	ND	ND	0.10
1-Hexanol	19.22	878	0.29	ND	ND	ND	ND
3-Methyl-1-heptanol	34.86	1274	0.16	ND	ND	ND	ND
Aldehydes							
Formaldehyde	2.99	<500	2.32	2.16	1.81	1.36	1.05
Acetaldehyde	3.27	<500	4.53	1.52	2.19	2.76	0.87
2-Methyl propanal	5.18	545	0.16	0.24	0.08	0.06	ND
3-Methyl butanal	8.58	645	0.11	0.25	0.04	ND	ND
2-Methyl butanal	9.14	656	0.08	0.10	ND	ND	ND
n-Pentanal	11.12	688	0.17	0.16	0.19	ND	ND
n-Hexanal	16.12	791	2.40	0.91	ND	ND	0.15
n-Heptanal	19.73	892	ND	2.11	ND	ND	ND
2-Methyl pentanal	19.75	892	0.35	ND	ND	ND	ND
2-Ethyl butanal	20.15	904	ND	ND	ND	ND	0.14
2,4-Nonadienal	22.85	992	ND	2.14	ND	0.39	ND
n-Decanal	32.42	1198	ND	2.64	3.08	1.38	ND
Esters							
Formic acid, butyl ester	3.33	<500	ND	1.32	1.15	ND	ND
Acetic acid, methyl ester	4.76	525	2.00	ND	ND	ND	ND
Acetic acid, ethyl ester	7.14	614	2.56	2.32	2.61	2.29	2.33
Butanoic acid, 3-methyl-, methyl ester	15.63	781	ND	ND	ND	0.14	0.21
Butanoic acid, 2-methyl-, methyl ester	15.72	783	ND	ND	ND	0.19	0.52
Acetic acid, butyl ester	16.92	809	ND	ND	0.41	0.02	ND
Sulfurous acid, decylpentyl ester	23.38	1005	ND	0.11	0.08	0.11	ND
Sulfurous acid, 2-ethylhexyl hexyl ester	35.67	1298	ND	0.32	0.05	ND	ND

Table 5 – (continued)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
Hydrocarbons							
Pentane	4.27	500	ND	ND	2.49	1.92	ND
Cyclopentene	5.32	551	ND	ND	0.06	0.05	0.07
2-Methyl pentane	5.62	564	ND	ND	0.29	ND	0.19
3-Methyl-pentane	6.04	581	0.46	0.40	ND	ND	ND
2-Butanone	6.28	590	3.61	0.26	1.66	0.83	3.68
n-Hexane	6.56	600	1.40	1.65	1.96	1.45	1.47
Methyl cyclopentane	7.73	628	0.43	0.17	0.73	0.81	0.14
Benzene	9.31	659	1.96	1.75	2.21	2.62	2.77
Cyclohexane	9.51	662	0.15	0.07	0.27	0.31	0.22
4-Methyl-1-hexene	11.39	693	0.23	ND	0.08	0.09	ND
n-Heptane	11.91	700	0.20	ND	ND	ND	0.32
Methyl benzene	15.05	770	7.52	10.69	8.60	7.68	5.45
4-Methyl heptane	15.34	776	0.78	ND	0.83	0.72	ND
2,3,4-Trimethyl pentane	15.37	776	ND	0.34	ND	ND	0.35
1-Octene	16.27	793	ND	0.46	ND	ND	ND
2,4-Dimethyl hexane	16.60	799	0.63	ND	0.91	0.70	ND
n-Octane	16.63	800	ND	0.41	ND	ND	0.35
2,3,4-Trimethyl hexane	17.56	829	0.19	ND	ND	ND	0.26
2,4-Dimethyl-1-heptene	18.22	849	0.67	0.26	0.91	0.72	0.34
Ethyl benzene	18.74	864	0.19	0.20	0.23	0.12	0.13
1,2-Dimethyl benzene	19.01	872	0.41	0.40	0.39	0.34	0.40
2,2,4-Trimethyl pentane	19.20	877	ND	ND	0.04	0.06	ND
n-Nonane	20.04	900	0.25	0.32	0.23	0.23	0.24
2,4-Dimethyl hexane	20.06	901	ND	ND	ND	0.15	ND
2,2,6,6-Tetramethyl heptane	21.08	935	ND	ND	ND	ND	0.04
2,3,4-Trimethyl heptane	21.95	964	0.15	ND	ND	ND	ND
2,2,6-Trimethyl octane	22.06	967	1.06	0.85	0.93	0.88	1.07
3,3,4-Trimethyl hexane	22.38	977	ND	ND	ND	ND	0.06
2,2,3,5-Tetramethyl heptane	22.49	981	0.42	0.35	0.38	0.25	0.34
3-Ethyl-2,2-dimethyl pentane	22.52	982	0.33	ND	ND	ND	ND
2,2,7-Trimethyl decane	22.59	984	0.24	ND	ND	0.18	0.24
2,2,7,7-Tetramethyl octane	22.64	985	ND	ND	ND	ND	0.12
2,2-Dimethyl octane	22.68	987	ND	ND	0.08	0.08	ND
Decane	23.12	1000	1.78	1.26	1.26	1.21	ND
3-Ethyl-3-methyl heptane	23.15	1001	ND	ND	ND	ND	0.94
3,3-Dimethyl undecane	23.39	1006	0.11	ND	ND	ND	ND
2,2,5-Trimethyl heptane	23.64	1011	0.14	ND	0.10	ND	ND
2,2,3-Trimethyl nonane	23.87	1016	0.40	0.31	0.35	ND	0.32
3,3,8-Trimethyl decane	23.92	1017	ND	0.15	ND	ND	ND
2,3,4-Trimethyl decane	23.98	1018	ND	ND	0.07	0.05	ND
2,2-Dimethyl decane	24.14	1021	2.38	1.81	1.98	1.72	2.09
2,2,4-Trimethyl decane	24.47	1028	1.11	0.80	0.87	0.74	0.96
Butyl cyclohexane	24.57	1030	0.13	0.11	0.08	ND	ND
2,3,5-Trimethyl decane	24.78	1034	4.47	3.27	3.66	3.30	3.94
3,4,5-Trimethyl heptane	25.03	1039	0.16	0.11	0.13	0.11	0.17
3-Methyl decane	25.27	1044	0.25	0.17	0.20	0.16	0.20
2,6-Dimethyl octane	25.41	1046	0.94	0.66	0.76	0.64	0.73
2,2,6-Trimethyl decane	25.59	1050	3.26	2.27	2.59	2.32	2.68
2,2,3,4,6,6-Hexamethyl heptane	25.74	1053	3.43	2.33	2.62	2.29	2.77
2,2,9-Trimethyl nonane	25.95	1057	0.18	0.02	0.03	0.02	ND
2,2-Dimethyl-3-decene	26.08	1059	0.32	0.13	0.14	0.17	0.21
2,2,4,6,6-Pentamethyl heptane	26.25	1062	0.20	0.04	0.15	0.08	0.08
4-Methyl dodecane	26.45	1066	2.63	1.68	1.95	1.67	2.00
2,2,7,7-Tetramethyl octane	26.66	1070	0.37	0.21	0.25	0.19	0.25
3,3,5-Trimethyl decane	26.97	1076	0.49	ND	0.21	ND	0.27
5-(2-Methylpropyl)-nonane	27.03	1077	1.29	1.09	1.05	0.68	0.74
5-Methyl-5-propyl nonane	27.35	1082	0.74	0.48	0.55	0.38	0.55
6-Ethyl-2-methyl octane	27.46	1084	ND	ND	ND	ND	0.14
2,2,3,4-Tetramethyl pentane	27.68	1088	ND	ND	ND	ND	0.03
3,7-Dimethyl nonane	28.14	1096	0.14	0.30	0.04	0.05	0.07

(continued on next page)

Table 5 – (*continued*)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
9-Methyl-2-undecene	28.25	1098	ND	0.07	ND	0.08	0.11
1,3-Dimethyl cyclopentane	28.28	1099	0.11	ND	0.08	ND	ND
2,2,6-Trimethyl octane	28.60	1106	ND	0.10	0.11	ND	0.13
4-Ethyl-2,2,6,6-tetramethyl heptane	28.65	1108	0.18	ND	ND	ND	ND
2,4-Dimethyl-2,6-octadiene	32.67	1206	0.30	ND	ND	ND	ND
5-Undecene	33.17	1222	ND	ND	ND	ND	0.05
1-Methyl-3-(1-methylethyl)-cyclopentane	33.55	1234	0.11	ND	ND	0.08	ND
Octyl cyclohexane	34.22	1255	0.10	ND	0.09	ND	ND
2,3,8-Trimethyl decane	35.69	1299	0.12	ND	ND	ND	ND
(3-Methylpentyl)-cyclohexane	37.86	1383	0.08	ND	ND	ND	ND
1,7-Dimethyl-4-(1-methylethyl)-cyclodecane	38.06	1391	0.03	ND	ND	ND	ND
Ketones							
Acetone	4.09	<500	43.62	18.15	9.90	8.88	46.18
2,3-Butanedione	5.87	574	0.94	ND	31.89	24.72	2.33
3-Methyl-2-butanone	9.12	655	ND	ND	ND	0.33	3.02
2-Pentanone	10.89	685	0.21	ND	0.10	0.17	0.42
3-Hydroxy-2-butanone	11.97	702	0.85	ND	128.34	69.71	0.78
3-Penten-2-one	12.33	710	0.32	ND	ND	ND	ND
3-Hydroxy-3-methyl-2-butanone	13.41	736	ND	ND	0.80	3.19	ND
4-Methyl-2-pentanone	13.78	744	ND	ND	0.08	0.12	0.28
3-Methyl-2-pentanone	14.40	757	ND	ND	0.20	0.50	3.89
4,4-Dimethyl-2-pentanone	15.94	787	ND	ND	ND	ND	0.06
2-Heptanone	19.43	883	ND	ND	ND	ND	1.17
5-Methyl-2-hexanone	19.46	884	ND	ND	0.07	0.73	2.38
Cyclohexanone	19.55	887	0.02	0.04	ND	ND	ND
3-Methyl-2-hexanone	20.17	905	0.11	ND	ND	ND	ND
6-Methyl-2-heptanone	21.41	946	ND	ND	ND	0.18	0.88
5-Methyl-2-heptanone	21.75	957	ND	0.25	0.34	ND	0.61
Miscellaneous							
Ethyl ether	4.40	507	ND	0.29	1.03	2.04	1.44
Dimethyl sulfide	4.49	512	0.55	0.20	0.35	ND	ND
Methylene chloride	4.71	523	0.40	0.57	1.27	0.78	1.70
Thiofuran	9.63	664	ND	0.06	0.08	0.01	ND
2-Ethyl furan	11.84	699	ND	0.73	ND	ND	0.18
Dimethyl disulfide	13.69	742	ND	ND	ND	ND	0.07
2,5-Dimethyl pyrazine	20.47	915	ND	ND	ND	0.24	3.04
2-Pentyl furan	22.84	991	ND	ND	ND	ND	0.33
Dihexyl sulfide	29.28	1124	0.10	ND	ND	ND	ND

Volatiles were collected at various fermentation time points and represented as peak area. The data represents the means of duplicates. The gas chromatographic retention data and mass spectral data were compared to those of authentic samples and library compounds, respectively. ND = not detected.

Table 6 – Volatile compounds present in the *Neulchan* cultivar.

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
Acids							
Acetic acid	7.34	619	0.36	ND	ND	ND	ND
2-methyl propanoic acid	15.72	784	0.06	ND	0.17	0.41	1.84
2-Ethyl butanoic acid	17.91	841	ND	ND	ND	ND	0.13
Alcohols							
Methanol	3.33	<500	0.19	ND	0.50	ND	0.99
Ethanol	3.82	<500	5.51	28.53	12.02	25.96	2.45
Isopropyl Alcohol	4.31	502	1.21	ND	ND	ND	ND
1-Propanol	5.57	562	0.64	ND	ND	ND	ND
2-Ethyl cyclobutanol	6.03	580	0.03	ND	ND	ND	ND
2-Butanol	6.82	607	0.23	ND	ND	ND	ND
2-Methyl-2-propanol	7.60	625	ND	ND	ND	0.42	ND
2-Methyl-1-propanol	8.13	636	0.24	ND	0.22	0.17	ND

Table 6 – (continued)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
4-Methoxy-1-butanol	11.15	690	0.11	ND	ND	ND	ND
1-Methoxy-2-propanol	11.19	690	0.04	ND	ND	ND	ND
3-Methyl-2-butanol	12.17	708	0.11	ND	ND	ND	ND
3-Methyl-3-buten-1-ol	13.81	745	ND	ND	ND	ND	ND
3-Methyl-1-butanol	14.14	752	0.20	ND	0.32	0.34	0.29
2-Methyl-1-butanol	14.25	755	0.34	ND	0.11	0.15	0.13
1-Pentanol	15.61	782	0.04	ND	ND	ND	ND
2,3-Butanediol	16.15	792	0.09	ND	80.13	46.01	ND
2-Methyl-3-hexanol	18.49	858	ND	ND	0.07	ND	ND
5-Methyl-1-hexanol	19.72	892	ND	ND	0.24	ND	ND
1-Hepten-3-ol	22.30	976	ND	ND	ND	ND	0.32
Aldehydes							
Acetaldehyde	3.27	<500	0.19	3.15	1.65	2.92	2.54
2-Methyl propanal	5.17	545	0.02	0.15	0.11	0.14	0.12
3-Methyl butanal	8.57	645	0.02	0.16	ND	0.26	0.12
2-Methyl butanal	9.13	656	0.02	ND	ND	ND	ND
n-Pentanal	11.07	688	0.02	ND	ND	ND	ND
n-Hexanal	16.12	791	0.23	0.30	ND	ND	0.07
2-Heptenal	19.63	890	ND	ND	ND	ND	0.19
Benzaldehyde	21.78	959	ND	ND	ND	1.55	ND
Esters							
Acetic acid, methyl ester	4.79	527	0.06	ND	ND	0.53	ND
Propanoic acid, 2-hydroxy-2-methyl-, ethyl ester	4.81	528	ND	0.27	0.35	ND	ND
Acetic acid, ethyl ester	7.12	614	0.41	2.12	4.72	4.97	4.39
Propanoic acid, 2-methyl-, methyl ester	11.10	689	ND	ND	ND	ND	0.64
Propanoic acid, 2-methyl-, ethyl ester	14.89	781	ND	ND	0.11	ND	0.07
Butanoic acid, 3-methyl-, methyl ester	15.59	952	ND	ND	ND	0.21	0.32
Propanoic acid, 2-methyl-, pentyl ester	21.57	962	ND	ND	ND	ND	0.19
Acetic acid, methoxy-, ethyl ester	21.87	964	ND	ND	ND	ND	0.16
Benzoic acid, pentyl ester	21.93	527	0.01	ND	ND	ND	ND
Hydrocarbons							
Pentane	4.27	500	ND	4.45	5.08	2.56	ND
2-Methyl butane	4.28	501	ND	ND	ND	ND	0.24
n-Hexane	6.56	600	0.10	1.38	1.17	1.14	1.11
Methyl cyclopentane	7.72	627	ND	0.20	0.40	0.20	0.23
Methoxy ethane	8.15	637	ND	ND	ND	ND	0.35
Benzene	9.29	659	0.46	4.31	4.17	11.08	5.62
Methyl benzene	15.03	770	0.55	6.51	6.39	5.67	7.44
2,3,4-Trimethyl pentane	15.30	776	ND	0.25	ND	ND	0.36
4-Methyl heptane	15.34	777	ND	ND	0.45	0.28	ND
3-Methylene heptane	16.19	793	0.10	ND	ND	ND	ND
n-Octane	16.60	800	ND	0.59	0.94	1.03	0.83
3-Methyl hexane	16.62	801	0.02	ND	ND	ND	ND
3,3-Dimethyl hexane	17.55	830	ND	ND	0.08	0.08	ND
2,4-Dimethyl-1-heptene	18.20	849	ND	0.29	0.58	0.50	0.31
3,7-Dimethyl-1-octene	18.23	850	0.00	ND	ND	ND	ND
Ethyl benzene	18.72	864	0.02	0.08	0.14	0.17	0.20
1,2-Dimethyl benzene	18.99	872	0.03	0.13	0.34	0.27	0.79
1-Octene	19.73	892	0.03	ND	ND	ND	ND
1,3-Dimethyl benzene	19.77	894	0.04	0.10	0.14	0.23	0.14
n-Nonane	20.01	900	0.02	0.15	ND	0.21	0.31
2,2,6-Trimethyl octane	22.04	967	0.10	0.93	1.30	1.03	0.62
3-Ethyl-2,2-dimethyl pentane	22.49	982	0.03	0.29	0.42	0.74	0.24
2,2,7-Trimethyl decane	22.58	984	0.03	0.17	0.28	0.31	ND
2,2,3,5-Tetramethyl heptane	22.69	988	ND	0.07	0.12	0.12	ND
1,2,3-Trimethyl benzene	22.90	994	ND	ND	ND	ND	ND
Decane	23.09	1000	ND	1.34	2.69	1.16	0.73
3-Ethyl-3-methyl heptane	23.12	1001	0.08	ND	ND	ND	ND
3,3-Dimethyl undecane	23.37	1006	0.01	ND	ND	ND	ND
3,3,4-Trimethyl heptane	23.46	1008	ND	ND	ND	ND	0.01
3,3,5-Trimethyl heptane	23.54	1009	ND	0.11	ND	ND	0.03

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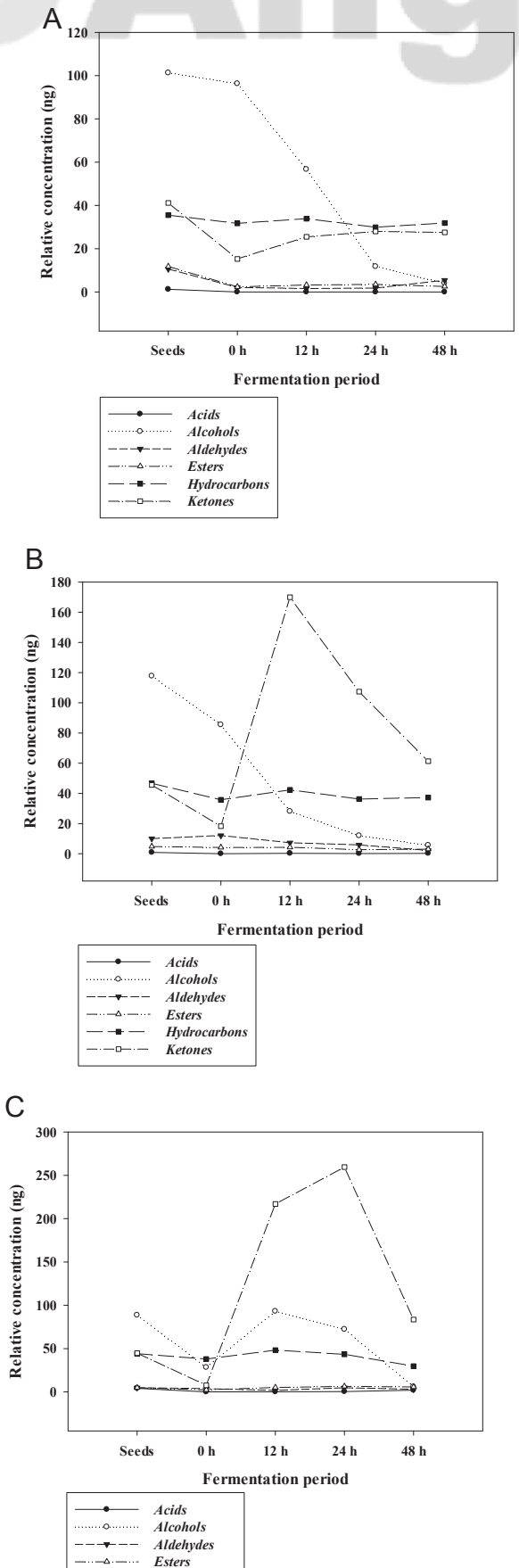
Table 6 – (*continued*)

Compounds	Retention time (min)	Retention index	Relative concentration (ng)				
			Soybean seed	Fermentation time			
				0 h	12 h	24 h	48 h
2,3,4-Trimethyl decane	23.60	1011	ND	ND	ND	ND	0.03
2,2,5-Trimethyl heptane	23.71	1013	ND	0.05	0.15	0.12	ND
2,2,3-Trimethyl nonane	23.84	1016	0.06	0.34	0.54	0.54	0.19
4-Methyl decane	23.99	1019	0.01	ND	ND	ND	ND
2,2-Dimethyl decane	24.12	1021	0.33	1.98	2.82	1.91	1.25
2,2,4-Trimethyl decane	24.43	1027	0.13	0.85	1.15	ND	0.43
Butyl cyclohexane	24.57	1030	ND	0.11	0.13	0.12	ND
2,3,5-Trimethyl decane	24.75	1034	0.62	3.60	5.22	3.65	2.48
3,4,5-Trimethyl heptane	24.99	1039	0.02	0.13	0.17	ND	0.12
2,3,6,7-Tetramethyl octane	25.26	1044	ND	0.18	0.24	0.16	ND
2,6-Dimethyl octane	25.38	1046	0.10	0.67	0.93	0.52	0.39
2,2,6-Trimethyl decane	25.56	1050	0.42	2.53	3.58	2.10	1.63
2,2,3,4,6,6-Hexamethyl heptane	25.71	1052	0.41	2.59	3.62	2.36	1.58
2,2,9-Trimethyl nonane	25.88	1056	ND	0.02	0.04	1.20	ND
2,2-Dimethyl-3-decene	26.04	1059	0.03	0.19	0.28	ND	0.10
3,3,7-Trimethyl decane	26.30	1063	ND	ND	ND	ND	1.23
2,2,4,6,6-Pentamethyl heptane	26.35	1064	0.02	0.16	0.19	ND	ND
4-Methyl dodecane	26.44	1066	0.30	1.88	2.60	1.70	ND
5-Ethyl-2,2,3-trimethyl-heptane	26.65	1070	ND	ND	ND	0.38	ND
2,2,7,7-Tetramethyl octane	26.66	1070	0.03	ND	0.23	ND	ND
3,3,8-Trimethyl decane	26.88	1074	0.04	0.25	0.35	0.25	ND
5-(2-Methylpropyl)-nonane	26.99	1076	0.09	0.49	0.68	0.55	0.46
5-Methyl-5-propyl nonane	27.32	1082	0.07	0.53	0.75	0.40	0.24
3,7-Dimethyl nonane	27.92	1093	0.01	0.13	0.03	0.06	0.06
3-Methyl-2-undecene	28.20	1097	0.01	ND	ND	ND	ND
9-Methyl-2-undecene	28.26	1098	ND	0.13	0.07	0.06	ND
1,3-Dimethyl cyclopentane	28.28	1099	0.02	ND	ND	ND	ND
2,2,5,5-Tetramethyl hexane	28.44	1102	ND	ND	ND	ND	0.08
2,2,6-Trimethyl octane	28.62	1107	ND	0.14	0.14	ND	ND
2,2,9-Trimethyl decane	28.65	1108	0.02	ND	ND	ND	ND
Dodecane	32.49	1200	0.10	ND	ND	0.60	ND
Pentyl cyclohexane	32.53	1201	ND	ND	ND	0.16	0.19
Ketones							
Acetone	4.09	<500	3.79	7.43	11.47	23.97	45.96
1-Buten-1-one	5.54	561	ND	ND	0.08	ND	ND
2,3-Butanedione	5.86	574	ND	ND	33.73	64.51	11.29
2-Butanone	6.25	589	0.42	0.13	1.06	2.16	3.54
3-Methyl-2-butanone	9.08	655	ND	ND	ND	0.44	1.82
2-Pentanone	10.83	685	0.06	ND	0.15	0.39	0.71
3-Pentanone	11.33	692	0.03	ND	ND	ND	ND
3-Hydroxy-2-butanone	11.99	703	0.21	0.19	171.16	160.01	4.63
3-Hydroxy-3-methyl-2-butanone	13.39	736	0.02	ND	0.71	7.34	7.99
4-Methyl-2-pentanone	13.72	743	ND	ND	ND	0.08	0.21
3-Methyl-2-pentanone	14.34	756	ND	ND	0.20	0.72	3.11
5-Methyl-2-hexanone	18.36	854	ND	ND	ND	0.59	2.77
6-Methyl-2-heptanone	21.32	944	ND	ND	ND	ND	1.26
5-Methyl-2-heptanone	21.65	955	ND	ND	ND	ND	1.07
3-Pentanone	28.56	1105	ND	ND	ND	ND	0.01
Miscellaneous							
Dimethyl sulfide	4.51	513	0.05	0.10	0.12	ND	ND
Methylene Chloride	4.71	523	0.05	0.32	0.42	0.80	1.17
2-Methyl furan	6.99	611	0.13	ND	ND	ND	ND
2-Ethyl furan	11.79	699	ND	1.84	ND	ND	0.31
Dimethyl disulfide	13.66	742	ND	ND	0.10	0.06	0.14
2,3,5-Trimethyl furan	17.06	815	ND	ND	ND	0.04	0.06
2,5-Dimethyl pyrazine	20.45	915	0.01	ND	ND	1.05	5.44
2-Pentyl furan	22.81	991	0.02	0.57	1.27	1.35	0.87
Trimethyl pyrazine	23.17	1002	ND	ND	ND	ND	4.69
Tetramethyl pyrazine	26.58	1069	ND	ND	ND	0.45	0.63

Volatiles were collected at various fermentation time points and represented as peak area. The data represents the means of duplicates. The gas chromatographic retention data and mass spectral data were compared to those of authentic samples and library compounds, respectively. ND = not detected.



fermentation and then gradually decreased afterward. This trend was demonstrated in all cultivars, but with varying magnitudes, and was similar to another study that highlighted that this alcohol is produced in the late fermentation stage of tempeh, another fermented soybean food, rather than the early period [28]. In terms of numbers of volatile compounds, hydrocarbons are the most prevalent group of volatiles in *Cheonggukjang*. Specifically, 62, 71, and 62 hydrocarbons were produced during the fermentation processes in *Saedanbaek*, *Daewon*, and *Neulchan* cultivars, respectively. Although this class of compounds has a restricted use as food ingredients, they are widely present in nature and used as important flavor materials [29]. Lastly, various pyrazines, compounds responsible for pungent and unpleasant *Cheonggukjang* flavors, were detected at the end of fermentation. Interestingly, the high-oil cultivar (i.e., *Neulchan*) had much higher signals compared to the high-protein cultivar (i.e., *Saedanbaek*). More specifically, three pyrazines were detected in *Neulchan* (2,5-dimethyl pyrazine, trimethylpyrazine, and tetramethylpyrazine), whereas only 2,5-dimethyl pyrazine was detected in the *Saedanbaek* sample. The peak area for this compound was approximately 14-folds higher in *Neulchan*.

Owing to the large numbers of volatiles detected in the system, we further categorized compounds into several classes: acids, alcohols, aldehydes, esters, hydrocarbons, and ketones. Changes in volatile compounds of *Cheonggukjang* samples are depicted in Figure 3. We were able to find the significant reduction in alcohols in the *Saedanbaek* samples throughout the fermentation periods. In contrast, ketones were gradually increased. Differences between seed samples and initiation of fermentation (i.e., 0 hour) are likely driven by heat treatment, meaning boiling beans (Figure 3). In the *Daewon* cultivar, similar trends were demonstrated. Alcohols were decreased throughout the fermentation processes whereas ketones were significantly increased at 12 hours of fermentation. Later, however, such increases were diminished over time. Lastly, of the volatile compounds analyzed, alcohols and ketones were also two major classes of volatiles that showed changes in the *Neulchan* cultivar; ketones decreased initially, but significantly increased up to 24 hours of fermentation. However, at this point, it is difficult to predict which soybean cultivar may confer more favorable sensory attributes for consumers because there are potential associations between different volatile chemicals [30]. Therefore, further comprehensive sensory evaluation might help to better understand and evaluate consumers' preferences for different soybean cultivars.

This study was conducted at the request of the soybean industry, to reexamine and update compositional information of *Cheonggukjang* made with novel Korean soybean cultivars. Given the paucity of studies on: (1) time course effects of fermentation on nutritional characteristics, (2) impacts of this

Figure 3 – Changes of volatile compounds in (A) *Saedanbaek*, (B) *Daewon*, and (C) *Neulchan*. [Symbols: ●, acids; ○, alcohols; ▼, aldehydes; △, esters; ■, hydrocarbons; and □, ketones.].

probiotic strain (i.e., *B. subtilis* CSY191) on soybean products including *Cheonggukjang*, and (3) characteristics of the soybean cultivars investigated in this study (i.e., *Saedanbaek*, *Daewon*, and *Neulchan*), results herein provide important preliminary data relating to the complete profiles of fatty acids and volatile compounds of these soybeans to monitor potential influences of the fermentation processes on one of the most commonly consumed Korean fermented foods. It is further expected that the findings of this research will be used for the nutrient database of *Cheonggukjang* and permit soybean researchers (e.g., breeders and geneticists) to develop significant relationships between important nutrients in fermented soybeans more easily. Although the fermentation period was not a strong correlate to changes in fatty acid profiles, we noted that profiles of volatiles in *Cheonggukjang* changed over time and were different between cultivars; thus, further sensory evaluation might be needed to determine if such differences influence consumers' preferences. Furthermore, additional studies are warranted to determine the associations between *B. subtilis* CSY191 fermentation and other nutritional components (e.g., amino acids) and their health-promoting potential in animal models.

Conflict of interest

All contributing authors declare no conflicts of interest.

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