

Metabolomic Insight into Soy Sauce through ^1H NMR Spectroscopy

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Soy sauce, a well-known seasoning in Asia and throughout the world, consists of many metabolites that are produced during fermentation or aging and that have various health benefits. However, their comprehensive assessment has been limited due to targeted or instrumentally specific analysis. This paper presents for the first time a metabolic characterization of soy sauce, especially that aged up to 12 years, to obtain a global understanding of the metabolic variations through ^1H NMR spectroscopy coupled with multivariate pattern recognition techniques. Elevated amino acids and organic acids and the consumption of carbohydrate were associated with continuous involvement of microflora in aging for 12 years. In particular, continuous increases in the levels of betaine were found during aging for up to 12 years, demonstrating that microbial- or enzyme-related metabolites were also coupled with osmotolerant or halophilic bacteria present during aging. This work provides global insights into soy sauce through a ^1H NMR-based metabolomic approach that enhances the current understanding of the holistic metabolome and allows assessment of soy sauce quality.

KEYWORDS: Metabolomics; metabolites; soy sauce; fermentation; NMR; PCA

INTRODUCTION

Soy sauce is a well-known seasoning in Korea, China, and Japan. Soy sauce is produced through a microbial process and contains many metabolites from the enzymatic degradation of source material during fermentation or aging. Manufacturing processes differ for the production of Japanese soy sauce, made with equal amounts of soybeans and wheat, and that of traditional Korean soy sauce, made of soybeans only. Moreover, microflora involved in the fermentation also vary between the two types. To date, amino acids, organic acids, and amines have been targeted for evaluating and characterizing fermented foods through liquid chromatographic (LC) or mass spectrometric (MS) approaches (1–4). However, global profiling to characterize metabolites in soy sauce has not been reported.

Metabolomic or metabonomic studies are a rapidly emerging area of “-omics” research and are defined as the comprehensive and quantitative analysis of all metabolites (5). Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are mostly used to generate global metabolite profiles in metabolomics (6). An advantage of NMR is that the samples do not require any physical or chemical treatment prior to the analysis, whereas MS usually requires that the metabolites be separated from the sample before detection by liquid chromatography (5). Although MS is more sensitive than NMR, NMR

is the more attractive tool for metabolomic studies because the nondestructive nature of NMR enables observation of the dynamics as well as separation of metabolites in biological samples; in contrast, MS disrupts the structures and interactions of molecular complexes. NMR spectroscopic analysis coupled with multivariate statistical chemometric methods offers a powerful new approach for assessing metabolic function. Pattern recognition and related multivariate statistical approaches can be used to discern significant patterns in complex data sets with the aim of classifying objects by identifying inherent patterns in a set of indirect measurements. Pattern recognition methods, such as principal components analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal projection to latent structures discriminant analysis (OPLS-DA), reduce the dimensionality of complex data sets and thereby facilitate the visualization of inherent patterns in the data and accelerate the interpretation.

^1H NMR-based metabolomic studies have been applied in the food sciences (7), including assessments of green tea (8), rosemary (9), honey (10), and grape wine (11, 12). In particular, we reported the dependence of wine metabolites on grape variety and growing area and on yeast strains using ^1H NMR-based metabolomic studies (11, 13, 14). Because soy sauce also consists of metabolites produced from soybean material by fermentative microorganisms, comprehensive profiling of the metabolites could provide a better understanding of metabolic behaviors in soy sauce during fermentation and better evaluation of the quality of the soy sauce product.

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In the present study, we report a statistical evolution of metabolites in Korean traditional soy sauces during aging from 1 to 12 years and a comparative evaluation of their quality to industrial soy sauces, using ^1H NMR-based metabolomics coupled with multivariate statistical analysis.

MATERIALS AND METHODS

Soy Sauces. All traditional Korean soy sauces were obtained directly from 21 different earthenware jars at each fermentation period: boiled ($n=5$) and unboiled ($n=5$) soy sauces aged for 1 year and soy sauces aged for 2 years ($n=5$), 4 years ($n=5$), and 12 years ($n=1$). Manufacturing processes of Korean traditional soy sauce are described in Figure S-1 in the Supporting Information. Commercial Korean ($n=5$, Sempio Co. Ltd. and CJ Co. Ltd., Seoul, Korea) and Japanese ($n=5$, Kikkoman Co. Ltd., Tokyo, Japan) soy sauces were purchased from a local market after careful selection of five different expiration dates to ensure that the commercial soy sauces were produced in different batches (Table 1).

pH Value and Salt Content. The pH was measured with a Corning 440 pH-meter (Corning Co. Ltd.). Salt content was determined according to AOAC procedures (15).

Total Number of Bacteria. Total viable cell counts were determined on triplicate pour plates of 1 mL of diluted soy sauce samples in Plate Count Agar (Difco). The plates were incubated for 3 days at 30 °C. Results are reported as log colony-forming units (CFU) per milliliter of soy sauce.

^1H NMR Spectroscopic Analysis of Soy Sauces. Soy sauce samples were prepared by mixing 120 μL of soy sauce with 420 μL of phosphate buffer (0.2 M sodium phosphate, pH 5.0) and 60 μL of D_2O (99.9% deuterium oxide) and then centrifuging at 13000g for 10 min. Supernatants (550 μL) were transferred into 5 mm NMR tubes. D_2O provided a field frequency lock. ^1H NMR spectra were acquired on a Varian Inova-600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) operating at 599.84 MHz ^1H frequency and a temperature of 298 K, using a triple-resonance 5 mm HCN salt-tolerant cold probe. A NOESYPRE-SAT pulse sequence was applied to suppress the residual water signal. For each sample, 16 transients were collected into 32K data points using a spectral width of 9615.4 Hz with a relaxation delay of 1.5 s, an acquisition time of 4.00 s, and a mixing time of 400 ms. Chemical shifts were referenced to that of α -glucose (^1H , δ 5.23). A 0.3 Hz line broadening function was applied to all spectra prior to Fourier transformation (FT). Signal assignment for representative samples was facilitated by acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and spiking experiments.

NMR Data Preprocessing and Multivariate Statistical Analysis.

All NMR spectra were manually phased and baseline corrected by VnmrJ software 2.1B (Varian Inc.) and then converted to ASCII format. The ASCII format files were imported into MATLAB (R2006a, Mathworks, Inc., 2006). The NMR spectral data were reduced into 0.001 ppm spectral buckets, and all spectra were aligned using the Correlation Optimized Warping (COW) method (11, 16). The region corresponding to water (4.6–4.8 ppm) was removed prior to the spectral alignment. The aligned spectra were normalized by two methods: normalization to the total spectral area and normalization through division of each spectrum by median spectrum. The resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis.

The mean center was applied for all multivariate analysis by SIMCA-P version 12.0 (Umetrics). PCA, an unsupervised pattern recognition method, was performed to examine the intrinsic variation in the data set. Hotelling's T^2 region, shown as an ellipse in the score plots, defines the 95% confidence interval of the modeled variation (17). The quality of the model is described by R^2 and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the model and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (14).

Statistical Analysis. All data were analyzed by analysis of variance (ANOVA), and significant differences in mean values among data were determined at $p < 0.05$ by Duncan's multiple-range tests using SPSS 12.0 (SPSS Inc., Chicago, IL).

Chemicals. All chemical reagents were of analytical grade from Sigma (St. Louis, MO).

RESULTS

Total Microflora, pH, and Salt Content in Soy Sauce. Salt content, pH, and total number of microflora are summarized in Table 1. After boiling for 30 min, the total number of microflora decreased significantly and remained constant until 12 years of aging. However, among boiled soy sauces, soy sauce aged for 4 years had about 2 times more total microflora. The reason for the higher number of total microflora in 4-year-aged soy sauce was not identified in the present study; there were no abnormal changes in salt content or pH, which increased slightly as the aging period increased in boiled soy sauces. Abnormal metabolic variations in 4-year-aged soy sauce are considered under Discussion.

^1H NMR Spectroscopy of Soy Sauce. Figure 1 shows representative one-dimensional (1D) ^1H NMR spectra of soy sauces aged for 1 year without boiling (A) and soy sauces aged for 1 (B), 2 (C), 4 (D), and 12 (E) years after boiling; each was manufactured with Meju starter according to traditional Korean methods. Typical ^1H NMR spectra of commercial Japanese (Kikkoman) (F) and Korean (CJ) (G) soy sauces are also presented in Figure 1. Thirty-seven metabolites were identified through analyses of 2D NMR (Figure S-2 in the Supporting Information) and spiking experiments. As shown in Figure 1, spectra from soy sauces were dominated by a number of metabolites, namely, leucine, isoleucine, valine, lactate, alanine, acetate, arginine, betaine, tyramine, phenylalanine, formate, α -glucose, β -glucose, lysine, γ -aminobutyrate (GABA), fucose, ethanol, succinate, tyrosine, proline, choline, uracil, hypoxanthine, aspartate, methionine, pyroglutamate, glutamate, glycine, trimethylamine, malonate, phosphocholine, oligosaccharides (O1, O2, and O3), and glycerol. Visual inspection of the spectra revealed huge amounts of ethanol in commercial soy sauces. Moreover, butyrate and propionate were observed in CJ soy sauces only.

Normalization Effect of Soy Sauce NMR Spectra on PCA Models. We investigated the effect of the normalization of soy sauce ^1H NMR spectra on PCA models. Commercial Korean soy sauces from CJ and Sempio manufacturers and traditional Korean soy sauce aged for 1 year were selected for the investigation because the former contained huge amounts of ethanol, whereas the latter contained little ethanol as observed by inspection of the 1D NMR spectra in Figure 1. Two methods were applied for normalizing the NMR spectra: normalization to total spectral area and normalization by dividing each spectrum by the median spectrum. Figure S-3 in the Supporting Information clearly shows a normalizing effect on the PCA model. The PCA models were generated after normalizing soy sauce to total spectral area (Figure S-3A in the Supporting Information) and after normalization by dividing each ^1H NMR spectrum by median spectrum (Figure S-3B in the Supporting Information). Each normalization was carried out in the absence (solid symbols) and presence (open symbols) of ethanol peaks. Because the ^1H NMR spectra of commercial soy sauces were dominated by huge amounts of ethanol, the ethanol peaks were excluded prior to PCA modeling to avoid any effect of ethanol on the model following normalization. On the basis of the results, PCA score plots showed that division by median spectrum is an appropriate normalization method in metabolomic study, particularly in samples including an abnormal spectral peak with a large area or high intensity, such as ethanol in the commercial soy sauces.

To examine the metabolic variations among soy sauces, a PCA strategy was applied to the entire data set. The PCA score plots

Table 1. Salt Content, pH, and Total Number of Bacteria in Soy Sauces

soy sauce	bacteria (log CFU/g)	salt (%)	pH
unboiled ^a	6.43 ± 0.07 a ^c	24.86 ± 0.16 b	5.00 ± 0.08 cd
1 year ^b	2.79 ± 0.39 c	20.17 ± 1.14 c	4.87 ± 0.06 cd
2 years	2.30 ± 0.07 d	27.45 ± 0.54 a	4.89 ± 0.02 cd
4 years	4.35 ± 0.02 b	29.11 ± 0.15 a	5.15 ± 0.04 bc
12 years	2.84 ± 0.13 c	29.05 ± 0.23 a	5.37 ± 0.02 ab
CJ	4.81 ± 4.76 abcd	25.28 ± 0.44 b	4.92 ± 0.05 cd
Sempio	— ^d	25.30 ± 0.29 b	5.15 ± 0.04 bc
Kikkoman	—	17.84 ± 0.12 d	4.75 ± 0.03 e

^a Represents soy sauce aged for 1 year without boiling. ^b Represents soy sauces aged for 1, 2, 4, and 12 years after boiling for 30 min. ^c Different letters indicate significant differences between samples in Duncan's multiple-range test ($p = 0.05$). ^d Not detected.

showed clear metabolic differences between traditional Korean (unboiled, 1, 2, 4, and 12 years) and commercial soy sauces (CJ, Sempio, and Kikkoman) (Figure 2A) and year-to-year differences between traditional Korean soy sauces during the brine fermentation or aging (Figure 2B). Movements of the plots from the left to the right in Figure 2B indicate continuous metabolic changes in traditional Korean soy sauces during fermentation or aging for up to 12 years.

Variations in Metabolites of Unboiled and Boiled Soy Sauces. To investigate the metabolic differences between boiled and unboiled traditional Korean soy sauces that were aged for 1 year, metabolites observed in the ¹H NMR spectra of all soy sauces from the same manufacturer were used to construct multivariate statistical models. Unboiled soy sauces (U) were clearly differentiated from boiled soy sauces (B) by PCA score plots with high statistical values of Rx^2 (0.85) and Q^2 (0.77), as shown in Figure 3A. The PCA loading plots reveal the metabolites responsible for the differentiation in the complementary PCA score plot. The upper section of the PCA loading plot indicates relatively higher levels of metabolites in unboiled soy sauces (U) compared with boiled soy sauces (B), whereas the lower section indicates lower levels. The differentiation between unboiled (U) and boiled (B) soy sauces was caused by higher levels of leucine, isoleucine, valine, lactate, alanine, lysine, GABA, acetate, methionine, pyroglutamate, glutamate, tyramine, betaine, glycine, trimethylamine, oligosaccharides (O1 and O2), and phenylalanine and by lower levels of fucose, aspartate, α - and β -glucoses, oligosaccharide (O3), tyrosine, and formate in unboiled soy sauce (U), as shown in Figure 3B. To see significant differences in metabolites, PCA column loadings plots were generated after selection of typical metabolites in the PCA line loading plot given in Figure 3B. The PCA column loading plots highlight the significant changes in methionine, aspartate, and U4 (Figure 3C); choline, betaine, and U5 (Figure 3D); and formate (Figure 3E); smaller bars indicate higher confidence limits.

Boiling mainly affects the microorganisms involved in brine fermentation as well as the color of soy sauce. This is confirmed by a larger total number of microorganisms in unboiled soy sauces (U) than in boiled soy sauces (1 year), as shown in Table 1. The large number of microflora in unboiled soy sauces (U) might be responsible for the differences in many metabolites and result in more intravariation, indicating that the lack of boiling would provide soy sauce of uncontrolled quality compared with soy sauces aged for 1, 2, and 4 years after boiling. These intravariations are indicated by relatively scattered clusters of unpasteurized soy sauce samples as shown in the PCA score plots (Figure S-4 in the Supporting Information).

Evolution of Metabolites during Aging. Figure 4A shows clear differentiation between soy sauces aged for 1 and 2 years by the first PCA component (PC1). Two-year-aged soy sauces (2 years)

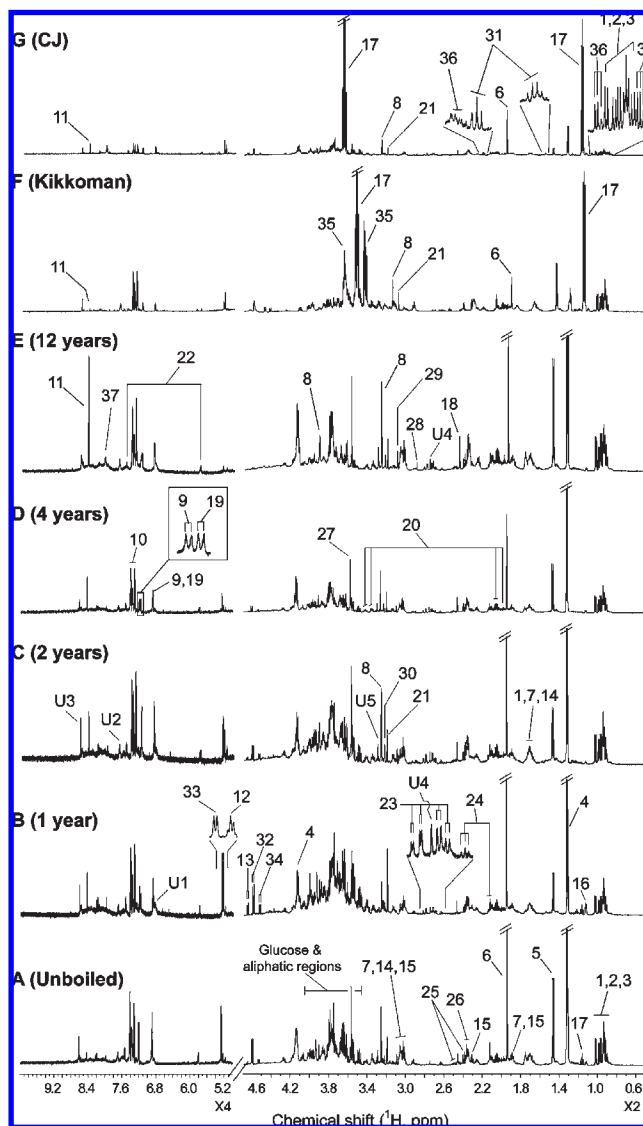


Figure 1. Typical 600 MHz ¹H NMR spectra of soy sauces unboiled (A) and aged for 1 year (B), 2 years (C), 4 years (D), and 12 years (E) obtained from a traditional Korean manufacturer. F and G represent commercially available soy sauces obtained from CJ and Kikkoman, respectively. Unpasteurized soy sauces were aged for 1 year. Peaks: 1, leucine; 2, isoleucine; 3, valine; 4, lactate; 5, alanine; 6, acetate; 7, arginine; 8, betaine; 9, tyramine; 10, phenylalanine; 11, formate; 12, α -glucose; 13, β -glucose; 14, lysine; 15, γ -aminobutyrate (GABA); 16, fucose; 17, ethanol; 18, succinate; 19, tyrosine; 20, proline; 21, choline; 22, uracil; 23, aspartate; 24, methionine; 25, pyroglutamate; 26, glutamate; 27, glycine; 28, trimethylamine; 29, malonate; 30, phosphocholine; 31, butyrate; 32, oligosaccharide (O1); 33, oligosaccharide (O2); 34, oligosaccharide (O3); 35, glycerol; 36, propionate; 37, hypoxanthine; U1, U2, U3, U4, and U5, unknown.

were characterized by higher levels of leucine, isoleucine, valine, lactate, alanine, glutamate, pyroglutamate, succinate, aspartate, lysine, GABA, glycine, malonate, betaine, tyrosine, phenylalanine, and formate (Figure 4C). In addition, levels of fucose, alcohol, choline, acetate, α - and β -glucoses, oligosaccharides (O), and tyramine were decreased in 2-year-aged soy sauces compared to those in 1-year-aged soy sauces, as shown in the PCA loading plot (Figure 4C). The changes in metabolites in 4-year-aged soy sauces compared with 2-year-aged soy sauces were similar to the changes between 1- and 2-year-aged soy sauces, indicating a dependence of metabolites on the aging period

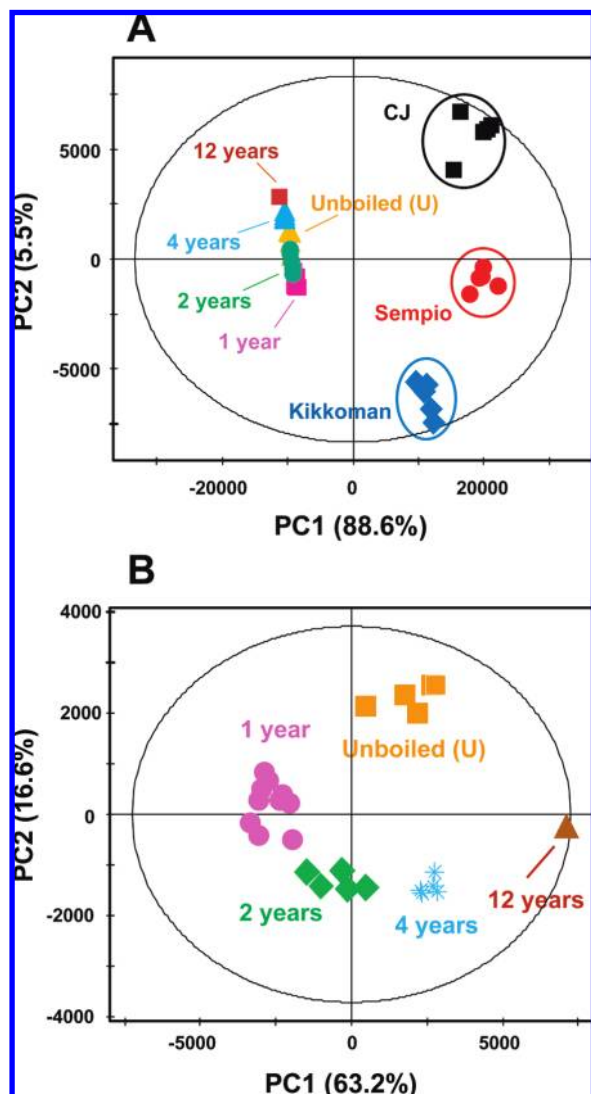


Figure 2. PCA score plot derived from the 600 MHz ^1H NMR spectra of traditional Korean and commercial Korean and Japanese soy sauces (**A**). Unboiled (U), 1, 2, 4, and 12 years represent traditional Korean soy sauces; unboiled soy sauces (U) were aged for 1 year without boiling, whereas 1, 2, 4, and 12 year soy sauces were boiled for 30 min prior to aging. CJ, Sempio, and Kikkoman represent commercially available soy sauces. PCA score plot in panel **B** was regenerated only with traditional Korean soy sauces from panel **A**.

(**Figure 4**). That is, these metabolites varied as the aging period increased; amino acids increased while carbohydrates decreased, strongly indicating continued involvement of enzymes and microflora in the brine fermentation until 4 years. This was confirmed by the total number of microflora measured (**Table 1**). The patterns of changes in metabolites were consistent with those in 12-year-aged soy sauce (**Figure 5**), even though the patterns in 12-year-aged soy sauces were not statistically relevant due to sample size (one batch); 4-year-aged soy sauces comprised five batches. However, aspartate levels were higher in 2-year-aged soy sauces than 4-year-aged soy sauces, and choline levels were lower in 4-year-aged soy sauces (**Figure 4D**).

Metabolic Differences between Traditional and Commercial Soy Sauces. To investigate metabolic differences between traditional and commercial soy sauces, we selected traditional Korean soy sauces aged for 4 years and commercial soy sauces produced by both CJ and Kikkoman. These soy sauces were clearly differentiated in the PCA score plots with high statistical values of R^2

and Q^2 (**Figure S-5A,C** in the Supporting Information), demonstrating significant differences in their metabolites. Higher levels of alcohol, acetate, α - and β -glucoses, and oligosaccharide (O) in CJ soy sauces were responsible for the differentiation between 4-year-aged and CJ soy sauces (**Figure S-5B** in the Supporting Information). On the other hand, 4-year-aged soy sauces were dominated by higher levels of leucine, isoleucine, valine, lactate, alanine, lysine, glutamate, succinate, malonate, phosphocholine, betaine, glycine, glycerol, tyrosine, tyramine, phenylalanine, and formate compared to CJ soy sauces. Comparison of 4-year-aged and Kikkoman soy sauces revealed that Kikkoman soy sauces were characterized by relatively higher levels of alcohol, glycerol, α - and β -glucoses, glutamate, methionine, and phenylalanine but lower levels of lactate, lysine, acetate, succinate, glycine, trimethylamine, oligosaccharides (O1, O2, and O3), aspartate, choline, phosphocholine, betaine, tyrosine, tyramine, and formate (**Figure S-5D** in the Supporting Information).

DISCUSSION

Metabolites Related to Choline Metabolism. With increasing aging period, choline levels decreased, whereas betaine and glycine levels increased; the highest levels of betaine and glycine were found in 12-year-aged soy sauces. The lactic acid bacterium (LAB) *Tetragenococcus halophila* grows during soy sauce fermentation, in which the salt concentration ranges from 12 to 26% (19), and *T. halophila* is the dominant bacterium in cured anchovies processed under reduced water activities (20). These halophilic bacteria can tolerate high salt concentrations, which suggests that they have a high osmotic adjustment capacity that allows them to maintain optimal cell turgor pressure. Osmoregulation has been also reported in nonhalophilic bacteria such as *Escherichia coli* (21), *Lactobacillus plantarum* (22), and *Bacillus subtilis* (23). These organisms therefore contribute to the biopreservation of the products. Betaine is reported to serve as the major effective osmoprotectant in these bacteria, improving salinity tolerance (24). Most bacteria can accumulate betaine through conversion of its precursor, choline, as a microbial stress response to high-osmolality environments (21, 25). Before the conversion of choline to betaine, choline is imported into the cell (26). In the present study, continuous increases in betaine levels provide evidence of the involvement of salt-tolerant microflora in the aging of soy sauce until 12 years. Greater accumulation of betaine in soy sauce (35.59 mg/100 g of food) than in raw soybean (2.08 mg/100 g of food) may also indicate synthesis of betaine by halophilic bacteria during soy sauce fermentation (27). Betaine (trimethylglycine or glycine betaine) also plays an important role in cell biochemistry, donating a methyl group in the conversion of homocysteine to methionine in mammals (28, 29). It also serves as an osmoprotective agent to protect against kidney and bacterial cell damages (30). Recently, alleviation of alcoholic and nonalcoholic liver injury by betaine supplementation has also been reported (31, 32).

Choline is an essential nutrient found in foods, including dried soybeans (47.27 mg/100 g) and soy sauce (31.01 mg/100 g) (27). The U.S. Institute of Medicine has made recommendations for dietary choline intake (33), because a choline-deficient diet causes liver steatosis and damage in humans (34), and growth retardation, renal dysfunction, and hemorrhage or bone abnormalities in animals (35, 36). The metabolic pathway related to choline and betaine is summarized in **Figure 6**. The levels of betaine, glycine, choline, and phosphocholine were significantly higher in Korean traditional soy sauces than in commercial soy sauces. This suggests that Korean traditional soy sauces may be more beneficial with respect to health than commercial products.

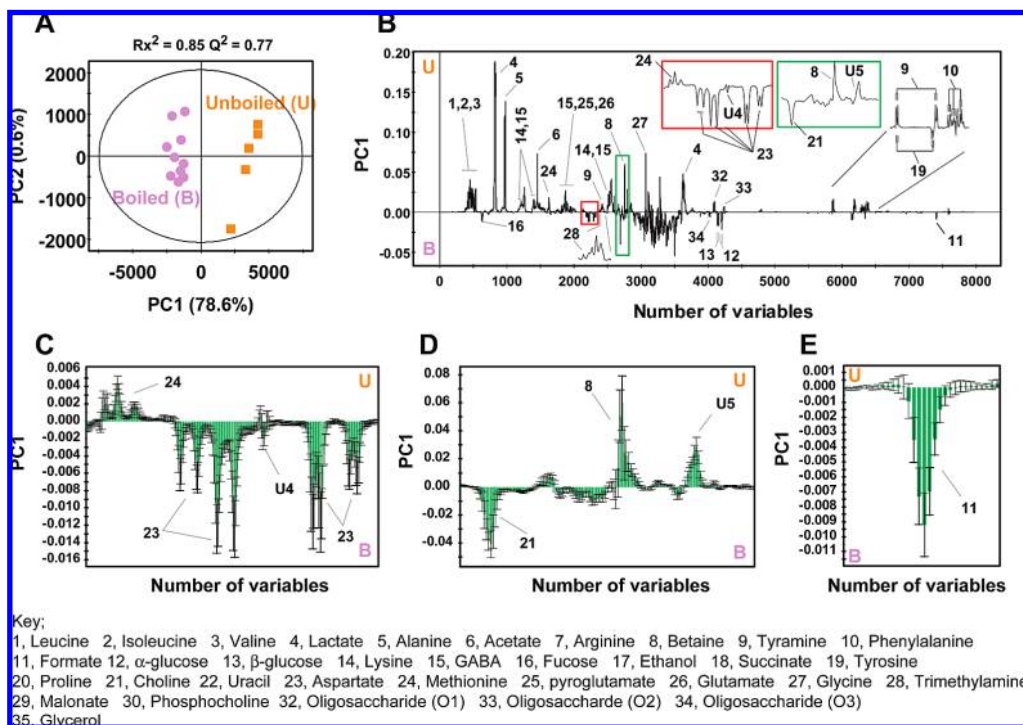


Figure 3. PCA score (A) and loading (B) plots derived from 600 MHz ^1H NMR spectra of traditional Korean soy sauces that were boiled (B) and unboiled (U), followed by aging for 1 year. Upper section of the loading plot represents relatively higher levels of metabolites in unpasteurized soy sauces, whereas the lower section represents lower levels. The Rx^2 (0.85) and Q^2 (0.77) values reveal high goodness of fit and predictability of the PCA model. The boxes inside panel B highlight the changes in methionine, aspartate, choline, betaine, and formate levels. PCA column loading plots reveal significant differences in methionine, unknown compound (U5), and aspartate (C), choline and betaine (D), and formate (E). The smaller the bar, the higher the confidence limit in the PCA column loading plots. Other metabolites were also considered whether their levels were significant or not. U4 and U5 represent unknown compounds.

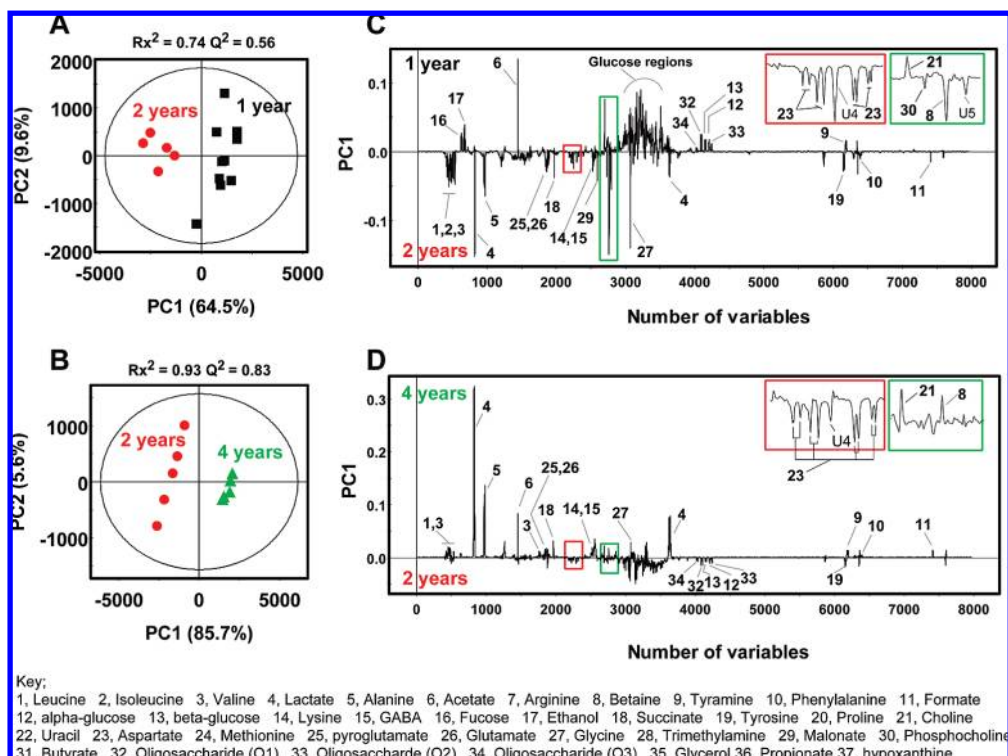


Figure 4. PCA score (A and B) and loading (C and D) plots derived from the ^1H NMR spectra of traditional Korean soy sauces aged for 1, 2, and 4 years, demonstrating significant metabolic evolutions as aging period increased. The fitness and predictability of the models are indicated by Rx^2 and Q^2 values of 0.74 and 0.93 and of 0.56 and 0.83, respectively. U4 represents unknown compounds.

Lactate. The highest levels of lactate were found in unboiled soy sauces, as expected on the basis of their large total number of

microflora. Among boiled soy sauces, including 1-, 2-, 4-, and 12-year-aged soy sauces, lactate levels were elevated as aging or

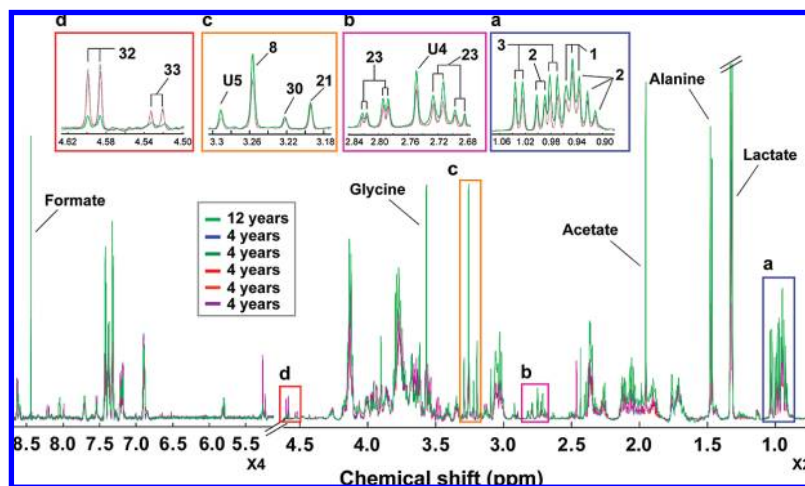


Figure 5. Normalized ^1H NMR spectra of 4 ($n=5$) and 12 ($n=1$) year aged soy sauces. Expanded panels a–d highlight elevated levels of amino acids and betaine and low levels of oligosaccharides in 12-year-aged soy sauce compared to those in 4-year-aged soy sauces; other metabolites also changed as the aging period increased, but their changes are not highlighted here. Peaks: 1, leucine; 2, isoleucine; 3, valine; 8, betaine; 21, choline; 23, aspartate; 30, phosphocholine; 32, oligosaccharide (O1); 33, oligosaccharide (O2); U4 and U5, unknown.

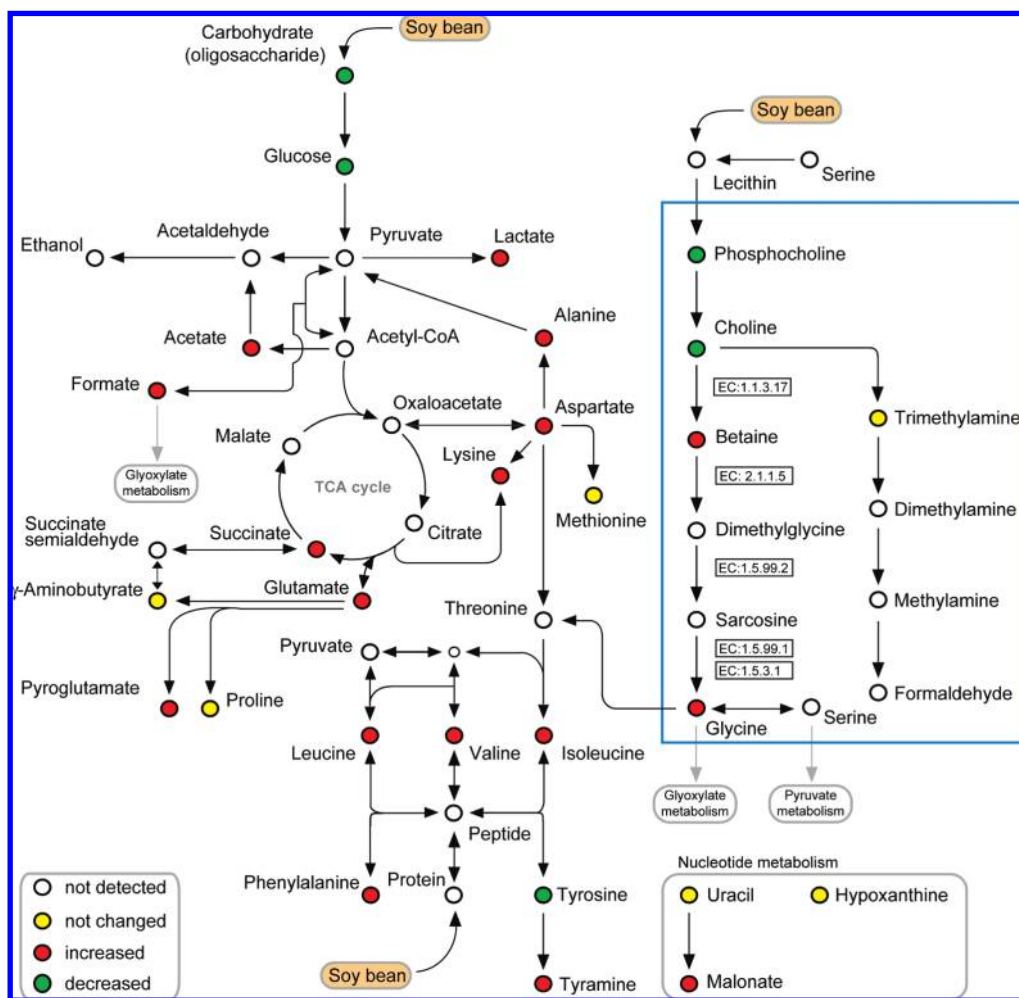


Figure 6. Schematic representation of metabolites produced during brine fermentation or aging of soy sauce. Identified metabolites from ^1H NMR spectroscopy are colored; increased metabolites are shown in red, and decreased metabolites are marked in green. Yellow represents no significant change. Degradation of soybean material by proteolysis or peptidase mainly contributed to the increased amount of amino acids during aging even though halophilic microflora synthesize the amino acids. The blue box highlights the significant accumulation of betaine and glycine in choline catabolism to protect against high cellular osmolality. Key enzymes in choline catabolism include EC 1.1.3.17, choline oxidase; EC 2.1.1.5, betaine-homocysteine methyltransferase; EC 1.5.99.2, dimethylglycine dehydrogenase; EC 1.5.3.1, sarcosine oxidase; and EC 1.5.99.1, choline oxidase.

fermentation period increased, indicating that halophilic or osmotolerant LAB, such as *T. halophilus*, were involved in the brine fermentation of the soy sauce.

Glutamate, Pyroglutamate, Succinate, and GABA. Levels of glutamate, pyroglutamate, succinate, and GABA also increased with up to 12 years of aging or fermentation. Glutamate is the major amino acid, along with aspartate, in raw soybean and fermented soy sauce (18) and serves as a key intermediate in amino acid metabolism of LAB because most aminotransferases utilize glutamate as the donor of amino groups. In addition to its origination from raw soybean material, glutamate is also produced by glutamate dehydrogenase from 2-oxoglutarate, a TCA cycle intermediate (37). Glutamate is used for production of GABA by glutamate decarboxylase. GABA is then transaminated to succinic semialdehyde by GABA transaminase, and finally succinic semialdehyde is oxidized to succinate by its dehydrogenase. During brine fermentation, the dehydrogenation of 2-oxoglutarate could contribute to high levels of glutamate, because glutaminases, which convert glutamine into glutamate, are produced by microorganisms and are inactivated at acidic pH and high salt (38). It is also well-known that fungi and LAB accumulate GABA via decarboxylation of glutamate (39,40). It is therefore likely that GABA and succinate levels, as well as glutamate levels, increase during the fermentation or aging of soy sauce. In general, pyroglutamate is known to be a derivative of glutamate or glutamine and is negatively associated with the flavor of soy sauce because it causes flavorless soy sauce (38,41). Decreasing the conversion of glutamate or glutamine into pyroglutamate would therefore improve the quality of soy sauce. Although a high amount of glutamate was responsible for increased pyroglutamate in aged soy sauces, the boiling process appeared to minimize the conversion of glutamate to pyroglutamate, as pyroglutamate levels were highest in unboiled soy sauces.

Formate and Acetate. Formate is an intermediate produced from pyruvate in normal metabolism and serves as a preservative and antibacterial agent in livestock feed, causing the feed to retain its nutritive value longer. Formate and acetate are produced by osmotolerant LAB and thus inhibit the growth of halophilic yeasts, such as *Saccharomyces rouxii* and *Torulopsis versatilis*, in “shoyu” (Japanese fermented soy sauce) and of *Shigella* (42–44). Increased levels of formate and acetate with increasing aging period up to 12 years in traditional Korean soy sauces were consistent with increases in lactate levels, revealing the involvement of osmotolerant LAB in the aging of soy sauce. Therefore, formate and acetate produced in soy sauce may contribute to the inhibition of the growth of undesirable microorganisms.

Tyrosine, Tyramine, and Aspartate. Tyramine is a monoamine and is one of the biogenic amines formed by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones (45). Tyramine is often produced by microbial decarboxylation of tyrosine in foods and vegetables during fermentation (46). Low levels of tyramine are found in commercial fermented foods, such as “miso”, a semisolid fermented soy paste in Japan, and kimchi, a traditional Korean fermented cabbage (46). Furthermore, tyramine is also the dominant amine in soy sauce (47). Many factors are involved in the production of biogenic amines. Tyramine, putrescine, and cadaverine contents in “tempeh”, Javanese fermented soybean, depend on the processing conditions of the soaked soybeans, the type of fermentative microorganism, and boiling and storage temperatures (48). In addition to the effect of microfloral type on the aminodecarboxylase activity responsible for the decarboxylation of tyrosine to tyramine, pH level and salt content are important factors influencing the activity. Higher tyramine levels are found in anchovies when the pH is low (49). Aminodecarboxylase activity is therefore

stronger in an acidic environment, with the optimum pH between 4.0 and 5.5 (45).

The highest levels of tyramine were observed in unboiled soy sauce, whereas tyrosine was not detected, indicating complete decarboxylation of tyrosine to tyramine (Figure 3B). This also demonstrates that a large number of microflora are involved in the fermentation of unboiled soy sauces and thus may produce larger amounts of aminodecarboxylase compared to that in boiled soy sauces. As the aging period increased up to 12 years, tyramine levels among boiled soy sauces increased, whereas tyrosine levels decreased. In addition, tyramine and tyrosine levels were significantly higher in traditional Korean soy sauces than those in commercial Korean and Japanese soy sauces (Figure S-5 in the Supporting Information), suggesting that tyrosine degradation from raw soy material and consequent enzyme decarboxylation contributed to the high levels of tyramine in soy sauces aged for a long time. Unexpectedly, aspartate levels were higher in soy sauces aged for 2 years than in soy sauces aged for 4 years (Figure 4). Some strains of *T. halophilus* convert aspartate to alanine through decarboxylation (50–53) (Figure 6). Although aspartate is converted to alanine, their levels increased simultaneously as the aging period increased in the present study due to the degradation of protein from raw soybean. However, alanine levels were higher in 4-year-aged soy sauces than in 2-year-aged soy sauces. Moreover, alanine levels were highest in soy sauces aged for 12 years. Therefore, it could be assumed that the large total number of microflora contributed to effective decarboxylation of aspartate to alanine, even though alanine levels were lower in 4-year-aged soy sauces than in 12-year-aged soy sauces (Table 1).

Glycerol and Ethanol. Yeasts, such as *Saccharomyces cerevisiae* in grape wine and *Zygosaccharomyces rouxii* in soy sauce, produce ethanol and glycerol (54, 55). The main differences in the manufacture of traditional Korean and Japanese soy sauces are the starters and the materials: meju and soybean in Korean soy sauce and “koji” and a mixture of soybean and wheat in Japanese soy sauce. Moreover, several types of microflora are involved in the fermentation of traditional Korean soy sauce, in which the microflora are spontaneously inoculated into the meju from the natural environment. In contrast, yeast, mold, and LAB considered to have high enzymatic activity, such as *Aspergillus sojae*, *Aspergillus oryzae*, *Pediococcus halophilus*, *Z. rouxii*, and *Candida* sp., are inoculated into koji, the starter for Japanese soy sauce fermentation (18, 43, 56).

Glycerol levels were approximately 4 or 2.5 times higher in industrial Japanese soy sauce than in traditional Korean soy sauces (Figure 1F and Figure S-5D in the Supporting Information), revealing that yeasts with high enzyme activity, such as *Z. rouxii*, are involved in the fermentation of the high amount of starch in the mixture of soybean and wheat in industrial Japanese soy sauce. In fact, most commercial Korean soy sauces are manufactured according to the Japanese method using koji inoculated with yeast and bacteria selected for their high enzymatic activities (18). However, for this study, we purchased commercial Korean soy sauces manufactured according to the traditional Korean method with meju starter. Therefore, it is also likely that glycerol was higher in commercial Japanese soy sauces (Kikkoman) than in commercial Korean soy sauces (CJ and Sempio) mainly because of differences in the source materials and microflora in Korean and Japanese commercial products.

Although alcohol or ethanol levels were higher in Korean industrial soy sauces (CJ), we did not further consider the difference in ethanol levels, because edible ethanol is added to the final soy sauce product to improve its preservation. Ethanol levels were low in the 1-year-aged unboiled and 1-year-aged

boiled traditional Korean soy sauces and were not observed in 2- and 4-year-aged traditional Korean soy sauces, indicating its evaporation from the traditional Korean soy sauce during long-term storage in the semiopen “onggi” vessels (see the Supporting Information) or its very low production due to the limited amount of starch in soybean material compared to that in the wheat material used in Japanese soy sauce.

Leucine, Isoleucine, Valine, Lysine, and Alanine. In general, amino acids originate from soybean material through proteolysis and peptidase activity of microorganisms involved both in making the meju starter and in the soy sauce fermentation. These amino acids are used as a nitrogen source for growth of the microorganism. After maximum growth of the microorganism, these amino acids are synthesized during fermentation. For example, significant consumption and synthesis of valine in grape wine yeast have been reported during wine fermentation (14). In the present study, the amounts of amino acids increased as aging time increased (Figures 4 and 5) even though the total number of microflora did not change significantly, except in 4-year-aged soy sauces (Table 1). Therefore, degradation of protein from soybean material by enzymes related to amino acids likely contributes to the elevated amino acid levels in soy sauces during prolonged aging, suggesting that the aged traditional soy sauce is more attractive as a dietary source of amino acids than commercial soy sauce.

Carbohydrate. α - and β - glucose and oligosaccharide levels were lowest in 12-year-aged soy sauce, revealing their usage as an energy source during the long fermentation or aging (Figures S-4 and S-5 in the Supporting Information). This is in good agreement with the oligosaccharide consumption time course by wine yeast in grape wine aged for 6 months following alcoholic fermentation (14). Moreover, polysaccharides in soy sauce are known to increase the production of immunoglobulin A (Ig A) in vitro and in vivo (57), and *T. halophilus* possesses immunomodulatory activity in vitro (58); thus, soy sauce could be a promising dietary source for enhancing host defenses because osmophilic or halophilic bacteria in soy sauces degrade starch into polysaccharides and oligosaccharides over a long period. Although these oligosaccharides were consumed by halophilic bacteria during aging, a significant amount remained in soy sauces.

In conclusion, through global profiling of the soy sauce metabolome, we found that traditional Korean soy sauces were clearly still undergoing aging or fermentation until 12 years, leading to carbohydrate consumption and elevated levels of a number of metabolites, including amino acids. Of these metabolites, formate, betaine, glycine, glucose, and oligosaccharides levels could be markers for soy sauces aged or fermented for a prolonged period because they are the main metabolites related to osmoprotection and growth/maintenance characteristics of the halophilic microflora involved in brine aging or fermentation of soy sauce. Recently, Yoshida et al. (59, 60) showed that hydrophilic metabolites related to proteolytic degradation and condensation of sugars and amino acids during fermentation could be found through metabolic profiling of hydrophilic compounds in miso and soy sauce by using a liquid chromatography–mass spectrometry (LC-MS) method. Moreover, NMR-based metabolomics was applied to monitor wine fermentation and to evaluate the fermentative characteristics of wine yeast strain (18). Therefore, metabolomics and global analysis of metabolites provide comprehensive insight into fermented foods and may facilitate a new understanding of fermentation in the future.

Supporting Information Available: Data for traditional Korean soy sauces. This material is available free of charge via the Internet at <http://pubs.acs.org>.

LITERATURE CITED

- (1) Je, J. Y.; Park, P. J.; Jung, W. K.; Kim, S. K. Amino acid changes in fermented oyster (*Crassostrea gigas*) sauce with different fermentation periods. *Food Chem.* **2005**, *91*, 15–18.
- (2) Erbas, M.; Ertugay, M. F.; Erbas, M. O.; Certel, M. The effect of fermentation and storage on free amino acids of tarhana. *Int. J. Food Sci. Nutr.* **2005**, *56*, 349–358.
- (3) Fernandezgarcia, E.; McGregor, J. U. Determination of organic acids during the fermentation and cold-storage of yogurt. *J. Dairy Sci.* **1994**, *77*, 2934–2939.
- (4) Ozogul, F.; Gokbulut, C.; Ozogul, Y.; Ozyurt, G. Biogenic amine production and nucleotide ratios in gutted wild sea bass (*Dicentrarchus labrax*) stored in ice, wrapped in aluminum foil and wrapped in cling film at 4 °C. *Food Chem.* **2006**, *98*, 76–84.
- (5) Nicholson, J. K.; Lindon, J. C. Systems biology—metabonomics. *Nature* **2008**, *455*, 1054–1056.
- (6) Lenz, E. M.; Wilson, I. D. Analytical strategies in metabonomics. *J. Proteome Res.* **2007**, *6*, 443–458.
- (7) Wishart, D. S. Metabolomics: applications to food science and nutrition research. *Trends Food Sci. Technol.* **2008**, *19*, 482–493.
- (8) Tarachiwin, L.; Ute, K.; Kobayashi, A.; Fukusakii, E. ¹H NMR based metabolomic profiling in the evaluation of Japanese green tea quality. *J. Agric. Food Chem.* **2007**, *55*, 9339–9336.
- (9) Xiao, C. N.; Dai, H.; Liu, H. B.; Wang, Y. L.; Tang, H. R. Revealing the metabolomic variation of rosemary extracts using ¹H NMR spectroscopy and multivariate data analysis. *J. Agric. Food Chem.* **2008**, *56*, 10142–10153.
- (10) Donarski, J. A.; Jones, S. A.; Charlton, A. J. Application of cryoprobe ¹H nuclear magnetic resonance spectroscopy and multivariate analysis for the verification of Corsican honey. *J. Agric. Food Chem.* **2008**, *56*, 5451–5456.
- (11) Son, H. S.; Kim, K. M.; Van den Berg, F.; Hwang, G. S.; Park, W. M.; Lee, C. H.; Hong, Y. S. H-1 nuclear magnetic resonance-based metabolomic characterization of wines by grape varieties and production areas. *J. Agric. Food Chem.* **2008**, *56*, 8007–8016.
- (12) Viggiani, L.; Morelli, M. A. C. Characterization of wines by nuclear magnetic resonance: A work study on wines from the Basilicata region in Italy. *J. Agric. Food Chem.* **2008**, *56*, 8273–8279.
- (13) Son, H. S.; Hwang, G. S.; Kim, K. M.; Ahn, H. J.; Park, W. M.; Van den Berg, F.; Hong, Y. S.; Lee, C. H. Metabolomic studies on geographical grapes and their wines using ¹H NMR analysis coupled with multivariate statistics. *J. Agric. Food Chem.* **2009**, *57*, 1481–1490.
- (14) Son, H. S.; Hwang, G. S.; Kim, K. M.; Kim, E. Y.; van den Berg, F.; P., P. W.; Lee, C. H.; Hong, Y. S. ¹H NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. *Anal. Chem.* **2009**, *81*, 1137–1145.
- (15) AOAC. *Official Methods of Analysis of AOAC International*, 16th ed.; AOAC International: Arlington, VA, 1995.
- (16) Larsen, F. H.; van den Berg, F.; Engelsen, S. B. In *An Exploratory Chemometric Study of H-1 NMR Spectra of Table Wines*, 1st Dataspec Conference, June 2005; Wiley: Chichester, U.K., 2005; pp 198–208.
- (17) Hotelling, H. The generalization of Student's ratio. *Ann. Math. Stat.* **1931**, *2*, 360–378.
- (18) Luh, B. S. Industrial production of soy sauce. *J. Ind. Microbiol.* **1995**, *14*, 467–471.
- (19) Roling, W. F.; Van Verseveld, H. W. characterization of *Tetragenococcus halophila* populations in Indonesian soy mash (kecap) fermentation. *Appl. Environ. Microbiol.* **1996**, *62*, 1203–1207.
- (20) Villar, M.; De Ruiz Holgado, A. P.; Sanchez, J. J.; Trucco, R. E.; Oliver, G. Isolation and characterization of *Pediococcus halophila* from salted anchovies. *Appl. Environ. Microbiol.* **1985**, *49*, 664–666.
- (21) Landfald, B.; Strom, A. R. Choline-glycine betaine pathway confers a high levels of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.* **1986**, *165*, 849–855.
- (22) Kets, E. P. W.; Groot, M. N.; Galinski, E. A.; de Bont, J. A. M. Choline and acetylcholine: novel cationic osmolytes in *Lactobacillus plantarum*. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 94–98.
- (23) Boch, J.; Kempf, B.; Schmid, R.; Bremer, E. Synthesis of the osmoprotectant glycine betaine in *Bacillus subtilis*: characterization of the gbsAB genes. *J. Bacteriol.* **1996**, *178*, 5121–5129.

- (24) Robert, H.; Le Marrec, C.; Blanco, C.; Jebbar, M. Glycine betaine, carnitine, and choline enhance salinity tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus halophilus*. *Appl. Environ. Microbiol.* **2000**, *66*, 509–517.
- (25) Boch, J.; Kempf, B.; Bremer, E. Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J. Bacteriol.* **1994**, *176*, 5364–5371.
- (26) Kempf, B.; Bremer, E. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **1998**, *170*, 319–330.
- (27) Zeisel, S. H.; Mar, M. H.; Howe, J. C.; Holden, J. M. Concentrations of choline-containing compounds and betaine in common foods. *J. Nutr.* **2003**, *133*, 1302–1307.
- (28) Finkelstein, J. D.; Harris, B. J.; Kyle, W. E. Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase. *Arch. Biochem. Biophys.* **1972**, *153*, 32–324.
- (29) Millian, N. S.; Garrow, T. A. Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. *Arch. Biochem. Biophys.* **1998**, *356*, 93–98.
- (30) Chambers, S. T.; Kunin, C. M. Isolation of glycine betaine and proline betaine from human urine: assessment of their role as osmoprotective agents for bacteria and the kidney. *J. Clin. Invest.* **1987**, *79*, 731–737.
- (31) Kim, S. J.; Jung, Y. S.; Kwon, D. Y.; Kim, Y. C. Alleviation of acute ethanol-induced liver injury and impaired, metabolomics of S-containing substances by betaine supplementation. *Biochem. Biophys. Res. Commun.* **2008**, *368*, 893–898.
- (32) Kwon, D. Y.; Jung, Y. S.; Kim, S. J.; Park, H. K.; Park, J. H.; Kim, Y. C. Impaired sulfur-amino acid metabolism and oxidative stress in nonalcoholic fatty liver are alleviated by betaine supplementation in rats. *J. Nutr.* **2009**, *139*, 63–68.
- (33) Institute of Medicine. *Dietary Reference Intakes for Folate, Thiamin, Riboflavin, Niacin, Vitamin B12, Panthothenic Acid, Biotin, and Choline*; National Academy Press: Washington, DC, 1998; Vol. 1.
- (34) Zeisel, S. H.; daCosta, K.-A.; Franklin, P. D.; Alexander, E. A.; Lamont, J. T.; Sheard, N. F.; Beiser, A. Choline, an essential nutrient for humans. *FASEB J.* **1991**, *5*, 2093–2098.
- (35) Newberne, P. M.; Rogers, A. E. Labile methyl groups and the promotion of cancer. *Annu. Rev. Nutr.* **1986**, *6*, 407–432.
- (36) Handler, P.; Bernheim, F. Choline deficiency in the hamster. *Proc. Soc. Exp. Med.* **1949**, *72*, 569.
- (37) Kumar, S.; Punekar, N. S. The metabolism of 4-aminobutyrate (GABA) in fungi. *Mycol. Res.* **1997**, *101*, 403–409.
- (38) Yoshimune, K.; Abe, T.; Moriguchi, M. A combination of glutaminase and pH control prevents the nonenzymatic conversion of L-glutamine into L-2-pyrrolidine-5-carboxylic acid in food processing. *J. Food Process. Eng.* **2006**, *29*, 327–336.
- (39) Siragusa, S.; De Angelis, M.; Di Cagno, R.; Rizzello, C. G.; Coda, R.; Gobetti, M. Synthesis of γ -aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.* **2007**, *73*, 7283–7290.
- (40) Kumar, S.; Punekar, N. S.; SatyaNarayan, V.; Venkatesh, K. V. Metabolic fate of glutamate and evaluation of flux through the 4-aminobutyrate (GABA) shunt in *Aspergillus niger*. *Biotechnol. Bioeng.* **2000**, *67*, 575–584.
- (41) Kuroshima, E.; Ohyama, Y.; Matsuo, R.; Sugimori, T. Biosynthesis and degradation of glutamic acid in microorganisms relating to the soy sauce brewing. *J. Ferment. Technol.* **1969**, *47*, 693–698.
- (42) Hentges, D. J. Influence of pH on the inhibitory activity of formic acid and acetic acids for *Shigella*. *J. Bacteriol.* **1967**, *93*, 2029–2030.
- (43) Noda, F.; Hayashi, K.; Mizunuma, T. Antagonism between osmophilic lactic acid bacteria and yeasts in brine fermentation of soy sauce. *Appl. Environ. Microbiol.* **1980**, *40*, 452–457.
- (44) Radler, F.; Brohl, K. The metabolism of several carboxylic acids by lactic acid bacteria. *Z. Lebensm. Unters. Forsch.* **1984**, *179*, 228–231.
- (45) Silla Santos, M. H. Biogenic amines: their importance in foods. *In. J. Food Microbiol.* **1996**, *29*, 213–231.
- (46) Stratton, J. E.; Hutkins, R. W.; Taylor, S. L. Biogenic amines in cheese and other fermented food—a review. *J. Food Prot.* **1991**, *54*, 460–470.
- (47) Stute, R.; Petridis, K.; Steinhart, H.; Biernoth, G. Biogenic amines in fish and soy sauces. *Eur. Food Res. Technol.* **2002**, *215*, 101–107.
- (48) Nout, M. J. R.; Ruikes, M. M. W.; Bouwmeester, H. M.; Beljaars, P. R. Effect of processing conditions on the formation of biogenic amines and ethyl carbamate in soybean tempeh. *J. Food Saf.* **1993**, *13*, 293–303.
- (49) Santos, C.; Pena, M. J.; Rivas, J. C. Changes of tyramine during storage and spoilage of anchovies. *J. Food Sci.* **1986**, *51*, 512–513, 515.
- (50) Abe, K.; Hayashi, H.; Malone, P. C. Exchange of aspartate and alanine—mechanism for development of a proton-motive force in bacteria. *J. Biol. Chem.* **1996**, *271*, 3079–3084.
- (51) Higuchi, T.; Uchida, K.; Abe, K. Aspartate decarboxylation encoded on the plasmid in the soy sauce lactic acid bacterium, *Tetragenococcus halophilus* D10. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 1601–1603.
- (52) Fernandez, M.; Zuniga, M. Amino acid catabolic pathways of lactic acid bacteria. *Crit. Rev. Microbiol.* **2006**, *32*, 155–183.
- (53) Christensen, J. E.; Dudley, E. G.; Pederson, J. A.; Steele, J. L. In *Peptidases and Amino Acid Catabolism in Lactic Acid Bacteria*; 6th Symposium on Lactic Acid Bacteria; Kluwer Academic: Dordrecht, The Netherlands, 1999; pp 217–246.
- (54) Reed, R. H.; Chudek, J. A.; Foster, R.; Gadd, G. M. Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Appl. Environ. Microbiol.* **1987**, *53*, 2119–2123.
- (55) Radler, F.; Shulz, H. Glycerol production of various strains of Saccharomyces. *Am. J. Enol. Vitic.* **1982**, *33*, 36–40.
- (56) Valyasevi, R.; Rolle, R. S. An overview of small-scale food fermentation technologies in developing countries with special reference to Thailand: scope for their improvement. *Int. J. Food Microbiol.* **2002**, *75*, 231–239.
- (57) Matsushita, H.; Kobayashi, M.; Tsukiyama, R. I.; Fujimoto, M.; Suzuki, M.; Tsuji, K.; Yamamoto, K. Stimulatory effect of shoyu polysaccharides from soy sauce on the intestinal immune system. *Int. J. Mol. Med.* **2008**, *22*, 243–247.
- (58) Masuda, S.; Yamaguchi, H.; Kurokawa, T.; Shirakami, T.; Tsuji, R. F.; Nishimura, I. Immunomodulatory effect of halophilic lactic acid bacterium *Tetragenococcus halophilus* Th221 from soy sauce moromi grown in high-salt medium. *Int. J. Food Microbiol.* **2008**, *121*, 245–252.
- (59) Yoshida, H.; Mizukoshi, T.; Hirayama, K.; Miyano, H. Comprehensive analytical method for the determination of hydrophilic metabolites by high-performance liquid chromatography and mass spectrometry. *J. Agric. Food Chem.* **2007**, *55*, 551–560.
- (60) Yoshida, H.; Yamazaki, J.; Ozawa, S.; Mizukoshi, T.; Miyano, H. Advantage of LC-MS metabolomics methodology targeting hydrophilic compounds in the studies of fermented food samples. *J. Agric. Food Chem.* **2009**, *57*, 1119–1126.

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