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, behenic, and lignoceric acids—and 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.

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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 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 (3000g) 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-Ngwinbi’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:

\[ R_i = \frac{P_{gi} \times W_{C17:0}}{P_{si} \times W_{si}} \]

where \( R_i \) refers to each relative response factor for fatty acid \( i \), \( P_{gi} \) is the peak area of each FAME \( i \) in the FAME standard solution, \( W_{C17:0} \) is the mass (mg) of the C17:0 FAME, \( P_{si} \) is the peak area of C17:0 FAME, and \( W_{si} \) 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:

\[ \% \text{ RSD} = \frac{\text{standard deviation} \times 100}{\text{mean}} \]

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

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

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]:

\[ RI_x = 100n + 100((t_{ex} - t_{so})/(t_{Rn+1} - t_{so})) \]

where \( RI_x \) is the RI of the observed compound, \( t_{ex} \) is the retention time of the observed compound, \( t_{so} \) 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]:

\[ \text{Quantification} = \frac{(PA_x)}{(PA)} \times \text{mass of the IS} \]

where \( PA_x \) is the peak area of observed compound and \( PA \) 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
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 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

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**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).

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

| Fatty acids | Weight percentage (%) | Accepted value* | Analytical value* | Bias | % of accepted value* | % RSD* |
|------------|------------------------|-----------------|------------------|------|---------------------|--------|
| 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-7  | 50.46 ± 5.50           | 51.36 ± 2.75    | −0.89            | 101.77 ± 3.60 | 5.35 |
| C18:2 ω-6  | 1.04 ± 0.03            | 1.04 ± 0.04     | 0.00             | 103.60 ± 0.40 | 4.22 |
| C18:3 ω-3  | 25.92 ± 2.10           | 25.31 ± 1.12    | 0.61             | 97.63 ± 2.63 | 4.42 |
| C20:0      | 0.40 ± 0.01            | 0.38 ± 0.02     | 0.02             | 96.25 ± 0.12 | 5.20 |
| C20:1 ω-9  | 0.29 ± 0.02            | 0.28 ± 0.01     | 0.01             | 96.42 ± 0.10 | 3.56 |
| C22:0      | 2.55 ± 0.25            | 2.51 ± 0.08     | 0.04             | 98.30 ± 0.43 | 3.19 |
| 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.  
* 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 | Fermentation time of Daewon cultivar | Fermentation time of Neulchan cultivar |
|-------------|--------------|------------------------------------------|--------------------------------------|----------------------------------------|
|             |              | 0 h                                      | 12 h                                 | 24 h                                   | 48 h                                   |
|             |              | 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 | 58.52 ± 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                            |

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 ω-9 and C18:2 ω-6 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 ω-6, C18:1 ω-9, 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 relation 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 ω-9 ($r = -0.84$; $p < 0.05$), which is biologically plausible considering the catalytic activity of oleoyl-phosphatidylcholine desaturase; 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 fermentation. 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 relation 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].

**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 ω-6 | C20:1 ω-9 | C22:0 | C24:0 | SFA | MUFA | PUFA | IV |
|-------|-------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-------|-------|-----|------|------|---|
| 0.40  | 0.69  | 0.23      | -0.84*    | -0.38     | -0.73     | -0.96*    | -0.42     | -0.84*    | 0.89* | 0.90* | 0.50 | 0.67 | -0.90* | -0.94* | 0.92* | 0.32 | 0.72 | -0.78 | -0.95 | 0.91* | 0.82* | 0.11 | 0.67 | -0.71 | -0.82* | 0.74 | 0.89* |
| 0.94* | 0.17  | 0.74      | -0.84*    | -0.94*    | 0.89*     | 0.96*     | 0.95*     | 0.66      | -0.38 | 0.46 | 0.70 | -0.84* | -0.97* | 0.92* | 0.94* | 0.81* | 0.94* | 0.63 | 0.64 | 0.13 | 0.99* | -0.65 | -0.80* | 0.60 | 0.68 | 0.65 | 0.70 | 0.44 | 0.65 |
| 0.87* | 0.35  | 0.95*     | -0.83*    | 0.96*     | -0.82*    | -0.91*    | -0.83*    | -0.93*    | -0.62* | -0.94* | 0.86* | 0.79 | 0.96* | -0.82* | 0.88* | 0.78 | 0.88* | 0.55 | 0.88* | 0.93* | -0.98* | 0.95* | -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 $p < 0.05$. 

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 fermentation.
| 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                       | 0.71         | 0.70              | 0.59 |      |
| Ethanol                                       | 3.83                 | <500            | 54.49                       | 93.52        | 7.07              | 1.74 | 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                          | 21.21        | ND                | ND   | ND   |
| 2,3-Butanediol                                | 16.28                | 794             | ND                          | 27.70        | 7.64              | ND   | ND   |
| 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                          | 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                          | 0.12         | ND                | ND   | ND   |
| Butanoic acid, 2-methyl-, methyl ester        | 15.73                | 784             | ND                          | ND           | ND                | ND   | 0.22 |
| Sulfurous acid, decylpentyl ester             | 23.38                | 980             | 0.11                        | ND           | ND                | ND   | 0.06 |
| 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 |
| Cyclopentane                                  | 5.41                 | 557             | ND                          | 0.04         | 0.38              | ND   | 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                          | 0.11         | ND                | 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 |
| Soybean seed Fermentation time | | | | | | |
| 1-Octene | 19.74 | 892 | ND | 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 | ND | 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 | 0.72 | 0.79 | 0.78 |
| 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 | ND | 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 | ND | 0.11 | ND |
| 2,2,3-Trimethyl nonane | 23.88 | 991 | 0.40 | 0.27 | 0.37 | 0.26 | ND |
| 2-Bromo-octane | 23.98 | 993 | 0.09 | ND | ND | 0.05 | 0.04 |
| 2,8,8-Trimethyl decane | 24.00 | 994 | ND | ND | ND | 0.16 | ND |
| 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 | ND | 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 |ND |
| 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 | 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 | 0.13 | ND |
| 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**

| Ketones | | | | | | |
| Acetone | 4.10 | <500 | ND | ND | 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 | 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 | 0.17 |
| 3-Methyl-2-pentanone | 14.42 | 758 | ND | ND | ND | 1.12 |
| Cyclopentanone | 15.68 | 783 | ND | ND | ND | ND |
| Cyclohexanone | 19.51 | 886 | ND | ND | ND | ND |
| 3-Methyl-2-hexanone | 20.15 | 903 | 0.17 | 0.14 | 0.14 | 0.12 | 0.12 |
| 6-Methyl-5-hepten-2-one | 22.40 | 958 | 0.20 | ND | ND | ND | ND |

**Miscellaneous**

| Miscellaneous | | | | | | |
| Dimethyl sulfide | 4.52 | 517 | 11.82 | 0.13 | 0.11 | 0.16 | ND |

(continued on next page)
| 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           | ND   | 0.24 | 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   |

Volatile compounds 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.

| Compounds                      | Retention time (min) | Retention index | Relative concentration (ng) | Soybean seed | Fermentation time |
|-------------------------------|----------------------|-----------------|-----------------------------|--------------|------------------|
|                               |                      |                 |                             |              | 0 h | 12 h | 24 h | 48 h |
| Acetic acid                   | 7.54                 | 623             | 0.95                        | ND           | 0.23 | ND   | ND   |
| 2-Ethyl butanoic acid         | 18.00                | 842             | ND                          | 0.07         | 0.16 | 0.22 |
| Methanol                      | 3.34                 | <500            | 10.12                       | 1.50         | ND   | ND   |
| Ethanol                       | 3.83                 | <500            | 86.51                       | 84.82        | 3.30 | 2.63 |
| 1-Propanol                    | 5.59                 | 563             | 14.08                       | ND           | ND   | ND   |
| 2-Butanol                     | 6.84                 | 607             | 2.79                        | ND           | ND   | ND   |
| 2-Methyl-1-propanol           | 8.17                 | 637             | 1.29                        | ND           | ND   | ND   |
| 1-Butanol                     | 10.53                | 679             | 1.31                        | ND           | 0.12 | 0.16 |
| 1-(1-Methylethoxy)-2-propanol | 11.16                | 689             | 0.25                        | 0.07         | 0.13 | 0.08 |
| 3-Pentanol                    | 13.53                | 738             | ND                          | ND           | ND   | ND   |
| 3-Methyl-3-buten-1-ol         | 13.96                | 748             | ND                          | ND           | ND   | ND   |
| 3-Methyl-1-butanol            | 14.18                | 752             | 0.45                        | ND           | ND   | ND   |
| 2-Methyl-1-butanol            | 14.26                | 754             | 1.12                        | ND           | ND   | ND   |
| 1-Pentanol                    | 15.62                | 781             | 0.25                        | ND           | ND   | ND   |
| 5-Methyl-2-heptanol           | 16.22                | 793             | ND                          | ND           | ND   | ND   |
| 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   |
| 1-Hexanol                     | 19.22                | 878             | 0.29                        | ND           | ND   | ND   |
| 3-Methyl-1-heptanol           | 34.86                | 1274            | 0.16                        | ND           | ND   | ND   |

Volatile compounds 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.
| 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                        |
| Cyclopentene                   | 5.32                 | 551            | ND                        |
| 2-Methyl pentane               | 5.62                 | 564            | ND                        |
| 3-Methyl-pentane               | 6.04                 | 581            | ND                        |
| 2-Butanone                     | 6.28                 | 590            | ND                        |
| n-Hexane                       | 6.56                 | 600            | ND                        |
| Methyl cyclopentane            | 7.73                 | 628            | ND                        |
| Benzene                        | 9.31                 | 659            | ND                        |
| Cyclohexane                    | 9.51                 | 662            | 0.15                      |
| 4-Methyl-1-hexene              | 11.39                | 693            | 0.23                      |
| n-Heptane                      | 11.91                | 700            | 0.20                      |
| Methyl benzene                 | 15.05                | 770            | 7.52                      |
| 4-Methyl heptane               | 15.34                | 776            | 0.78                      |
| 2,3,4-Trimethyl pentane        | 15.37                | 776            | ND                        |
| 1-Octene                       | 16.27                | 793            | ND                        |
| 2,4-Dimethyl hexane            | 16.60                | 799            | 0.63                      |
| n-Octane                       | 16.63                | 800            | ND                        |
| 2,3,4-Trimethyl hexane         | 17.56                | 829            | ND                        |
| 2,4-Dimethyl-1-heptene         | 18.22                | 849            | 0.67                      |
| Ethyl benzene                  | 18.74                | 864            | 0.19                      |
| 1,2-Dimethyl benzene           | 19.01                | 872            | 0.41                      |
| 2,4,4-Trimethyl pentane        | 19.20                | 877            | ND                        |
| n-Nonane                       | 20.04                | 900            | 0.25                      |
| 2,4-Dimethyl hexane            | 20.06                | 901            | ND                        |
| 2,2,6,6-Tetramethyl heptane    | 21.08                | 935            | ND                        |
| 2,3,4-Trimethyl heptane        | 21.95                | 964            | ND                        |
| 2,2,6-Trimethyl octane         | 22.06                | 967            | 1.06                      |
| 3,3,4-Trimethyl hexane         | 22.38                | 977            | ND                        |
| 2,2,3,3-Tetramethyl heptane    | 22.49                | 981            | 0.42                      |
| 3-Ethyl-2,2-dimethyl pentane   | 22.52                | 982            | 0.33                      |
| 2,2,7-Trimethyl decane         | 22.59                | 984            | 0.24                      |
| 2,2,7,7-Tetramethyl octane     | 22.64                | 985            | ND                        |
| 2,2-Dimethyl octane            | 22.68                | 987            | ND                        |
| Decane                         | 23.12                | 1000           | 1.78                      |
| 3-Ethyl-3-methyl heptane       | 23.15                | 1001           | ND                        |
| 3,3-Dimethyl undecane          | 23.39                | 1006           | 0.11                      |
| 2,2,5-Trimethyl heptane        | 23.64                | 1011           | ND                        |
| 2,2,3-Trimethyl nonane         | 23.87                | 1016           | 0.40                      |
| 3,3,8-Trimethyl decane         | 23.92                | 1017           | ND                        |
| 2,3,4-Trimethyl decane         | 23.98                | 1018           | ND                        |
| 2,2-Dimethyl decane            | 24.14                | 1021           | 2.38                      |
| 2,2,4-Trimethyl decane         | 24.47                | 1028           | 1.11                      |
| Butyl cyclohexane              | 24.57                | 1030           | 0.13                      |
| 2,3,5-Trimethyl decane         | 24.78                | 1034           | 4.47                      |
| 3,4,5-Trimethyl heptane        | 25.03                | 1039           | 0.16                      |
| 3-Methyl decane                | 25.27                | 1044           | 0.25                      |
| 2,6-Dimethyl octane            | 25.41                | 1046           | 0.94                      |
| 2,2,6-Trimethyl decane         | 25.59                | 1050           | 3.26                      |
| 2,2,3,4,6,6-Hexamethyl heptane | 25.74                | 1053           | 3.43                      |
| 2,2,9-Trimethyl nonane         | 25.95                | 1057           | 0.18                      |
| 2,2-Dimethyl-3-decene          | 26.08                | 1059           | 0.32                      |
| 2,2,4,6,6-Pentamethyl heptane  | 26.25                | 1062           | 0.20                      |
| 4-Methyl dodecan               | 26.45                | 1066           | 2.63                      |
| 2,2,7,7-Tetramethyl octane     | 26.66                | 1070           | 0.37                      |
| 3,3,5-Trimethyl decane         | 26.97                | 1076           | 0.49                      |
| 5-(2-Methylpropyl)-nonane      | 27.03                | 1077           | 1.29                      |
| 5-Methyl-5-propyl nonane       | 27.35                | 1082           | 0.74                      |
| 6-Ethyl-2-methyl octane        | 27.46                | 1084           | ND                        |
| 2,2,3,4-Tetramethyl pentane    | 27.68                | 1088           | ND                        |
| 3,7-Dimethyl nonane            | 28.14                | 1096           | 0.14                      |

(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 | ND   | 0.11 | ND   |
| 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   | 0.08 |
| 1-Methyl-3-(1-methylethyl)-cyclopentane | 33.55            | 1234            | 0.11 | ND   | ND   | ND   | 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-butanoone            | 11.97                | 702             | 0.85  | ND    | 128.34| 69.71 | 0.78  |
| 3-Penten-2-one                   | 13.33                | 710             | 0.32  | ND    | ND    | ND    | ND    |
| 3-Hydroxy-3-methyl-2-butanoone   | 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  | ND    | 0.04  | ND    | ND    |
| 3-Methyl-2-hexanone              | 20.17                | 905             | 0.11  | ND    | ND    | ND    | ND    |
| 6-Methyl-2-heptanone             | 21.41                | 946             | ND    | ND    | 0.18  | 0.88  | ND    |
| 5-Methyl-2-heptanone             | 21.75                | 957             | 0.25  | ND    | 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    |
| Methylenic 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    | 0.33  | ND    |
| 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                            |                      |                 | 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   | 0.13 | ND   |
| Alcohols                         |                      |                 | 0.36 | ND   | ND   | ND   | ND   |
| 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   |
| Compounds | Retention time (min) | Retention index | Relative concentration (ng) | Soybean seed | Fermentation time |
|-----------|----------------------|-----------------|-----------------------------|--------------|------------------|
| 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 | 0.35 |
| 3-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 | ND | 0.07 | ND |
| 5-Methyl-1-hexanol | 19.72 | 892 | ND | ND | ND | 0.24 | ND |
| 1-Hepten-3-ol | 22.30 | 976 | ND | ND | ND | ND | 0.32 |

**Table 6 – (continued)**

| Compounds | Retention time (min) | Retention index | Relative concentration (ng) | Soybean seed | Fermentation time |
|-----------|----------------------|-----------------|-----------------------------|--------------|------------------|
| Aldehydes |                      |                 |                             |              |                  |
| Acetdehyde | 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 | 0.07 | ND |
| 2-Heptanal | 19.63 | 890 | ND | ND | ND | ND | 0.19 |
| Benzaldehyde | 21.78 | 959 | ND | ND | ND | 1.55 | ND |

**Esters**

| Compounds | Retention time (min) | Retention index | Relative concentration (ng) | Soybean seed | Fermentation time |
|-----------|----------------------|-----------------|-----------------------------|--------------|------------------|
| Acetic acid, methyl ester | 4.79 | 527 | 0.06 | ND | ND | ND | 0.53 |
| 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 | ND | 0.11 | ND | 0.07 |
| Butanoic acid, 3-methyl-, methyl ester | 15.59 | 952 | ND | ND | ND | ND | 0.21 | 0.32 |
| Propanoic acid, 2-methyl-, penty ester | 21.57 | 962 | ND | ND | ND | ND | ND | 0.19 |
| Acetic acid, methoxy-, ethyl ester | 21.87 | 964 | ND | ND | ND | ND | 0.16 |
| Benzoic acid, penty ester | 21.93 | 527 | 0.01 | ND | ND | ND | ND |

**Hydrocarbons**

| Compounds | Retention time (min) | Retention index | Relative concentration (ng) | Soybean seed | Fermentation time |
|-----------|----------------------|-----------------|-----------------------------|--------------|------------------|
| 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 | 1.37 | 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 | ND | 0.28 |
| 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 | ND | 0.08 | 0.08 |
| 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 |
| 3,4,4-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 |

(continued on next page)
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 – (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 | 2.62 | 1.91 | 1.25 |
| 2,2,4-Trimethyl decane            | 24.43        | 1027             | 0.13 | 0.85 | 1.15 | ND  | 0.45 |
| 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-decane             | 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   |
| 5-Methyl dodecane                 | 26.44        | 1066             | 0.30 | 1.88 | 2.60 | 1.70 | ND   |
| 2,2,7,7-Tetramethyl octane        | 26.66        | 1070             | ND  | ND  | ND  | ND  | 0.08 |
| 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-butanol 9.08 655 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-butanol 11.99 703 0.21 0.19 171.16 160.01 4.63
3-Hydroxy-3-methyl-2-butanol 13.39 736 0.02 ND 0.71 7.34 7.99
4-Methyl-2-pentanone 13.72 743 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 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 ND

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 0.20 0.72 3.11
2-Ethyl furan 11.79 699 ND 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 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 ND 0.45 0.63
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, Daeuwon,* and *Neulchon*), 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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